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Feeding behavior of *Diaphorina citri* (Hemiptera: Liviidae) and its acquisition of '*Candidatus* Liberibacter asiaticus', on huanglongbing-infected *Citrus reticulata* leaves of several maturity stages

Xiaozhu Luo[1], Alan L Yen[2, 3], Kevin S Powell[4], Fengnian Wu[1], Yanjing Wang[1], Lixia Zeng[1], Yuzhi Yang[1] and Yijing Cen[1,]*

Abstract

The Asiatic citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the main vector of the Asiatic form of huanglongbing (HLB), the putative cause of which is a phloem-limited bacterium '*Candidatus* Liberibacter asiaticus' ('*C*Las') (α-Proteobacteria). Nymphal and adult *D. citri* prefer to feed on young leaves of their host plants. Adults feeding on mature leaves are not considered very important by some Chinese farmers in the management of HLB. This study examined feeding by adult *D. citri* on '*C*Las'-infected citrus leaves of several maturity stages. *Diaphorina citri* adults from a '*C*Las'-free colony were tested for feeding behavior and the efficiency of their acquisition of '*C*Las' from new shoots, and young and mature leaves. Probing and feeding behavior were monitored using the electrical penetration graph (EPG) technique, and pathogen acquisition efficiencies were tested by qPCR. The results showed that some EPG variables were significantly influenced by host-plant leaf maturity. The duration of waveform C (pathway phase) on new shoots was significantly longer than that on young leaves and mature leaves. In contrast, the duration of waveform E2 (phloem ingestion) was significantly shorter on new shoots and young leaves than on mature leaves. However, the duration taken for stylets of adult *D. citri* to reach the phloem and commence ingestion was not related to leaf maturity status. The qPCR results indicated that 23 of the 24 adults for which E2 waveforms were recorded harbored '*C*Las'. In addition, the minimum period of E2 waveform of these individuals was only 2 min. Proportions of '*C*Las'-positive adults feeding on mature leaves, young leaves and new shoots, were 55%, 40% and 35%, respectively. The main EPG variables were not significantly different between the males and females. Our results suggest that the acquisition of '*C*Las' by adult *D. citri* is highly efficient, even when feeding on mature leaves. Therefore to effectively manage both vector and pathogen, *D. citri* populations should be monitored carefully, even when the trees stop producing new growth.

Key Words: Asian citrus psyllid; HLB; leaf age; electrical penetration graph; pathogen acquisition; citrus

Resumen

El psílido de los cítricos asiático, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), es el vector principal de la forma asiática de Huanglongbing (HLB), la supuesta causa de que es una bacteria limitada al floema '*Candidatus* Liberibacter asiaticus' ('*C*Las') (α-Proteobacteria). Las ninfas y adultos de *D. citri* prefieren alimentarse de las hojas jóvenes de sus plantas hospederas. Los adultos que se alimentan de las hojas maduras no son considerados muy importantes por parte de algunos agricultores chinos en cuanto al manejo de HLB. Este estudio examinó la alimentación de adultos de *D. citri* en hojas de cítricos de varios estados de madurez. infectadas con '*C*Las'. Se probaron adultos de *Diaphorina citri* de una colonia libre de '*C*Las' para su comportamiento de alimentación y su eficiencia para la adquisición de '*C*Las' en nuevos brotes y hojas jóvenes y maduras. Se realizó un monitoreo del comportamiento de prueba de plantas y su alimentación por medio de la técnica Gráfico de Penetración Eléctrica (GPE), y la eficiencia de adquisición de patógenos fueron probados por qPCR. Los resultados mostraron que algunas variables de GPE influyó significativamente en la madurez de la hoja de la planta hospedante. La duración de la forma de onda C (fase de vía) en nuevos brotes fue significativamente más larga que en las hojas jóvenes y hojas maduras. En contraste, la duración de la forma de onda de E2 (ingestión floema) fue significativamente más corta en nuevos brotes y hojas jóvenes que en las hojas maduras. Sin embargo, la duración tomada por las estiletes de adultos de *D. citri* para llegar al floema y comenzar la ingestión no fue relacionada con el estado de madurez de la hoja. Los resultados de qPCR indicaron que 23 de los 24 adultos en que registraron formas de onda E2 tenian '*C*Las'. Además, el período mínimo de forma de onda de E2 de estos individuos fue sólo 2 minutos. La proporcióon de los adultos '*C*Las ' positivos se alimentan de las hojas maduras, hojas jóvenes y nuevos brotes, fue 55%, 40% y 35% respectivamente. Las principales variables de GPE no fue-

