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Characterization of electrical penetration graphs of *Bucephalogonia xanthophis*, a vector of *Xylella fastidiosa* in citrus

M. P. Miranda[1], A. Fereres[2], B. Appezzato-da-Gloria[3] & J. R. S. Lopes[1]*

[1]*Departamento de Entomologia, Fitopatologia e Zoologia Agricola, ESALQ/Universidade de São Paulo, CP. 9, Piracicaba, SP 13418-900, Brazil,* [2]*Departamento de Protección Vegetal, Instituto de Ciencias Agrarias (ICA, CSIC), C/Serrano, 115 dpdo, 28006 Madrid, Spain, and* [3]*Departamento de Ciências Biológicas, ESALQ/Universidade de São Paulo, CP. 9, Piracicaba, SP 13418-900, Brazil*

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Abstract

The sharpshooter *Bucephalogonia xanthophis* (Berg) (Homoptera: Cicadellidae) is a vector of the xylem-limited bacterium, *Xylella fastidiosa* (Wells, Raju, Hung, Weisburg, Mandelco-Paul, and Brenner), which causes citrus variegated chlorosis. Despite the importance of citrus variegated chlorosis, the probing behavior of vectors on citrus and its implications for transmission of *X. fastidiosa* have not been studied. Here we studied electrical penetration graph (EPG-DC system) waveforms produced by *B. xanthophis* on *Citrus sinensis* (L.) Osbeck (Rutaceae), and their relationships with stylet activities and xylem ingestion. Electrical penetration graph waveforms were described based on amplitude, frequency, voltage level, and electrical origin of the observed traces during stylet penetration on plant tissues. The main waveforms were correlated with histological observations of salivary sheaths in plant tissues and excretion analysis, in order to determine stylet activities and their precise position. Six waveforms and associated activities are described: (S) secretion of salivary sheath and intracellular stylet pathway, (R) resting during stylet pathway, (Xc) contact of stylets with xylem vessels, (Xi) active xylem ingestion, (N) interruption within the xylem phase (during Xc or Xi), and (W) withdrawal of stylet from the plant. The sharpshooter spent 91.8% of its probing time with its stylet in the xylem, where the main activity was ingestion (Xi: 97.5%). During a probe, the most likely sequence of events is secretion of salivary sheath and pathway (S) through epidermal and parenchyma cells (all individuals), followed by contact with xylem (Xc) (67.6% of all individuals) and ingestion (Xi) (88.3% of those that exhibit waveform Xc). The mean time to contact the xylem (Xc) and initiate ingestion (Xi) after onset of the first probe was 27.8 and 34.2 min, respectively. However, sustained xylem ingestion (Xi > 5 min) was established after 39.8 min, on average. This information is basic for future studies on the transmission mechanisms of *X. fastidiosa* and in order to establish control strategies aimed at interfering with this process.

Introduction

Citrus variegated chlorosis (CVC) is a serious disease caused by the gram-negative, xylem-limited bacterium, *Xylella fastidiosa* (Wells, Raju, Hung, Weisburg, Mandelco-Paul, and Brenner) (Lee et al., 1993), which is naturally transmitted to citrus plants by xylem-feeding leafhoppers (Homoptera: Cicadellidae) of the subfamily Cicadellinae, commonly known as sharpshooters. In the State of São Paulo, Brazil's major citrus producer, over 40% of the sweet orange trees are diseased, resulting in losses of US$ 100 million per year (Lopes et al., 2004). During the 1990s, CVC spread rapidly to various citrus-growing regions in Brazil, primarily due to shipment of infected nursery trees from São Paulo and other affected areas. However, since strict regulations were implemented to produce certified healthy nursery trees inside vector-proof greenhouses,

*Correspondence: João Roberto Spotti Lopes, Departamento de Entomologia, Fitopatologia e Zoologia Agricola – ESALQ/Universidade de São Paulo, Avenue Pádua Dias, 11, Piracicaba, SP 13418-900, Brazil. E-mail: jlopes@esalq.usp.br

CVC spread between and within citrus groves has been caused mainly by the vectors (Lopes, 1999; Yamamoto & Lopes, 2004).

Of 11 sharpshooter species known to transmit *X. fastidiosa* to citrus plants (Redak et al., 2004), *Bucephalogonia xanthophis* (Berg) is considered a key vector for CVC, because of its prevalence in young citrus groves (Yamamoto et al., 2001). Despite the important role of this sharpshooter as a vector, little is known about its feeding behavior or the mechanisms involved in *X. fastidiosa* transmission to citrus trees.

The development of the electrical penetration graph (EPG) technique was a major breakthrough in the study of plant pathogen transmission by homopteran insects, whose feeding activities take place inside plants. The technique was originally devised by McLean & Kinsey (1964), by using an alternating current (AC) system. Later, Tjallingii (1978) developed a new system based on direct current (DC) that registers voltage variations caused by resistance (R) and electromotive forces (emf). With these EPG systems, detailed information on feeding behavior was obtained for over 50 homopteran species, mostly from the suborder Sternorrhyncha (Backus, 1994). The electrical penetration graph has been useful in studies on host plant resistance to sap-feeding insects (Ullman et al., 1988; Garzo et al., 2002; Alvarez et al., 2006), on evaluation of genetically modified plants (Liu et al., 2005), on the mode of action of insecticides (Nisbet et al., 1993; Harrewijn & Kayser, 1997), and on the identification of stylet activities that are critical for virus transmission by aphids (Prado & Tjallingii, 1994; Martin et al., 1997; Collar & Fereres, 1998).

