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Electropenetrography application and molecular-based virus detection in mealybug (Hemiptera: Pseudococcidae) vectors of *Cacao swollen shoot virus* on *Theobroma cacao* L.

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ABSTRACT

Cacao swollen shoot virus (CSSV) is a peril exclusive to the West African cacao-growing countries; causing the Cacao swollen shoot virus disease. This study was set out (1) to analyse the feeding behaviour of two West African and one non-West African mealybug species, *Planococcus citri* (Risso) and *Pseudococcus longispinus* (Targioni Tozzetti) and *Ps. viburni* (Signoret) respectively on CSSV-free cacao. and (2) to provide molecular-based information on the ability of these mealybugs to acquire and transmit the 'New Juaben' CSSV strain from CSSV-infected cacao. Electrical penetration graph (EPG) analysis established that these three mealybug species performed both extracellular (C, E1e, F, G and Np waveforms) and intracellular (E1 and E2 waveforms) feeding activities on cacao which were typical of stylet-possessing, phloem-feeding, virus transmitting hemipterans. Waveform F reported in this study is the first for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* feeding on cacao. The competitive feeding efficiency of *Ps. viburni* on cacao highlights its potential as a 'new' vector of CSSV. PCR-based results show that *Pl. citri*, *Ps. longispinus* and *Ps. viburni* can acquire CSSV after a 72-h access acquisition period (AAP). DNA sequences of CSSV were detected in leaf tissues of the test plants after a 30-day post 72-h inoculation access period (IAP) by the viruliferous mealybug individuals. It is the first report, with molecular evidence, of *T. cacao* serving as an acceptable host to *Ps. viburni*.

1. Introduction

Mealybugs (Hemiptera: Coccoidea: Pseudococcidae) cause direct harm to a wide range of crops with phloem-ingesting nymphs and adults bringing about decreased vigour and defoliation while their excretion of honeydew can serve as a substrate for damaging sooty mould growth (e.g. (Calatayud et al., 2002; Morandi Filho et al., 2015)). It is the indirect damage mealybugs can cause as pathogen vectors that makes their movement between crop plants potentially so destructive. With their piercing-sucking mouthparts capable of injecting viruses into specific plant tissues mealybugs have been shown to be the main vehicle for the dispersion of grapevine leafroll-associated virus GLRaV (Charles et al., 2009), pineapple mealybug wilt-associated virus PMWaV (Sether et al., 1998) and Piper yellow mottle virus (Lockhart et al., 1997). However, in terms of scale by far the most devastating impact of mealybugs to date has been their role in the spread of the *Cacao swollen shoot virus* (CSSV) among the West African cacao (*Theobroma cacao*) crop. Using caging trials on healthy cacao seedlings it was originally thought that CSSV could be spread by all available cacao sucking Hemiptera including cacao psyllids (*Mesohomotoma tessmanni* Aulmann), Aphis (*Toxoptera aurantii*) and Thrips (*Heliothrips rubrocinctus*) (Posnette, 1941), but these were discounted as vectors when visual symptom-based transmission trials (Box, 1945) using the mealybug species *Ferrisia virgata* Cockerell, *Pseudococcus exitiabilis* Laing. and *Planococcus citri* Risso indicated that Pseudococcidae were the only Hemipteran family that could transmit the virus. The electrical penetration graph (EPG) technique allows for the quantification of complex insect-plant interactions exhibited by feeding hemipterans. While the approach was pioneered in the analysis of aphids and whiteflies, EPG is increasingly being utilised to elucidate the feeding behaviour of pseudococcids. The first use of EPG to record and characterise the feeding behaviour of mealybugs was with cassava mealybugs (*Phenacoccus manihoti* Matile-Ferrero) on cassava (*Manihot esculenta* Crantz), *Talinum* (*Talinum triangularae* Jacq.) and poinsettia