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ron significativamente diferentes entre los machos y hembras. Nuestros resultados sugieren que la adquisición de '*C*Las'por adultos de *D. citri* es muy eficiente, incluso cuando se alimentan de las hojas maduras. Por lo tanto, para el manejo eficaz de poblaciones del vector y patógenos de *D. citri*, se debe realizar un monitoreo cuidadoso, incluso cuando los árboles dejan de producir un nuevo crecimiento.

Palabras Clave: sílido asiático de los cítricos; HLB; edad de hoja; gráfico de la penetración eléctrica; adquisición de patógenos, cítricos

The Asiatic form of huanglongbing (HLB) is the most destructive disease of citrus and is a threat to the industry worldwide (Bové 2006). It is associated with a phloem-limited bacterium '*Candidatus* Liberibacter asiaticus' ('*C*Las') (α-Proteobacteria), which is most commonly transmitted in Asia and the Americas by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Sternorrhyncha: Liviidae) (Bové 2006; Grafton-Cardwell 2013). In China, the pomelo psyllid, *Cacopsylla citrisuga* Yang and Li (Psyllidae) has also been recorded as a vector of '*C*Las' (Cen et al. 2012a, 2012c).

The first electrical monitoring system, the electrical penetration graph (EPG), for recording feeding behavior of Hemipteran insects was designed by McLean & Kinsey (1964) using an AC system. Tjallingii (1978, 1985) improved this system, and invented the DC-EPG system which has been widely used for Aphididae. The EPG technique has more recently been adapted to characterize feeding behavior of the European pear psylla (*Cacopsylla pyri* (Förster)) (Civolani et al. 2011, 2013), the tomato-potato psyllid (*Bactericera cockerelli* (Sulc) (Triozidae) (Pearson et al. 2010, 2014; Sandanayaka et al. 2011, 2013, 2014; Butler et al. 2012) and Asian citrus psyllid (Bonani et al. 2008, 2010; Yang et al. 2011; Cen et al. 2012b; Ammar et al. 2013; Serikawa et al. 2011, 2012, 2013).

Bonani et al. (2010) characterized the main EPG waveforms of *D. citri* adults probing and feeding on 'Pera' sweet orange (*C*. × *aurantium* L.) leaves. Other studies characterized both a 'walking' and a stationary 'non-probing' waveform exhibited by *D. citri* (Serikawa et al. 2011; Youn et al. 2011). Cen et al. (2012b) compared the EPG waveforms of *D. citri* adults feeding on healthy and '*C*Las'-infected citrus leaves, they found that *D. citri* adults spent more time searching for suitable feeding sites on severely infected leaves in contrast to pathogen-free and less severely infected leaves. Additionally, the duration of xylem feeding was significantly longer, but the duration of phloem feeding was markedly shorter on infected plants than on healthy plants (Cen et al. 2012b). The presence and physical location of stylet tracks in leaves has also been described in studies on the feeding behavior of *D. citri*: Yang et al. (2013) found that adult *D. citri* preferred to feed on immature in preference to mature calamondin (*Citrus* × *microcarpa* Bunge) leaves on healthy plants. Ammar et al. (2013) found that changes in stylet morphometrics of *D. citri* nymphs and adults feeding on 'Valencia' orange leaves were affected by impacts of '*C*Las' on citrus leaf vein structure.

In this study we used the DC-EPG technique to compare the feeding behavior of *D. citri* adults on HLB-infected leaves within 3 leaf-maturity categories. Acquisition efficiencies of '*C*Las' by these adults were tested by qPCR, and the relationship between '*C*Las' acquisition and probing behavior (EPG waveforms) on host leaves of several maturity stages was analyzed. The result will provide information for improved control measures for HLB and *D. citri*, especially when the leaves express relatively mild symptoms.