In the Auchenorrhyncha, most EPG studies have been carried out with some groups of planthoppers (Delphacidae) and leafhoppers (subfamilies Deltocephalinae and Typhlocybinae). The feeding behavior of various species of these taxa has been studied in relation to host plant resistance (Kimmins, 1989; Rapusas & Heinrichs, 1990; Calderon & Backus, 1992; Mesfin et al., 1995) and virus transmission (Wayadande & Nault, 1993). More recently, feeding behavior studies have been performed on xylem-feeding leafhoppers (subfamily Cicadellinae) that are vectors of *X. fastidiosa*. This is due to the increasing impact of diseases caused by *X. fastidiosa* on cultivated crops, especially grape in USA and citrus in Brazil (Hopkins & Purcell, 2002). However, EPG information is available for only two sharpshooter species that are vectors of this pathogen in grape, *Graphocephala atropunctata* (Signoret) (Almeida & Backus, 2004) and *Homalodisca coagulata* (Say) (Backus et al., 2005; Joost et al., 2006). Waveforms associated with stylet activities of both sharpshooters were characterized by using the AC-EPG system, and the information gained is important for understanding the mechanisms of the transmission of *X. fastidiosa* in grapes.

Despite the great importance of CVC, the feeding behavior of sharpshooter vectors and its implications for transmission of *X. fastidiosa* have not been studied on citrus. Therefore, the objectives of this article were (1) to describe EPG waveforms produced by the vector *B. xanthophis* on citrus and (2) to investigate their relationship with stylet activities and xylem ingestion, based on correlations with excretion and histological information obtained from dissection of stylet-penetrated plant tissues. Unlike previous EPG studies with sharpshooters, we used the DC-EPG system in order to provide an alternative analysis of probing and feeding activities for this group of leafhoppers. The DC system has proven useful for characterizing the feeding behavior of other hoppers (Kimmins, 1989; Lett et al., 2001).

Materials and methods

Insects and plants

A healthy colony of *B. xanthophis* was maintained on plants of *Vernonia condensata* Baker (Asteraceae), as described by Marucci et al. (2003). For the EPG study, fourth and fifth instars were withdrawn from the colony and caged on healthy sweet orange seedlings, *Citrus sinensis* (L.) Osbeck cv. Pêra (Rutaceae), in an acclimatized room at 25 ± 2 °C and L14:D10 photoperiod, to complete their development. Only adult females aged 5–8 days were used in the study. Test plants used for the EPG recordings were healthy sweet orange seedlings of the same height (20–25 cm) and cultivar used to rear the adults. These seedlings were grown in 500-ml pots with a substrate of soil, bovine manure, and sand (3:1:1), inside a vector–proof greenhouse.

Electrical penetration graph

The EPG recordings were obtained using a DC-monitor, GIGA-8 model (Tjallingii, 1978, 1988), adjusted to 75× gain. The analog signal was digitalized through a Di-710 board (Dataq® Instruments, Akro, OH, USA) in a Pentium4® computer, where the data were acquired, stored, and analyzed, using the software Probe 3.0 for Windows (Laboratory of Entomology, Wageningen University, The Netherlands).

In order to establish the electrical circuit, sharpshooters and test plants were connected to electrodes of the DC monitor. The sharpshooters were anesthetized with CO_2 for 3 s and immediately immobilized under a dissection microscope, using a vacuum chamber similar to that described by van Helden & Tjallingii (2000). The tip of a 3-cm long, 37-μm diameter gold wire (Sigmund Cohn, Mount Vernon, NY, USA) was placed on the insect pronotum and glued with silver conductive paint (Ted Pella, no. 16034; Pelco® Colloidal Silver, Ted Pella, Redding, CA, USA). Before

tethering the insect, the other tip of the gold wire was attached with silver paint to a copper electrode measuring 3 cm in length × 1 mm in diameter. This electrode was connected to the EPG head stage amplifier and the tethered insect was placed on the stem of a potted citrus seedling. Another copper electrode (10 cm long × 2 mm wide) was inserted into the pot substrate. The system was assembled inside a Faraday cage, in an acclimatized room (25 ± 2 °C) with artificial light provided by six fluorescent bulbs (240 W).

Each sharpshooter was monitored for 8 h (08:00–16:00 hours) and 20 individuals were analyzed. Electrical penetration graph waveforms were described based on standard characteristics, amplitude spectrum analysis (maximum and minimum), frequency (Hz), voltage level (extra- or intracellular), and electrical origin [resistance (R) or electromotive force (emf)]. In order to determine the electrical origin, voltage adjustments to positive and negative levels were done in different periods for each waveform. Waveform amplitude and frequency were estimated based on the average of 60 observations for each waveform (three observations per recorded insect).

After determination of the typical waveform categories on citrus, some EPG parameters were calculated to describe stylet penetration by *B. xanthophis* in citrus stems during the 8-h access period: (1) number of individuals that produced a specific waveform (NPW), (2) number of times the waveform was produced per insect (NWI), (3) duration of waveform per insect (DWI), (4) time to first xylem contact from start of first probe, (5) time to first xylem ingestion from start of first probe, (6) time to first xylem ingestion >5 min (successful probe) from start of first probe, (7) time to first xylem contact from start of first successful probe, and (8) time to first xylem ingestion from start of first successful probe.

We also studied typical sequences of waveform events during stylet penetration, by calculating the likelihood of a certain waveform being followed by any other waveform type. We considered only sequences of events with probabilities higher than 2% (Wayadande & Nault, 1996; Almeida & Backus, 2004).

Plant tissue histology

In order to correlate EPG waveforms with salivary sheath termini in the plant, adult females of *B. xanthophis* were monitored on sweet orange seedlings and their probing was artificially terminated when one of the distinct EPG waveforms of interest was observed. The insects were removed from the plant and the stem sections containing the salivary sheaths were cut and treated with acid fucsin (1%) for 3 min for visualization of the salivary sheath termini. The stem sections were washed in distilled water and dried on filter paper, and then fixed in Karnovsky solution (Karnovsky, 1965) and dehydrated at room temperature in an ethanolic series (10–100%). For infiltration and blocking, the Historesin Kit (Leica, Heidelberg, Germany) was used. Serial transverse sections (10-μm thick) were cut on a rotary microtome, colored with toluidine blue (Sakai, 1973), and then mounted in synthetic resin 'Entellan' for photomicrography with a photomicroscope Leica DM LB.