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(*Euphorbia pulcherrina* Wild) (Calatayud et al., 1994). Subsequent EPG studies with mealybugs included citrus mealybug (*Planococcus citri* Risso) feeding on grapevine (Cid and Fereres, 2010), solenopsis mealybug, *Phenacoccus solenopsis* (Tinsley) feeding on cotton (Huang et al., 2012), and alternative hosts (Huang et al., 2014). Overall, EPG analysis has proven to be effective for the characterisation of the hemipteran feeding patterns that are associated with the transmission of semi- versus non-persistent plant viruses (Moreno et al., 2012). The aims of this study, therefore, were to characterise the feeding behaviour and test the acquisition ability of citrus mealybug (*Pl. citri* Risso), longtailed mealybug (*Ps. longispinus* (Targioni Tozzetti)) and obscure mealybug (*Ps. viburni* Signoret) on CSSV-free and CSSV-infected *T. cacao* var. Amelonado, respectively.

2. Material and methods

2.1. Plant source and nursery management

CSSV-free cacao seedlings were grown from seeds from ripe cacao pods collected from the International Cacao Quarantine Centre (ICQC), Reading, UK. The seeds were sown in a compost (75%) – vermiculite (25%) mix in lightweight seed trays (34.4 cm × 21.4 cm × 5.2 cm) and maintained at 25 ± 2 °C and 60 ± 5% relative humidity (RH). At 14 days, germinated seedlings were transplanted into plastic pots (14 cm × 12 cm) and the established plants were then fertilized at four-weekly intervals with Sangral™ water soluble fertilizer (NPK (3:1:1) + 2MgO + Trace Elements (TE), Part Number: F1500172S) (William Sinclair Holdings Plc, Lincoln, UK). CSSV-infected plants carrying the viral isolate 'New Juaben' were generated from imbibed cacao seeds on which viruliferous mealybugs had been allowed to feed at the Cacao Research Institute Ghana (CRIG). The cacao seeds were then sent to the University of Reading where they were raised in the nursery unit.

2.2. Collection, identification and mass rearing of mealybugs

Gravid females of *Pl. citri* and *Ps. longispinus* mealybugs were collected from the plant quarantine facility at the Royal Botanic Gardens, Kew, UK; *Ps. viburni* were collected from the Tropical Glasshouse at the University of Reading, UK. Leaves carrying the mealybugs were gently tapped at the petiole to disrupt possible feeding and induce the retraction of stylets by the mealybugs, at least 2 min before they were collected with a fine paintbrush and stored in 2 ml round-base Eppendorf tubes. Mealybug species identity validation was based on a combination of morphological analyses and DNA barcoding using the cytochrome *c* oxidase (CO1) gene (Wetten et al., 2016). Mealybug lines were established from single gravid females of *Pl. citri*, *Ps. longispinus* and *Ps. viburni*, each placed on sprouting potatoes in individual 0.9L snap-closure boxes which were maintained inside dark incubators at 25 ± 1 °C, 55 ± 5% RH. Lines were sub-cultured every three weeks by transferring a single gravid female to a new culture box. The fidelity of the three mealybug lines was tested at regular intervals by High Resolution Melt Analysis (Wetten et al., 2016).

2.3. CSSV acquisition and inoculation by Pl. citri, Ps. longispinus and Ps. viburni on T. cacao

Capacities of *Pl. citri*, *Ps. longispinus* and *Ps. viburni* to acquire and transmit CSSV were tested using second-stage female instars. These instars were collected from the potato cultures and placed inside sealed petri dishes for a 24-h starvation period. Between 15 and 20 individuals were transferred to the abaxial surfaces of fully expanded true leaves of six-month old New Juaben CSSV-infected cacao seedlings and held in place to feed inside secure sprung traps for a 72-h virus acquisition access period (AAP) inside a controlled environment chamber (25 ± 2 °C, 55 ± 5% RH, 14 h dark photoperiod). After AAP, no less than 15 viruliferous mealybugs (virus-retention status of the mealybugs had previously been confirmed via CSSV-specific qPCR) were transferred into separate sprung traps and mounted on abaxial surfaces of fully expanded true leaves of six-month old CSSV-free cacao seedlings (virus-free status had previously been confirmed via CSSV-specific PCR) for a 72-h inoculation access period (IAP). The mealybugs were removed (alongside the traps) for destruction. Four weeks after the end of the IAP, leaf disc samples (diameter = 8 mm) were taken from the inoculated leaf on each of the test plants for PCR-based CSSV screening using Qiagen DNA extraction kit (Qiagen, UK) optimised for cacao leaf tissues. Forward and reverse primers and thermocycler settings for amplification of 'New Juaben' CSSV strain DNA was as described in Quainoo et al. (2008). PCR amplicons of the expected 375 bp size were purified using the QIAquick PCR purification kit (Qiagen, UK) then submitted for Sanger sequencing at Source Bioscience (Oxford, UK).