Materials and Methods

INSECTS AND PLANTS

A *D. citri* colony was sourced from *D. citri*-infested and '*C*Las'-free orange jasmine (*Murraya exotica* L.) plants on the campus of South China Agricultural University (SCAU; N 23°09' E 113°20'), Guangzhou, Guangdong Province, China. The colony was maintained for several generations on potted *M. exotica* in a controlled environment facility at 25 ± 1 °C, 60 ± 5% RH and a photoperiod of 14:10 h L:D. The colony was confirmed to be '*C*Las'-free through multiple sampling tests by qPCR. Each test included 30 samples, taken once per month; methods are described below. Adult insects were put in plastic tubes and keep starvation 4 h before the EPG test.

HLB-free, 2-year-old *Citrus reticulata* Blanco 'Sunki' (syn. *C. sunki* (Hayata) Hort. ex Tanaka) plants were grafted with buds from HLB-infected *C. reticulata* 'Shatangju' trees produced by the Citrus Huanglongbing Research Laboratory, SCAU. After 12 months, the diseased buds were removed. At this point the *C. reticulata* 'Sunki' plants exhibited symptoms of HLB and tested positive for '*C*Las' by nested PCR (methods are described below). The leaves were classified in 1 of the 3 maturity stages: (1) soft and light newly developed green shoots; (2) immature, soft, fully expanded and symptomless leaves; and (3) firm, fully expanded, HLB-symptomatic mature leaves (Fig. 1).

EPG DATA COLLECTION

A Giga-4 DC-EPG system (Wageningen University, The Netherlands) was used to record the feeding activities of adult *D. citri*. The EPG recordings were analyzed using Stylet version 01.20 software (EPG Systems, Wageningen, The Netherlands). After being immobolized in a refrigerator (-20 °C) for 30 s (Tang et al. 2011), the insects were immediately attached to a gold wire electrode with silver glue (EPG Systems, Wageningen, The Netherlands). Tethered psyllids were settled on the midrib of the leaf's abaxial surface. The plant electrode was inserted into the soil. The EPG experiments were conducted within a Faraday cage in a climate controlled laboratory at 25 ± 1 °C, 60 ± 5% RH during a 4 month period.

The EPG data was continuously recorded for 8 h. The waveforms recorded for *D. citri* probing behavior were characterized according to previous reports (Bonani et al. 2010; Yang et al. 2011). A total of 20 adult psyllids (10 males and 10 females) were allowed to feed on leaves of each maturity stage. Four typical waveforms: pathway phase, C; phloem salivation, E1; phloem ingestion, E2; xylem phase, G were recorded.

For the purposes of this study 4 key EPG variables were measured and analyzed (i.e., duration of pathway phase, C; duration of xylem

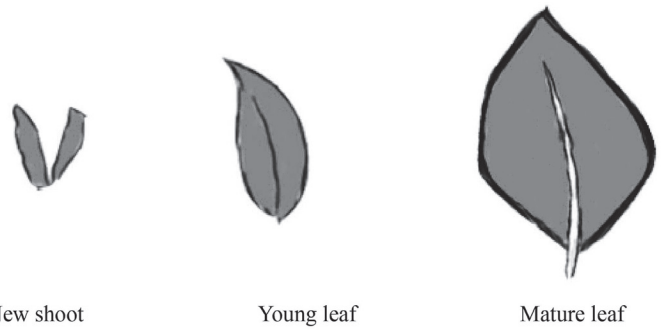


Fig. 1. Sketches of citrus leaves at several maturity stages.

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phase, G; duration of phloem salivation, E1; and the duration of phloem ingestion, E2). Waveform C was changeable (variable in amplitude and repetition rate) in EPG, but the other 3 measured waveforms (E1, E2, G) were typical of *D. citri* feeding on HLB-infected leaves (Fig. 2).

EXTRACTION OF PLANT AND INSECT DNA

DNA samples were extracted according to the manufacturer's instructions. Thus DNA was extracted from ca. 200 mg of symptomatic leaf tissue of 20 citrus leaves using E.Z.N.A.™ Plant DNA Kit supplied by OMEGA Company, Norcross, Georgia, USA. DNA of psyllids was extracted by a TIANamp Genomic DNA Kit provided by Tiangen Biotech (Beijing) Co., Ltd, Beijing, China.