Excretion analysis

Observations of excretory droplet production were carried out simultaneously with the EPG recordings of *B. xanthophis* females on sweet orange seedlings, in order to detect ingestion and to identify salivary sheath termini during the occurrence of each specific waveform. Excretion rates and chemical analysis (pH or amino acid concentration) of excreta produced during EPG recordings are useful to identify ingestion sites (e.g., phloem and xylem) (Walker, 2000).

The excretory activity of 11 insects was recorded by using a digital video camera (Sony DCR-HC21; Sony, Toyohashi, Japan) and the images were later analyzed to determine the rate of droplets produced per min. Excreted droplets were collected for chemical analysis by placing a stretched piece of parafilm® underneath the stem where the insect was feeding. Sampled droplets were analyzed for the presence of amino acids by reaction with ninhydrin (0.1%) on filter paper. For 10 insects, the pH of the excreted droplets was determined with indicator paper (Universalindikator pH; Merck, Darmstadt, Germany).

Results

Electrical penetration graph waveforms

The sharpshooter *B. xanthophis* produced six types of EPG waveforms on *C. sinensis* that are distinct from the DC-EPG waveforms described for other species of leafhoppers. These waveforms were named S, R, Xc, Xi, N, and W (Figure 1), according to their associated major behavioral activity or plant tissue, and following the naming conventions in earlier leafhopper EPG work, such as Khan & Saxena (1985), Rapusas & Heinrichs (1990), and Backus et al. (2005). The main characteristics of the observed waveforms and their correlations with stylet activities in the plant tissues are summarized in Table 1. S stands for salivation, a major activity during stylet pathway. R represents resting during stylet pathway and was named after Rapusas & Heinrichs (1990). Waveforms Xc and Xi represent xylem contact and active ingestion, respectively; the latter term was already used by Khan & Saxena (1985) to indicate xylem ingestion for a deltocephaline leafhopper. The term N was created by Backus et al. (2005) to indicate