2.4. EPG analysis of Pl. citri, Ps. longispinus and Ps. viburni behaviours on CSSV-free T. cacao

Each group of the 24-h EPG recording was performed at 25 ± 2 °C; 80 ± 5% RH and 12:12 L:D light cycle. Second instars of CSSV-free *Pl. citri*, *Ps. longispinus* and *Ps. viburni* individuals were used for EPG analysis. The selected insects were collected starved for 24 h inside petri dishes prior to the commencement of the EPG setup. After setting up the EPG device (DC-EPG Giga-8 System (EPG Systems, Wageningen, Netherlands)) as described by Cid and Fereres (2010) and Huang et al. (2012), the feeding behaviour of the mealybugs were monitored on real-time using the electrical penetration graph (EPG) technique (DC system) for 24 h beginning at 09:00 h and data stored for subsequent extraction, annotation and statistical analyses. EPG recordings data were rejected if there was an eventual dropping off from the leaf by dropping off from the leaf and additional recordings were conducted until 16 replicate data had been accumulated for each of three mealybug species (i.e. 16 individual mealybugs per species). The following EPG waveforms depicting various pre-, post- and active mealybug feeding behaviours were observed for the three mealybug species: C (intercellular activities during penetration i.e. pathway waveform); E1e (extracellular salivation); E1 (salivation in sieve element); E2 (phloem ingestion); F (derailed stylet mechanics); G (xylem ingestion); Np (non-probing); pd (potential drop; intracellular stylet tip puncture). Sequential and non-sequential parameters of the designated EPG waveforms were processed by Microsoft Excel macros developed by Consejo Superior de Investigaciones Científicas (CSIC, Madrid, Spain) (Sarria et al., 2009).

2.5. Statistical analysis

Sequential and non-sequential EPG results for all the mealybug species were analysed as mean ± standard error for *n* number of individuals out of a total of sixteen individuals monitored per species for each of the observed EPG waveforms. Shapiro-Wilk's test (Shapiro and Wilk, 1965) was used to estimate data departure from normal distribution and Bartlett's test (Snedecor and Cochran, 1989) was used to determine homogeneity of variances. Where applicable, the data were natural log-transformed, ln (x + 1), before a one-way analysis of variance (ANOVA) was performed using GenStat 16th edition (VSN International Ltd, Hemel Hempstead, UK). Each of the sixteen individual mealybugs per mealybug species was considered as a replicate for the ANOVA. A *post hoc* test for significant means difference was subsequently performed using the Fisher's Least Significant Differences at a 95% confidence limit.

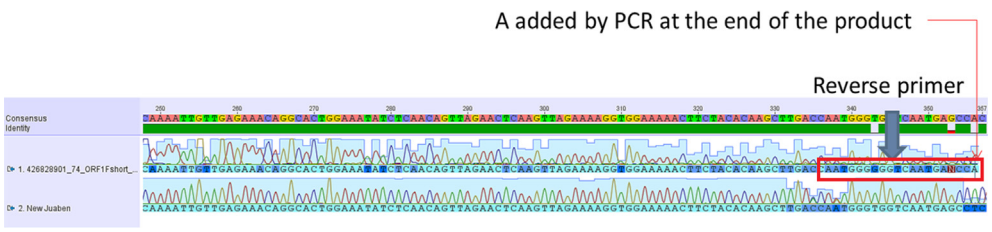

Fig. 1. Portion of read from Sanger sequencing of 375 bp fragment produced with CSSV Open Reading Frame 1 (ORF1) primers showing 100% homology with published sequence of the New Juaben strain of CSSV (NCBI accession number AJ608931).