MOLECULAR DETECTION OF PATHOGEN

Nested-PCR (Harakava et al. 2000) was used for detection of '*C*Las' in the citrus plants. The primer 1500R/27F was used for the first amplification, and OI1 /OI2c was used for the second amplification (Jagoueix et al. 1994). QPCR was used to detect '*C*Las' in the psyllid adults, using SYBR Green 1 Master Mix. The primers used in qPCR were HLBasf/HLBasr (Li et al. 2006). One µL DNA from plants and psyllids was used in the PCR tests. A samples was considered to be *C*Las-positive if 2/3 or 3/3 sample triplicates returned positive results.

STATISTICAL ANALYSIS

Statistical analysis was done according to previous EPG studies (Cen et al. 2012b; Sandanayaka et al. 2014). Before analysis, the homogeneity of the variances was checked using Levene's test. If the variances were not homogeneous, they were subjected to log or arcsine transformations to remove heteroscedasticity. The EPG variables were calculated by Microsoft Office Excel 2007 (Microsoft, San Francisco, California, USA), and the data were analyzed with SPSS 17.0 statistical software (SPSS Inc., Chicago, Illinois, USA). EPG variables between female and male *D. citri* were compared using a 2-tailed non-parametric Wilcoxon test ($\alpha = 0.05$). Duncan's multiple range test was used to compare EPG variables of *D. citri* at several leaf maturity stages ($\alpha = 0.05$).

Results

LEAF MATURITY STAGE AND *D. CITRI* FEEDING BEHAVIOR

The EPG waveform characteristics of pathway, phloem and xylem probing events are shown in Fig. 3A. On the HLB-infected plants, the pathway and xylem phases accounted for a large proportion of all feeding activities. The average duration of the pathway phase (C) on new shoots was significantly longer than on young leaves and mature leaves. In contrast, the average durations of the phloem phases (E1 and E2) on new shoots and young leaves were significantly shorter than on mature leaves, and the duration of the xylem phase (G) was longer on mature leaves than that on shoots and young leaves.

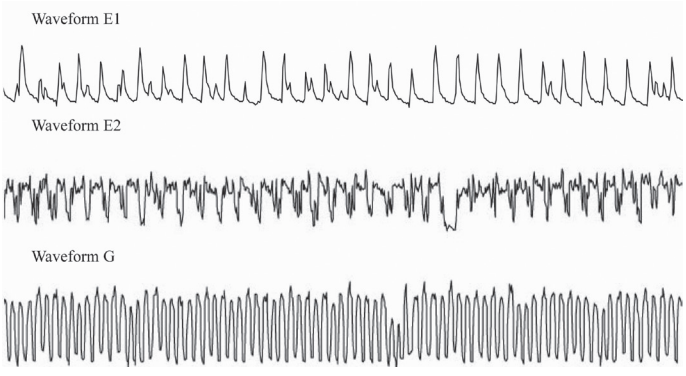


Fig. 2. Visual representation of EPG waveforms E1 (phloem salivation), E2 (phloem ingestion) and G (xylem ingestion) produced by adult *Diaphorina citri* feeding on HLB-infected *Citrus sunki* leaves.

The average durations of phloem salivation (E1) before phloem ingestion (E2) at the 3 maturity stages were in the following increasing order: new shoots < young leaves < mature leaves. Although the results suggest that the duration of phloem salivation increased with leaf maturity, there was no significant difference related to leaf age (Fig. 3B).

In contrast, the percentage of salivation in total phloem activities was significantly smaller on mature leaves than on young leaves and new shoots (Fig. 3C). However the durations of phloem salivation and ingestion on mature leaves were significantly longer than on new shoots (Fig. 3B).

The average time span from the start of probing to the first phloem salivation was more than 4 h, and the time span from the start of probing to the first phloem ingestion was 4.5 h approximately. There were no significant differences exhibited in these 2 variables for different leaf maturity stages. The results demonstrated that *D. citri* adults spent a relatively long time before commencing the phloem phase on HLB-infected hosts, regardless of leaf maturity stage (Fig. 3-D).