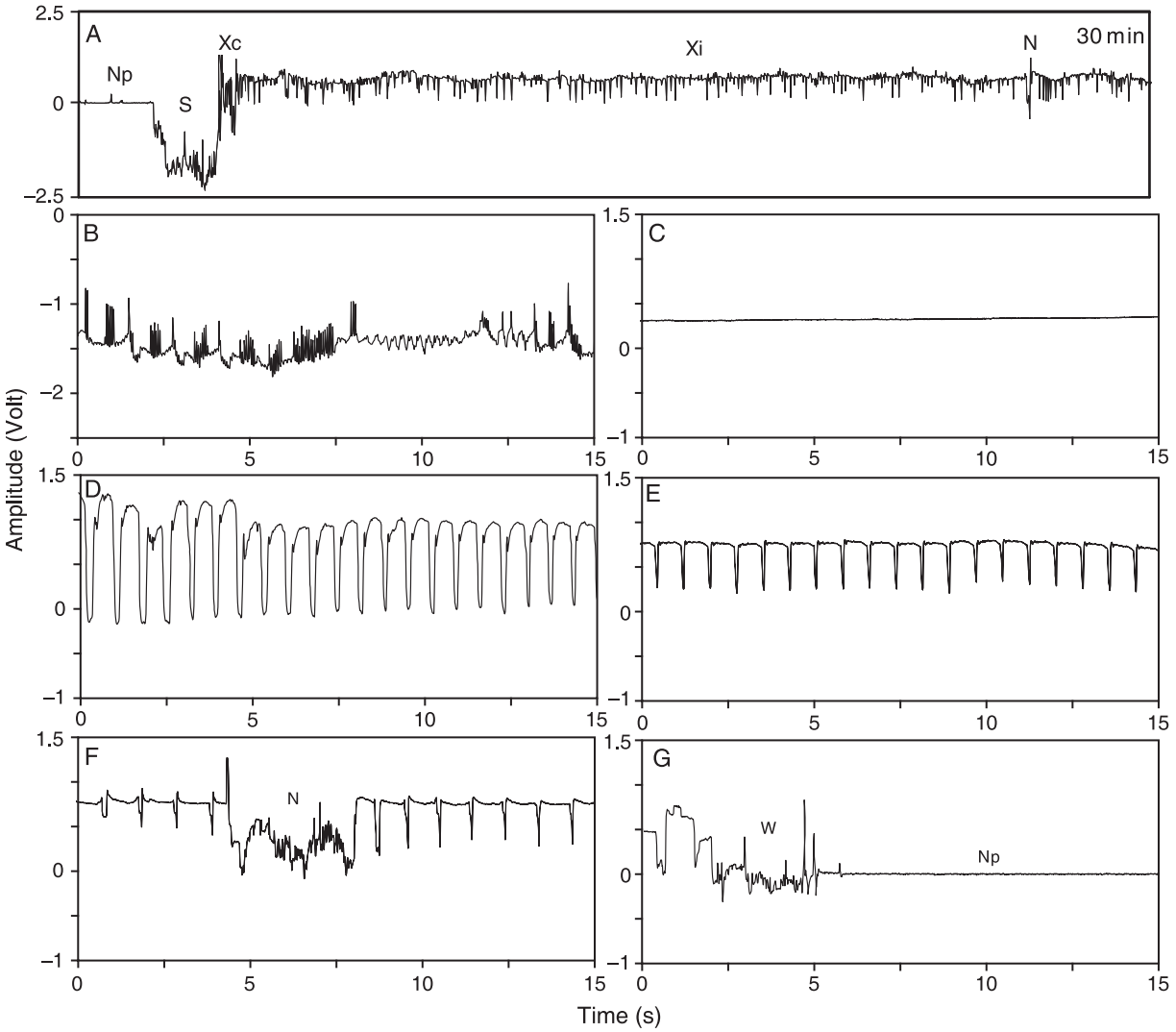

Figure 1 Electrical penetration graph waveforms produced by *Bucephalogonia xanthophis* on citrus stems. (A) Scheme of major waveforms in electrical penetration graph (EPG) record. (B) Waveform S. (C) Waveform R. (D) Waveform Xc. (E) Waveform Xi. (F) Waveform N produced inside of Xi. (G) Waveform W. Waveforms R and W are not shown in the general scheme because they were not always performed. NP, non-probing period.

an interruption in the ingestion phase for the sharpshooter *H. coagulata*. W is a new term that stands for stylet withdrawal.

No changes in waveform characteristics were observed when substrate voltage was adjusted to positive or negative, suggesting that the main electrical components of these waveforms have an electromotive force (emf) origin. Using the same procedure, Let et al. (2001) reported similar associations with emf origin for waveforms of the leafhopper *Cicadulina mbila* Naudé. An irregular waveform similar to electrical noise, but with a higher amplitude, was also observed for *B. xanthophis*; simultaneous video recording indicated that it was correlated with the insect walking on the plant. Because it was not related to probing, this irregular waveform was not considered in this study.

During the 8 h recordings, *B. xanthophis* performed an average of 5.65 ± 0.69 probes (n = 20), with a mean duration of 71.39 ± 10.29 min/probe (n = 113). Waveform S (Figure 1B) was always the first during stylet penetration in the plant. This waveform was characterized by an irregular trace, with high oscillations in frequency and amplitude (0.1–1.5 V), and always occurred at voltage levels (usually negative) lower than those observed for the other waveforms, which is indicative of intracellular penetration (Table 1). It was seen in all probes, with mean duration <2 min (Table 2). Waveform R (Figure 1C) generally occurred between two

Table 1 Summary of main characteristics and correlations of DC EPG waveforms described for *Bucephalogonia xanthophis* on citrus; emf, eletromotive force

	Waveform characteristics				Correlations		
EPG waveform	Amplitude (%)[1]	Frequency (Hz)	Voltage level[2]	Electrical origin	Stylet tips in plant tissue	Excretion	Activity
S	12.82	Mixed	i	emf	All tissues	No	Secretion of salivary sheath/intracellular penetration
R	–	0.1–0.2	e	emf	Parenchyma	No	Unknown
Xc	24.5	0.6–1.8	e	emf	Xylem	No	Unknown
Xi	15.72	0.2–2.5	e	emf	Xylem	Yes	Ingestion
N	7.2	Mixed	e/i	emf	Xylem	No	Unknown
W	–	Mixed	e/i	Unknown	All tissues	No	Stylet withdrawal

[1]Medium amplitude; 5 V = 100% amplitude.
[2]e, extracellular; i, intracellular.

S waveforms, as a regular flat trace and very low amplitude and frequency. This waveform was present in 75% of the recordings, with a mean duration of >5 min (Table 2).

Waveform Xc (Figure 1D) usually occurred between S and Xi, and it was characterized by a regular trace of relatively high amplitude peaks (0.5–2 V) compared to the other waveforms, particularly in the beginning. This waveform typically showed an extracellular (positive) voltage level and was observed in 100% of the recordings, with a mean duration of <1 min (Table 2).

Waveform Xi (Figure 1E) was also present in 100% of the recordings, always after waveform Xc, with a mean duration of >30 min (Table 2). This waveform usually had a regular trace, but variation due to oscillations occurred, usually at lower frequency at the beginning of the waveform (0.4–0.5 Hz), with a tendency to increase over time (0.8–1.3 Hz). Its amplitude range was the second highest among the waveforms described and its voltage level was positive in most cases, indicating extracellular activity (Table 1).

Waveform N (Figure 1F) represented a short potential drop that occurred within waveforms Xc and Xi, but more frequently in Xi. It was observed in all recordings, with a mean duration of <6 s (Table 2). Its voltage level indicated mainly extracellular activity, although sometimes it was negative. This waveform showed large frequency oscillations; amplitude was usually low in the beginning (0.1–0.4 V), but voltage levels sometimes increased towards the end of the waveform period (0.7–1 V).

Waveform W (Figure 1G) was present in 90% of the recordings, with a mean duration of <10 s (Table 2). It was associated with stylet withdrawal from the plant, because it was always followed by non-probing (Np) periods. However, it was not observed when the probe was artificially terminated by removing the insects abruptly from the plant. This waveform was characterized by an irregular trace with large oscillations in frequency and amplitude, but it differed from the EPG trace observed when the insect walked on the plant.