3. Results

3.1. Molecular evidence of CSSV transmission by Pl. citri, Ps. longispinus and Ps. viburni

Sanger sequencing showed that the 375 bp PCR products generated from mealybug-inoculated test plants (*T. cacao* var. Amelonado) were 100% matches for the 'New Juaben' CSSV sequences (NCBI accession number AJ608931) generated from the virus source plants (Fig. 1).

3.2. EPG waveforms of Pl. citri, Ps. longispinus and Ps. viburni behaviours on CSSV-free T. cacao

3.2.1. Sequential and non-sequential EPG waveforms

Details of relevant sequential and non-sequential EPG waveforms for the feeding behaviour of the three mealybug species monitored on CSSV-free *T. cacao* var Amelonado are presented in Table 1. EPG waveforms exhibited were averagely consistent, but with high degrees of variability in their relative proportions between individual mealybugs. In terms of the average time taken to make the first probe, *Ps. longispinus* (3 ± 1.2 min) and *Ps. viburni* (7.2 ± 2.4 min) individuals were more active than *Pl. citri* (15 ± 7.2 min). However, *Ps. longispinus* (19.8 ± 9.6 min) and *Ps. viburni* (42 ± 27.6 min) did achieve potential drop (pd) later than *Pl. citri* (12.6 ± 3.6 min).

3.2.2. Basic EPG waveforms: C, E1e, E1, E2, F, G and Np

After the initial non-probing phase (Np), waveforms C was the most frequently generated extracellular EPG patterns observed for all the mealybug species. This waveform represented the pathway phase when the insect would either be walking or sampling feeding sites yet to be probed. The fluctuating pattern of waveform C amplitude in response to voltage tunings depicted a resistance (R) electrical origin of the waveform. In this study, waveform C produced by *Pl. citri* (Fig. 2A) and *Ps. longispinus* (Fig. 3A) mealybugs on CSSV-free *T. cacao* leaves resembled those of *Pl. citri* and *Ps. longispinus* on grapevine leaves, in terms of the duration, frequency and electrical origin of the waveform. Based on these references, waveform C was also determined and annotated for *Ps. viburni* (Fig. 4A). The average proportion of waveform C (s_C in Table 1 presented as percentage in 24 h duration) for the three mealybug species ranged from 40.13 ± 6.71% (*Pl. citri*) to 51.33 ± 8.46% (*Ps. viburni*). Though the extracellular salivation waveform (E1e) observed for *Pl. citri* (Fig. 2C), *Ps. longispinus* (Fig. 3C) and *Ps. viburni* (Fig. 4C) closely resembled waveform C and intercellular waveform E1/E2, E1e was distinguishable by its relatively low amplitude, frequency and electrical origin – electromotive force (emf). Waveform E1e was often preceded and followed by pds and in some cases E1e was followed by Np phases. The observed G waveforms for *Pl. citri* (Fig. 2A), *Ps. longispinus* (Fig. 3A) and *Ps. viburni* (Fig. 4A) on cacao had a similar amplitude to waveform C, but was distinct with relatively high and uniform frequency. Waveform F was generated in response to stylet derailment i.e. the probing difficulty experienced by mealybugs while attempting to either puncture or penetrate leaf tissues. It was characterised by low amplitude and high frequency for each of the three mealybug species *viz.*, *Pl. citri* (Fig. 2C), *Ps. longispinus* (Fig. 3C) and *Ps. viburni* (Fig. 4C). The pattern of waveform G often continued unchanged in response to voltage alterations and in some cases varied which indicated that G had both emf and R electrical origins. There were essentially two forms of the intracellular waveform E – E1 and E2, resulting from sustained potential drops e.g. *Pl. citri* (Fig. 2B), *Ps. longispinus* (Fig. 3B) and *Ps. viburni* (Fig. 4B). These intercellular waveforms have been associated with phloem access, sieve element salivation and ingestion. Generally, E1 and E2 waveforms alternated sequentially. Though there were no known cues to the transition from E1 to E2 and vice versa, E1 waveform showed variable and an undetermined electrical origin whereas E2 originated from both emf and R electrical origins. E2 maintained a longer duration of occurrence than E1 and with lower frequency of peaks and waves. Overall, the average proportions of the waveforms performed by each of the three mealybug species followed the order, *Pl. citri*: C > G > F > Np > E2 = E1e > E1 (Fig. 5A), *Ps. longispinus*: C > G > F > Np > E2 > E1e > E1 (Fig. 5B) and *Ps. viburni*: C > G > Np > E2 > E1e > F > E1 (Fig. 5C). The ANOVA for the natural log transformed data (Table 2) only showed significant differences ($p \leq 0.05$) between the mealybugs for the following waveforms, E1e, E1 and G. *Pl. citri* and *Ps. viburni* significantly differed from *Ps. longispinus* for E1e. Also, *Ps. longispinus* and *Ps. viburni* significantly differed ($p \leq 0.05$) from *Pl. citri* for E1. *Ps. viburni* performed the longest waveform G, similar to *Pl. citri* but statistically different from *Ps. longispinus*.