Whether or not followed by phloem ingestion, the numbers of phloem salivation events did not differ significantly among different leaf maturity stages. The data indicated that phloem salivation happened frequently across all infected plants (Fig. 3-E). On the other hand, the percentage of *D. citri* adults with sustained phloem ingestion (E2 > 2 min) was less than 50% (26/60). Moreover, only a few *D. citri* adults showed sustained E2 when they reached the phloem for the first time (4/60).

DIAPHORINA CITRI GENDER AND *D. CITRI* FEEDING BEHAVIOR

Selected EPG variables closely related to the insect feeding behavior, host plant suitability and HLB disease transmission were chosen to compare the feeding behaviors between the 2 genders. The results showed that there were no significant differences between probing and feeding behavior of female and male adults with respect to leaf maturity ($P > 0.05$) (Table 1).

RELATIONSHIP BETWEEN ACQUISITION OF '*C*LAS' AND EPG WAVEFORMS

Of the 60 *D. citri* individuals monitored using EPG, 24 (40%) were characterized as having ingested phloem. Twenty of these 24 individuals also fed on xylem. All 24 phloem feeding adults were tested individually for '*C*Las' using real-time PCR. PCR showed that 23 out of the 24 individuals, which had ingested phloem were '*C*Las' positive. In contrast, only 2 out of the 36 individuals that did not ingest phloem were '*C*Las' positive. One individual fed on young leaves, another on new shoots. Both of these individuals recorded short-duration phloem salivation (Table 2). In addition, the shortest period of the E2 waveform of these *C*Las-positive individuals was only 2 min, and the longest period of the E2 waveform was 257 min.

Diaphorina citri acquisition of '*C*Las' from different leaf maturity stages of infected citrus was also analyzed. The numbers of *D. citri* individuals with phloem ingestion were 11, 7 and 6 on mature leaves, young leaves and new shoots, respectively. And the numbers that acquired '*C*Las' from mature leaves, young leaves and new shoots were

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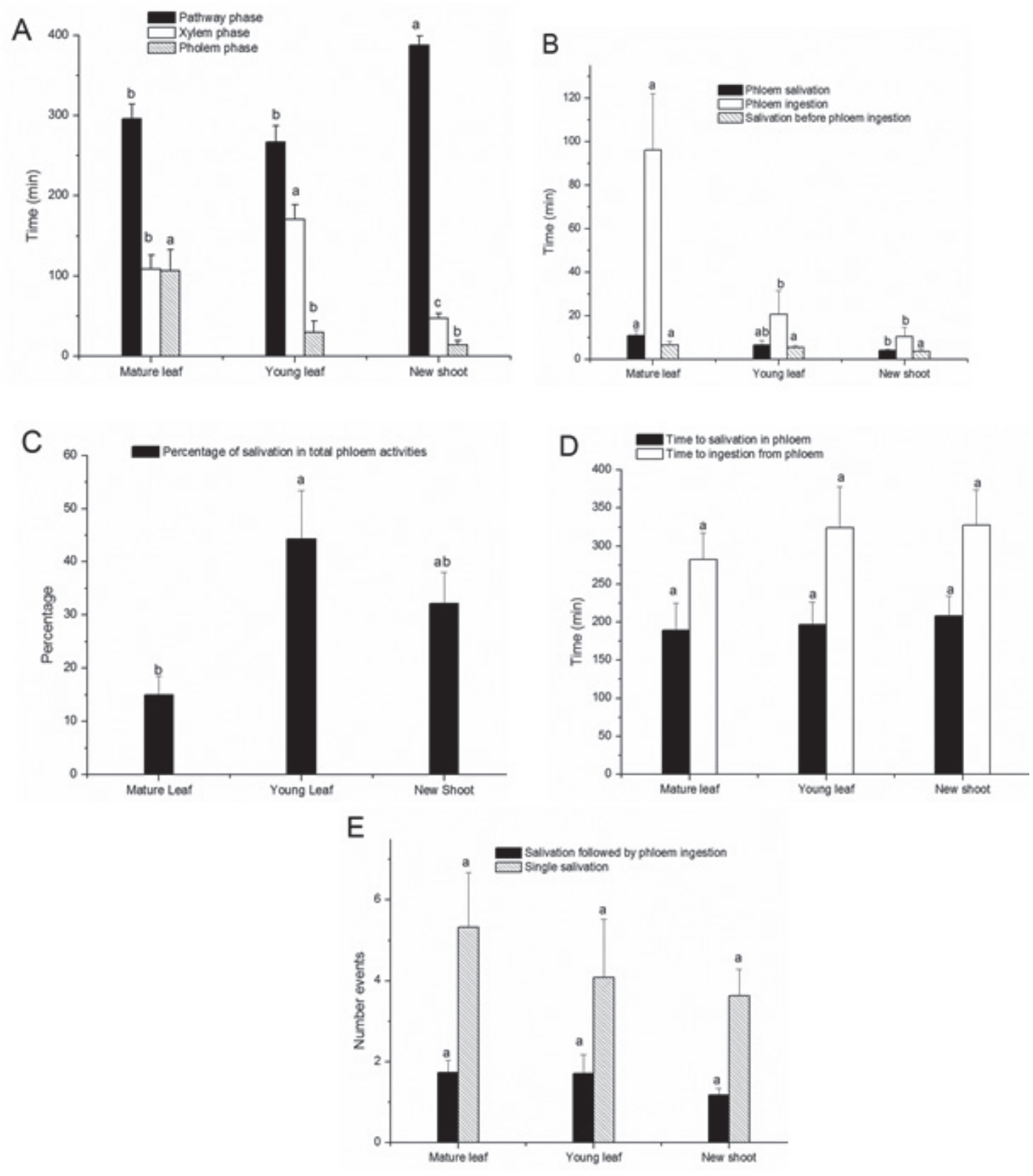