Table 2 EPG parameters used to study probing behavior of *Bucephalogonia xantophis* on *Citrus sinensis* during an 8-h access period to young stems. NPW, number of individuals that produced the specific waveform; NWI, number of times the waveform was produced per individual; DWI, duration of waveform per individual (min). Np, non-probing period

	NPW	NWI		DWI	
Waveform	(n = 20)	Mean ± SE (n = 20)	(Min–Max)	Mean ± SE (n)	(Min–Max)
Np	20	6 ± 0.71	(1–13)	11.54 ± 1.64 (120)	(0.03–100.06)
S	20	11.65 ± 1.59	(2–28)	1.58 ± 0.2 (233)	(0.05–19.23)
R	15	2.3 ± 0.58	(0–9)	6.57 ± 0.91 (46)	(0.44–25.95)
Xc	20	12.3 ± 1.35	(3–24)	0.54 ± 0.04 (246)	(0.05–4.97)
Xi	20	10.8 ± 1.1	(3–21)	33.18 ± 2.79 (216)	(0.23–245.76)
Xi <5 min	15	2.45 ± 0.56	(0–8)	2.8 ± 0.17 (49)	(0.23–4.83)
Xi >5 min	20	8.35 ± 0.73	(3–13)	42.1 ± 3.31 (167)	(5.52–245.76)
N	20	13.15 ± 1.76	(2–29)	0.09 ± 0.001 (263)	(0.05–0.20)
W	18	2.6 ± 0.42	(0–7)	0.14 ± 0.01 (52)	(0.03–0.55)

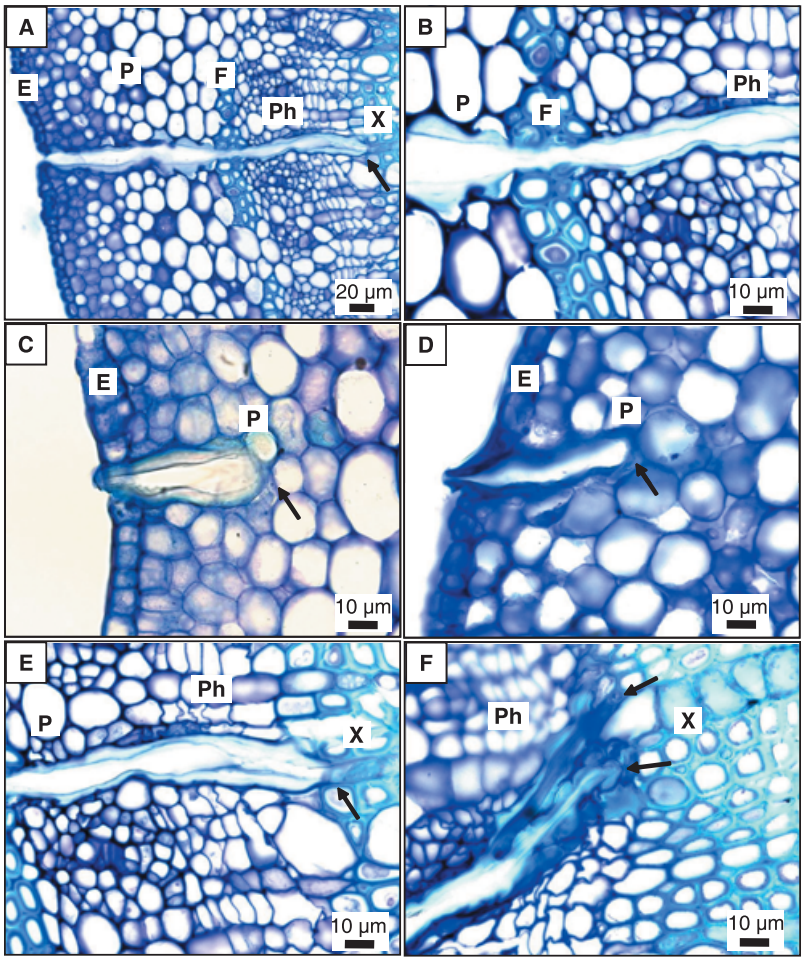

Figure 2 Cross-sections of citrus stem containing *Bucephalogonia xanthophis* salivary sheaths. (A) Salivary sheath ending in the xylem during Xc waveform (40×). (B) Detail of the cellular lyses provoked by the passage of the stylets (100×). (C, D) Salivary sheath ending in the parenchyma during S and R waveforms, respectively (100×). (E) Salivary sheath ending in the xylem during Xc waveform (100×). (F) Two branches of the salivary sheath ending in the xylem during Xi waveform (100×). E, epidermis; P, parenchyma; F, pericyclic fibers; Ph, phloem; X, xylem. Arrows indicate the end-point of the salivary sheaths.

Correlations between electrical penetration graph waveforms and salivary sheath termini in the plant

Histological analyses indicated that *B. xanthophis* initially secretes a small amount of sheath saliva on the stem surface, creating a salivary flange that marks the probing site, where stylet penetration starts through epidermal cells or (less often) stomata pores. After crossing the epidermis, the stylets move intracellularly across the parenchyma (chlorophyll-bearing and cortical), the phloem, and finally reach the xylem vessels (Figure 2A). In some samples, salivary sheaths were also observed across the pericyclic fibers, which are lignified structures that occur in small groups adjacent to the phloem. Cell lyses was clearly observed throughout the stylet pathway (Figure 2B).

Electrical penetration graph recordings were artificially terminated during waveforms S, R, Xc, and Xi for determination of salivary sheath termini within plant tissue. Sheaths produced during waveforms S ($n = 4$) and R ($n = 3$) always terminated in parenchyma cells (Figure 2C,D, respectively), whereas sheaths produced during waveforms Xc ($n = 4$) and Xi ($n = 6$) always terminated in secondary xylem vessels (Figure 2E,F, respectively). Among salivary sheaths that ended in the xylem, seven were unbranched and three had branches in the xylem tissue, with terminations inside the vessels (Figure 2F). In the case of Xc, all recordings were terminated when the waveform event was initiated for the first time in the probe. For Xi, three excisions were obtained by artificially terminating the probe a few minutes after this waveform event was observed for the first time; the other three excisions were prepared by terminating the probe a few minutes after the insects had initiated Xi for the second time (branched salivary sheaths were observed only in these latter excisions).