4. Discussion

4.1. Molecular evidence of CSSV transmission by Pl. citri, Ps. longispinus and Ps. viburni

The identification of CSSV infected cacao plants has been primarily based on visual symptoms. This could be misleading given that nutrient deficiencies can also present similar symptomatic effects on cacao plants as those of CSSV infection. Also, it is visually impossible to identify a viruliferous mealybug vector; those capable of transmitting CSSV between cacao plants. However, the use of molecular-based screening and detection method on both mealybug species exposed to CSSV infected cacao plants and mealybug-inoculated cacao plants represents an effective virus indexing approach. It is evident in the present study that the use of CSSV primer-specific PCR to test for the acquisition and transmission efficiencies of putative mealybug vector species of CSSV was reliable and robust. There is paucity of such report for cacao-CSSV-mealybug interactions. In general, it takes months to years for cacao to express symptoms of CSSV infection after inoculation by mealybug vectors, but in this study asymptomatic cacao plants could be tested and in a very short period (4 weeks) after infection. This would therefore encourage early detection and timely control of the spread of the virus. It is an approach that would benefit plant breeders and plant health managers in setting up breeding strategies against CSSV and its mealybug vectors in cacao.

4.2. EPG waveforms of Pl. citri, Ps. longispinus and Ps. viburni behaviours on CSSV-free T. cacao

This is the first EPG study of the feeding behaviour of mealybugs on *T. cacao*. It is discussed with reference to previous EPG studies with mealybugs, which is however very limited in literature. The application of EPG analysis has gained importance in monitoring the highly

Table 1
Characterisation of the probing and feeding behaviour of *Planococcus citri* (Risso), *Pseudococcus longispinus* (Targioni Tozzetti) and *Ps. viburni* (Signoret) on *Theobroma cacao* L.