Fig. 3. The major EPG variables of *Diaphorina citri* adults feeding on citrus leaves of several maturity stages. Mean (+SE) (A) durations of pathway phase (C), xylem phase (G) and phloem phase (E1+E2) (B) durations of phloem salivation (E1), phloem ingestion (E2) and salivation before phloem ingestion (E1 before E2) (C) percentage of salivation in total phloem activities (E1/E1+E2) (D) time from start to first salivation in phloem (Time to first E1) and to the first ingestion from phloem (Time to first E2) and (E) numbers of single E1 (single salivation) and E1 followed by E2 (salivation followed by ingestion). Within the same series, means capped by the same letter are not significantly different according to Duncan's Multiple Range test ($P > 0.05$).

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Table 1. The main EPG variables of female and male adult *Diaphorina citri* feeding on *Citrus sunki* leaves of several maturity stages.

	Mature leaf		Young leaf		New Shoot	
EPG Variable[1]	Female	Male	Female	Male	Female	Male
Duration of C (min)	316.90 ± 22.42 n = 10	274.90 ± 28.59 n = 10	281.50 ± 25.29 n = 10	253.20 ± 31.25 n = 10	382.20 ± 16.97 n = 10	392.90 ± 16.44 n = 10
Duration of G (min)	83.29 ± 20.17 *n* = 7	127.67 ± 26.23 n = 9	165.20 ± 27.33 n = 10	176.11 ± 26.26 n = 9	52.75 ± 7.47 n = 8	41.25 ± 9.33 n = 8
Duration of E1 (min)	6.56 ± 2.22 n = 9	15.75 ± 4.96 n = 8	5.50 ± 1.39 n = 8	8.50 ± 5.84 n = 4	3.63 ± 1.21 n = 8	4.33 ± 0.71 n = 9
Duration of E2 (min)	105.00 ± 44.27 n = 6	84.80 ± 27.03 n = 5	13.75 ± 10.43 n = 4	30.00 ± 23.07 n = 3	8.00 ± 4.04 n = 3	12.67 ± 8.67 n = 3
Duration of E1 before E2 (min)	4.83 ± 1.58 n = 6	8.60 ± 2.94 n = 5	6.25 ± 1.11 n = 4	4.20 ± 1.91 n = 3	2.67 ± 1.20 n = 3	4.63 ± 1.59 n = 3
Time to E1 (min)	216.78 ± 50.43 n = 9	158.50 ± 50.64 n = 8	201.13 ± 38.82 n = 8	187.75 ± 48.90 n = 4	224.75 ± 40.19 n = 8	193.00 ± 36.33 n = 9
Time to E2 (min)	280.67 ± 56.97 n = 6	284.80 ± 42.19 n = 5	318.75 ± 68.00 n = 4	331.33 ± 105.79 n = 3	277.00 ± 90.56 n = 3	377.33 ± 11.55 n = 3
No. of E1 followed by E2 events	1.17 ± 0.17 n = 6	2.40 ± 0.51 n = 5	1.75 ± 0.75 n = 4	1.67 ± 0.67 n = 3	1.33 ± 0.33 n = 3	1.00 ± 0.00 n = 3
No. of single E1 events	3.00 ± 0.76 n = 7	7.38 ± 2.27 n = 8	5.00 ± 2.12 n = 8	2.25 ± 0.75 n = 4	2.43 ± 0.61 n = 7	4.56 ± 1.00 n = 9
E1/(E1+E2) (%)	12.71 ± 6.73 n = 5	16.85 ± 3.90 n = 6	56.27 ± 12.23 n = 4	28.34 ± 7.69 n = 3	26.95 ± 1.04 n = 3	37.42 ± 12.03 n = 3