Correlations between electrical penetration graph waveforms and excretion

Among the waveforms described, excretion was observed only during Xi, with an average frequency of 24.54 ± 1.26 drops/min ($n = 11$). Thus, Xi is likely to represent active

Table 3 EPG sequential parameters (duration in min) used to study probing feeding behavior of *Bucephalogonia xantophis* on citrus during an 8-h access period to young stems, based on data of 20 individuals

Parameters	Mean ± SE (Min–Max)
Time to first Xc from start of first probe	27.81 ± 6.57 (2.49–99.98)
Time to first Xi from start of first probe	34.16 ± 6.89 (3.49–101.93)
Time to first Xi >5 min from start of first probe	39.75 ± 7.46 (3.49–101.93)
Time to first Xc from start of first successful probe[1]	8.81 ± 2.39 (0.28–40.40)
Time to first Xi from start of first successful probe[1]	15.71 ± 3.21 (3.49–53.48)

[1]'Successful probe' is the probe that ends in a period of xylem ingestion >5 min.

xylem ingestion. Excretion was observed only 3–5 min after the beginning of this waveform. No direct relationship was observed between number of Xi waveform peaks and number of excreted droplets. However, it was noted that an increase in the frequency of waveform Xi resulted in a higher frequency of excretion, suggesting that this frequency may be related to cibarial pump activity and active ingestion. During excretion, regular movements of the last abdominal segments were observed and a droplet was excreted after each movement. Droplets had a pH near 7 (n = 10) and no color reaction with ninhydrin, indicating that they have very low concentrations of (or no) amino acids, which suggests a xylem sap origin.

Time partitioning and sequence of events during stylet penetration

Of the 160 h of EPG recordings, *B. xanthophis* spent 15% of the time non-probing (Np), 7% on probing activities outside the xylem (waveforms S, R, and W), and 78% on probing in the xylem (Xc and Xi). Thus, this insect spent most of its probing time (92%) in the xylem, where the main activity was ingestion (Xi: 97.5% of the time). The xylem vessels were reached by the stylets after an average of 2.15 ± 0.37 probes (n = 20), although 50% of the individuals could contact this tissue in their first probe. On average, the time periods required to contact the xylem (Xc) and to initiate ingestion (Xi) after onset of the first probe were 27.81 ± 6.57 (n = 20) and 34.16 ± 6.89 min (n = 20), respectively. However, sustained xylem ingestion (Xi >5 min) was established only after 39.75 ± 7.46 min (n = 20), on average. By considering only the probes that resulted in sustained ingestion, shorter time periods were observed between the onset of stylet penetration and the performance of waveforms Xc and Xi (Table 3).

A scheme describing the likely sequence of events during a probe by *B. xanthophis* in citrus is given in Figure 3. Waveform N was not represented, because it occurs within Xc or Xi. All individuals begin stylet penetration with waveform S. Following a period in S, 18% of these individuals return to Np, 13% start waveform R, and 67.6% move to waveform Xc. Once in Xc, some individuals can return to S, but most (88.3%) start Xi. Among the insects that exhibit waveform Xi, 40.6% can restart Xc, 38.8% return to activities out of the xylem (S and R), and 20.3% withdraw their stylets from the plant (waveform W). Therefore, the most likely sequence of events during a probe by *B. xanthophis* was stylet penetration with waveform S, followed by Xc and then Xi in the xylem tissue, where this insect spends most of its probing time.

Discussion

This is the first DC-EPG study combined with histological analyses of stylet position within plant tissue and video observations of excretions for a leafhopper species from the subfamily Cicadellinae. With their anatomical adaptations, such as their swollen clypeus and many powerful muscles,

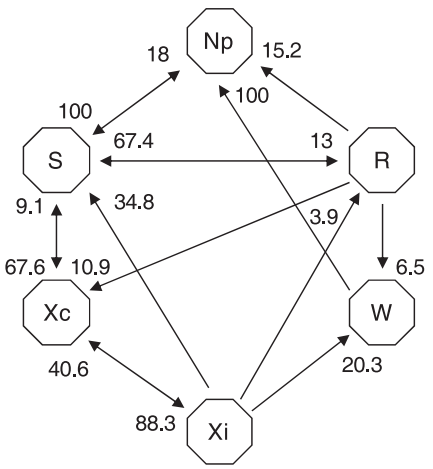

Figure 3 Transition scheme of waveform events for *Bucephalogonia xanthophis* on citrus seedlings. The values near the arrowheads correspond to the likelihood of a certain waveform being followed by any other waveform type. Probabilities <2% are not shown. Waveform N was not represented in the diagram, because it occurs within waveforms Xc or Xi.

this group of Auchenorrhyncha became specialized in xylem-sap ingestion (Nielson, 1985). These adaptations probably explain the distinct DC-EPG waveforms observed for *B. xanthophis* compared with those previously described for auchenorrhynchan groups that ingest from plant tissues other than xylem, such as planthoppers (Kimmins, 1989; Buduca et al., 1996) and leafhoppers in the subfamily Deltocephalinae (Lett et al., 2001).

Electrical penetration graph–direct current waveforms and correlations with stylet activities

Like in previous studies of sharpshooter probing behavior (Backus et al., 2005), we could easily divide *B. xanthophis* waveforms into three phases: pathway phase, ingestion phase, and interruption phase. Generally, both AC and DC-EPG types of waveforms can be similarly analyzed and compared at a compressed, coarse-structure level, a unifying principle first published in van Helden & Tjallingii (2000). We have tried to compare the DC-EPG waveform types from this study with AC-EPG waveforms from previous sharpshooter studies (e.g., Backus et al., 2005). However, correlation studies using both EPG systems (AC and DC) with the same sharpshooter and host plant species would be necessary to confirm the similarity in biological activities between such waveforms.

Waveform S

This waveform was associated with stylet pathway activities (i.e., pathway phase), during which sheath saliva secretion and stylet movement across plant tissues take place. Based on histological analyses, we verified that stylet penetration is intracellular and similar to that in other species of Auchenorrhyncha (Chang, 1978; Rapusas & Heinrichs, 1990; Spiller, 1990; Buduca et al., 1996; Backus et al., 2005). The salivary sheaths always terminated in the parenchyma when insect probing was artificially terminated during waveform S. However, this waveform also occurs in other plant tissues, because it is observed throughout stylet pathway until first contact with the xylem.

Waveform S is characterized by a potential drop compared with the other waveforms described in this study, and its voltage level was negative in most recordings. Potential drops have been well-characterized for sternorrhynchans (i.e., aphids, whiteflies, and mealybugs) along the stylet pathway, representing the insertion of the stylets into a living cell with minimal, repairable damage to the integrity of the cell membrane (Tjallingii, 1988; Janssen et al., 1989; Calatayud et al., 1994). However, this may not be the case for auchenorrhynchans. In planthoppers of the family Delphacidae, short potential drops were observed during the stylet pathway of *Nilaparvata lugens* (Stål) and at the onset of stylet penetration by *Peregrinus maidis* (Ashmead) (Spiller, 1990; Buduca et al., 1996). These planthoppers were observed to disrupt cell membranes after puncturing. Our histological observations indicated that *B. xanthophis* also severely affected the integrity of the cell membranes during waveform S. In contrast, the deltocephaline leafhopper *C. mbila* did not show any potential drop during the pathway waveform, although a waveform of active ingestion was observed in non-vascular tissues (Lett et al., 2001). Therefore, potential drop waveforms may be either present or absent in auchenorrynchan EPG recordings. If present, it is unlikely that they represent minimal membrane breakage, as in sternorrhynchans, due to the more damaging, intracellular probing behavior of these insects.

For some *B. xanthophis* individuals, an electrical noise with positive and irregular voltage was observed between Np (non-probing) and S, which was probably caused by contact and walking of the sharpshooter on the plant before stylet penetration. This noise is likely due to the robust body of *B. xanthophis*, whose mean length is 5.1 mm (Marucci et al., 2002).

In Cicadellinae, AC-EPG waveforms associated with the stylet pathway phase were characterized for *H. coagulata* on grape (Backus et al., 2005). This phase was subdivided in waveform family A, which represents the onset of stylet penetration with simultaneous secretion of the salivary sheath, and waveform family B, associated with the extension of an existing salivary sheath or formation of new ones searching for the xylem vessels. Waveform S described for the sharpshooter *B. xanthophis* in the present study is probably associated with similar behavioral activities described for the AC-waveform pathway phase by Backus et al. (2005). However, details of waveform types below pathway-phase level cannot be given at this time for *B. xanthophis*.

Waveform R

This waveform is associated with the parenchyma and shows a flat trace with an average duration longer than 5 min, without excretion. Thus, R possibly represents a resting period during stylet penetration in the plant. Similar behavior was found for the deltocephaline leafhopper *Nephotettix virescens* (Distant) (Rapusas & Heinrichs, 1990) using AC-EPG. Because this behavior has not been described in previously published sharpshooter EPG studies, we adopted the term 'R' for this waveform, following Rapusas & Heinrichs (1990).

Waveform Xc

Because xylem is extracellular apoplast, the DC-EPG waveforms recorded in this tissue show a voltage level that is typical of extracellular activity (Spiller et al., 1990). For *B. xanthophis*, the waveform Xc (xylem contact) was

usually observed with a positive voltage level and all salivary sheaths terminated in the xylem when the insects were disturbed and removed from the plant during this waveform. Therefore, Xc clearly represents the first contact between the stylets and the xylem for any waveforms we identified.

Phloem-feeding planthoppers eventually perform active ingestion from the xylem when feeding on a suitable host plant. The delphacids *P. maidis* and *N. lugens* show DC-EPG waveforms in the xylem that are similar in amplitude and frequency (Kimmins, 1989; Buduca et al., 1996). However, waveform Xc of *B. xanthophis* is not similar to the ones produced by these delphacids.

It is possible that waveform Xc represents an activity in response to a mechanical or chemical stimulus resulting from contact with xylem before initiating sap ingestion. Excretion was not observed during this short waveform, whose duration was usually less than 1 min. In EPG studies using the AC system, no xylem waveforms before active ingestion (waveform C) were observed for the sharpshooters *G. atropunctata* and *H. coagulata* (Almeida & Backus, 2004; Backus et al., 2005). Nevertheless, their waveform B1, representing the last waveform of the pathway phase in *H. coagulata*, was sometimes correlated with xylem contact (Backus et al., 2005). Hence, the waveform Xc that we observed for *B. xanthophis* may represent some unknown activity of Cicadellinae that is not detected by the AC system. Further studies will be important to determine the biological activities that occur during this waveform.

Waveform Xi

Xi was always preceded by Xc, staying at a similar voltage level. The histological observations showed that salivary sheaths end in the xylem when the Xi waveform is artificially terminated. This waveform was usually observed for long periods of time and was frequently associated with excretion. Considering that the xylem tension is negative (Holbrook et al., 1995), Xi represents active ingestion of xylem sap, mediated by cibarial pump activity.

For the sharpshooters *G. atropunctata* and *H. coagulata*, active xylem ingestion was associated with the AC-EPG waveform C (Almeida & Backus, 2004; Backus et al., 2005). For the latter species, salivary sheath termini were observed in the xylem in probes artificially terminated during this waveform (Backus et al., 2005). Based on the histology and excretion correlations, waveform Xi described here for *B. xanthophis* can be considered equivalent to waveform C reported for other sharpshooters using the AC system. Thus, it can be inferred that the ingestion phase occurs in the same location for both AC and DC waveforms.