Data in the table are mean (durations of events) ± SE. Values in the same row not followed by the * are not significantly different at the 5% level by non-parametric Wilcoxon test (mean values were compared on each leaf maturity stage respectively).

[1]C, pathway phase; E1, phloem salivation; E2, phloem ingestion; G, xylem phase

11, 8 and 7, respectively. Consequently both the rate of occurrence of phloem ingestion and '*C*Las' acquisition were highest on mature leaves.

Discussion

During normal leaf senescence chemical and physical changes occur in the leaf. Chlorophyll, lipid, nucleic acid and soluble protein content decrease with the changes of leaf physiology and biochemistry, and the incidence of starch granules in the sieve tubes increases (Ma 2007). Leaf tissues, particular phloem, are also damaged in citrus leaves as a consequence of '*C*Las' infection. This leads to abnormally large amounts of starch accumulation in the cell. Hence, psyllids have to make more saliva during the feeding process (Schneider 1968; Kim et al. 2009). Thus, the process of leaf senescence in HLB-infected mature leaves results in more complex physical and chemical processes than in infected young leaves or newly emerged shoots.

Walker (1985) reported that the cuticle of mature lemon (*C.* × *limon* (L.) Osbeck) leaves was much thicker than the cuticle of young lemon leaves and suggested that this may have impeded stylet penetration of the bayberry whitefly (*Parabemisia myricae* (Kuwana)) (Sternorrhyncha: Aleyrodidae). Our results showed that intervals from the beginning of EPG recording to the first phloem salivation and ingestion were not significantly different. These results are similar to those of a previous study by Cen et al. (2012b) who reported that the duration of events when *D. citri* adults fed on HLB-infected plants were significantly longer than for adults feeding on '*C*Las'- free controls. This may be attributed to structural changes caused by HLB infection, thus leading to prolonging the duration of searching for suitable feeding sites, regardless of leaf age.

In our study, within an 8-h period, *D. citri* spent a relatively long time probing before the first phloem salivation and ingestion, and salivated frequently prior to phloem ingestion. This behavior is similar to that in

Table 2. Relationship between probing behavior of *Diaphorina citri* and acquisition of *C*las when feeding on *Citrus sunki* leaves of several maturity stages.

	Individuals that performed these activities			*C*Las detection	
Waveform activities	Mature leaf	Young leaf	New shoot	Positive	Negative
C, G, E1, E2	40% (8/20)[1]	35% (7/20)[1]	25% (5/20)[1]	95% (19/20)[2]	5% (1/20)[3]
C, E1, E2	15% (3/20)[1]	—	5% (1/20)[1]	100% (4/4)[2]	—
C	—	5% (1/20)[1]	—	—	100% (1/1)[3]
C, G	15% (3/20)[1]	35% (7/20)[1]	15% (3/20)[1]	—	100% (13/13)[3]
C, E1	5% (1/20)[1]	—	10% (2/20)[1]	—	100% (3/3)[3]
C, G, E1	25% (5/20)[1]	25% (5/20)[1]	45% (9/20)[1]	10.5% (2/19)[2]	89.5% (17/19)[3]

[1]No. of *D. citri* that performed these activities/total no. of *D. citri* analyzed.
[2]No. of *C*Las positive *D. citri*/total no. of *D. citri* that performed these activities.
[3]No. of *C*Las negative *D. citri*/total no. of *D. citri* that performed these activities.