As observed for Xc, Xi amplitude and frequency characteristics are different from those DC-EPG waveforms recorded during active ingestion by delphacid planthoppers. For *B. xanthophis*, a frequency range of 0.2–2.5 Hz is observed during Xi, whereas for *P. maidis* and *N. lugens* the frequency of active ingestion waveforms is around 3–7 Hz (Kimmins, 1989; Buduca et al., 1996). The differences in DC-EPG waveforms observed during xylem-sap ingestion between *B. xanthophis* and these other planthoppers are possibly associated with differences in size and/or feeding site; Cicadellinae and Delphacidae are distantly related groups within the Auchenorrhyncha, whose species differ in body size and primary ingestion tissues of their host plants. Cicadelline species are generally larger and are specialized in xylem ingestion, whereas delphacids are usually phloem-ingesting (Sogawa, 1982; Nielson, 1985).

Xylem-ingesters, such as sharpshooters, are able to survive on a nutritionally poor food source. These insects usually show high conversion efficiency for all organic components (>98%) and they ingest high amounts of sap (100–300 times their body dry weight per day) (Brodbeck et al., 1993). *Bucephalogonia xanthophis* was shown to be a typical xylem feeder, spending most of its probing time on active xylem ingestion (Xi). The excreta produced during Xi did not contain amino acids and the frequency of excretion was ≈24 droplets/min. This high excretion rate and the lack of detectable levels of amino acids in the excreta are indicative of xylem sap ingestion, which has low concentrations of organic compounds (Andersen et al., 1989).

Backus et al. (2005) observed that the sharpshooter *H. coagulata* ingests almost exclusively from xylem (93%), although once their waveform C was correlated with phloem ingestion. DC-EPG waveforms for phloem ingestion are very characteristic and somewhat similar for distantly related groups of plant-sap-sucking homopterans. Phloem-related waveforms characterized for *C. mbila* are considered similar to those determined for aphids (Lett et al., 2001). A similar phloem-ingestion waveform was not observed for *B. xanthophis* in the present study, although its stylets tend to cross this tissue before reaching the xylem.

Waveform N

This waveform represents a brief interruption (≈5 s) within the xylem ingestion waveform. It was observed mainly within Xi, and occasionally in Xc or between Xc and Xi. For the sharpshooter *H. coagulata*, two similar waveforms were observed during the ingestion phase (waveform C) using the AC-EPG system: non-pathway N, which is possibly associated with secretion of watery saliva in the xylem, and pathway-like N (similar to pathway waveforms), which might represent a search for a new ingestion site within the xylem tissue (Backus et al., 2005).

For *B. xanthophis*, waveforms Xc and Xi are also occasionally interrupted by a short waveform S, with subsequent return to typical xylem activities. This behavior may represent a shift to a different vessel; this hypothesis is corroborated by the histological observation of a salivary sheath with two distinct branches ending in the xylem (Figure 2F). Thus, the short waveform S might correspond to the pathway-like N waveform observed for *H. coagulata*, whereas the interruption waveform could be equivalent to non-pathway N. The fact that both AC and DC waveform types represent an interruption within the ingestion phase in the same plant tissue (xylem) supports their similarity. Therefore, we decide to use the same designation N (Backus et al., 2005) for the interruption waveform observed for *B. xanthophis*.

Waveform W

This short and irregular waveform was observed only when the insect ended its probe. It is possibly associated with salivary sheath filling, which occurs when the insect naturally withdraws the stylets from the plant. When the probing process is artificially terminated, sharpshooters tend to withdraw their stylets without filling the sheath terminations with saliva (Chang, 1978; Lett et al., 2001; Backus et al., 2005). This was also observed for *B. xanthophis* in the present study; probes that were artificially terminated for the histological observations showed hollow sheaths and no W waveform was observed.

Characteristics of stylet penetration of *Bucephalogonia xanthophis* on citrus

Studies using conditional probability have been done in the past aiming to understand the sequence of events that occurs during stylet penetration by homopterans (Ullman & McLean, 1988; Wayadande & Nault, 1996; Almeida & Backus, 2004). For deltocephaline leafhoppers, it was observed that selection of ingestion sites does not occur casually; a typical series of behavioral events eventually leads to phloem ingestion (Wayadande & Nault, 1996). For *B. xanthophis*, we also verified a sequence of events with high probability of occurrence during a probe, leading to xylem sap ingestion. The most likely sequence was stylet penetration through epidermis and parenchyma (waveform S), followed by xylem contact (Xc) and then ingestion (Xi), where this insect spends most of its probing time (97.5%).

Usually, *B. xanthophis* does few probes before getting into the xylem, taking around 30 min to start ingestion after the onset of the first probe. Nevertheless, by considering only the probes that resulted in sustained xylem ingestion, the mean time to start ingesting dropped by half, and for several individuals it was even shorter than 4 min. During certain probes, the insects may exhibit only Xc or Xi waveforms for just a very short period (Xi <5 min). This may occur because the vessel is unsuitable for the insect to accept and continue ingesting. In such situations, the sharpshooter starts a new pathway phase or just withdraws its stylets from the plant. Probes with short periods of xylem sap ingestion were also observed for *G. atropunctata* and *H. coagulata* (Almeida & Backus, 2004; Backus et al., 2005).

The information obtained on stylet penetration of the vector *B. xanthophis* on citrus should serve as a basis for advanced studies on transmission mechanisms of *X. fastidiosa* and development of more effective methods to interfere with this process in order to reduce disease spread in citrus groves. We described the main EPG-DC waveforms associated with stylet pathway and activities in the xylem, where acquisition and inoculation of *X. fastidiosa* is supposed to occur. Further studies should be carried out to determine which EPG waveforms or stylet activities are involved in acquisition and inoculation of *X. fastidiosa*, as well as to estimate time thresholds for vector transmission of this pathogen in citrus.

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