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a previous report by Cen et al. (2012b) who found that '*C*Las'-infection prolonged the duration between initial probing and commencement of phloem activities. The average duration of salivation before phloem ingestion was notably shorter, albeit not statistically so, on new shoots than mature leaves, which may indicate that it is easier for *D. citri* adults to locate phloem in the tender leaf tissues. *Diaphorina citri* adults secreted more saliva and ingested more sap from phloem on mature leaves, which suggested that they could readily adapt to feeding on mature leaves to enhance survival. Annually immature leaves are present on citrus trees for relatively short intervals, thus, *D. citri* adults must feed on mature leaves in order to survive for most of the year. *D. citri* spent longer time in xylem ingestion activities on mature and young leaves than on new shoots. Since the sap of xylem mainly include mineral salts and water (Gollan et al. 1992), a possible hypothesis is that the cell sap concentration is greater in mature and young leaves than in new shoots, so *D. citri* needs supplementary supplies of water to maintain the salt and water balance in the body.

'*C*Las' acquisition by *D. citri* is associated with the feeding activity of both nymphal and adult psyllids feeing on host phloem. In this study, 23 out of the 24 *D. citri* adults that exhibited phloem ingestion (E2) waveforms were subsequently shown to be '*C*Las' positive, demonstrating these EPG waveforms are strongly associated with '*C*Las' acquisition. The result also illustrated that '*C*Las' acquisition was very high efficient. However 2 individuals that exhibited single E1 (phloem salivation) not followed by E2 phloem ingestion also tested '*C*Las' positive. A possible explanation is that phloem ingestion may occur over a relatively short duration and the waveform was not observed in the EPG recording due to the presence of other waveforms. Acquisition may also have occurred atypically during salivation.

In separate studies Li et al. (2009) and Kunta et al. (2014) used real-time PCR to quantify the distribution of the '*C*Las' in HLB-infected citrus plants. They found that the '*C*Las' concentration in mature leaves was higher than in young leaves. However, real-time PCR cannot discriminate between live and dead bacteria (Li et al. 2009), so the acquisition of '*C*Las' by real-time PCR cannot determine the quantity of live '*C*Las' cells directly.

Leaf age plays an important role in the feeding behavior of *D. citri*. Studies based on the presence of stylet tracks showed that adult psyllids preferred to feed on immature leaves, and the abundance of stylet tracks per leaf declined with increasing maturity of leaves (Yang et al. 2013). Bonani et al. (2008) reported a higher rate of '*C*Las' acquisition on asymptomatic young leaves than on symptomatic mature leaves during the same feeding period, and phloem probing was more frequent. Our contrasting results might be affected by different experimental conditions, such as the *D. citri* population, host plant species, plant age and HLB infection stage and inoculum level.

A potential shortcoming of the EPG technique is that the gold wire and silver glue can alter insect behavior (Caillaud 1999; Prado & Tjallingii 1999) due to a 'tethering effect'. No evidence of a tethering effect was apparent in this study. However, additional studies on *D. citri* feeding naturally are required to further validate the EPG results. Furthermore, 8 hours is short-time for monitoring insect behavior. Therefore, real-time video observation and microscopy should be combined with EPG to further elucidate the *D. citri* feeding behavior. Additionally, impacts of changes in leaf structure and functions resulting from '*C*las' infections also need to be elaborated.

The behavior of *D. citri* on young flushes has attracted more concern than on mature leaves. Measures to control and prevent HLB and *D. citri* focus primarily on young parts of the host plants (Xu et al. 1988; Grafton-Cardwell 2005). The reason is that *D. citri* females always lay eggs on the new growth, and tender flushes are essential for survival of nymphs. But our results indicated that mature leaves were as likely to be fed on by *D. citri* adults as young leaves and new shoots. The duration of phloem ingestion on mature leaves was even longer than on the other leaf maturity stages. These 2 observations combined may provide a reason why *D. citri* also is found on older leaves in spite of strong preference on young leaves. We suggest that in the future application of *D. citri* control measures more attention should be given to mature leaves especially when no new growth flushes occur.

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