Parameter Type	Variable	Explanation	Unit	*Planococcus citri*			*Pseudococcus longispinus*			*Ps. viburni*		
				n	Mean ± SE	Range	*n*	Mean ± SE	Range	*n*	Mean ± SE	Range
Nonsequential	s_C	Sum of C	h	*16*	9.63 ± 1.61	1.01–23.55	*16*	11.32 ± 2.17	0–23.98	*16*	12.32 ± 2.03	0.94–23.86
Nonsequential	s_Np	Sum of non-probing	h	*16*	1.02 ± 0.28	0.02–4.41	*16*	2.42 ± 1.3	0–21.01	*16*	1.35 ± 0.76	0–12.47
Nonsequential	a_C	Average C; with pd without E1e, F and G	min	*16*	40.57 ± 7.1	7.12–113.35	*16*	232.7 ± 118.91	0.04–1438.69	*16*	63.13 ± 20.41	11.33–357.91
Nonsequential	a_Np	Average non probing (period duration)	min	*16*	14.67 ± 8.07	0.73–132.45	*16*	31.37 ± 19.35	0.06–315.17	*16*	15.73 ± 9.03	0.23–149.6
Nonsequential	a_Pr	Average probe	min	*16*	351.93 ± 90.02	73.91–1434.49	*16*	622.69 ± 147.32	59.77–1439.95	*16*	565.38 ± 117.02	109.46–1439.78
Nonsequential	s_pd	Sum of pd	min	*16*	38.59 ± 8.44	6.08–119.07	*13*	24.9 ± 6.56	0–78.70	*16*	34.92 ± 6.44	3.06–82.36
Nonsequential	n_C	Number of C periods	no.	*16*	18.81 ± 3.38	1–56	*16*	11.63 ± 3.40	1–44	*16*	17.13 ± 3.05	1–44
Nonsequential	n_Np	Number of non-probing periods	no.	*16*	7.63 ± 1.29	1–18	*16*	5.5 ± 1.23	1–16	*16*	4.25 ± 0.80	1–12
Nonsequential	n_pd	Number of pd	no.	*16*	85 ± 19.13	14–275	*13*	49.75 ± 13.51	0–165	*16*	83.19 ± 15.81	8–208
Nonsequential	n_Pr	Number of probes	no.	*16*	7.63 ± 1.29	1–18	*16*	5.31 ± 1.24	1–16	*16*	4.13 ± 0.77	1–12
Nonsequential	d_1pd	Duration of the first pd	s	*16*	33.11 ± 10.05	11.02–180.43	*12*	21.84 ± 7.31	0–117.55	*15*	19.9 ± 3.43	0–46.83
Nonsequential	d_2pd	Duration of the second pd	s	*16*	24.55 ± 2.38	3.9–38.99	*13*	21.69 ± 3.80	0–44.42	*14*	20.39 ± 4.00	0–54.02
Nonsequential	d_pd5	Mean duration of the first 5 pd	s	*16*	25.8 ± 2.12	10.67–43.05	*13*	28.24 ± 3.92	11.43–68.55	*16*	20.13 ± 2.59	1.73–33.72
Nonsequential	s_E1e	Sum of E1e	h	*14*	0.47 ± 0.15	0–2.28	*6*	0.35 ± 0.17	0–2.30	*16*	0.42 ± 0.13	0.02–1.69
Nonsequential	s_G	Sum of G	h	*14*	8.17 ± 1.94	0–22.88	*11*	5.53 ± 2.29	0–24.00	*15*	8.92 ± 2.15	0–23.02
Nonsequential	a_E1e	Average E1e	min	*14*	2.14 ± 1.15	0–19.23	*6*	1.53 ± 0.76	0–11.87	*16*	2.35 ± 0.57	0.52–8.14
Nonsequential	a_G	Average G	min	*14*	185.47 ± 75.33	0–1061.76	*11*	251.63 ± 124.06	0–1439.91	*15*	193.44 ± 86.69	0–1371.44
Nonsequential	n_E1e	Number of E1 extracellular (E1e) periods	no.	*14*	19.69 ± 5.40	0–75	*6*	8.75 ± 4.72	0–64	*16*	10.94 ± 2.55	1–32
Nonsequential	n_G	Number of G	no.	*14*	5.19 ± 1.58	0–26	*11*	2.88 ± 1.14	0–15	*15*	9.69 ± 2.31	0–30
Nonsequential	nPr_1G	Number of probes before the first G	no.	*14*	1.81 ± 0.42	0–7	*11*	1.81 ± 0.79	0–13	*15*	1.13 ± 0.15	0–3
Nonsequential	s_F	Sum of F	h	*9*	4.1 ± 1.48	0–18.40	*7*	3.96 ± 1.61	0–19.39	*4*	0.29 ± 0.20	0–3.17
Nonsequential	a_F	Average F	min	*9*	97.68 ± 30.69	0–326.13	*7*	130.88 ± 59.62	0–799.91	*4*	15.14 ± 11.84	0–190.1
Nonsequential	n_F	Number of F	no.	*9*	1.5 ± 0.54	0–8	*7*	0.88 ± 0.33	0–4	*4*	0.56 ± 0.33	0–5
Nonsequential	n_Pr_1pd	Number of probes before 1st pd	no.	*9*	0.56 ± 0.13	0–1	*8*	0.5 ± 0.13	0–1	*10*	0.63 ± 0.13	0–1
Sequential	t_1E	Time to 1st E (always E1; from the 1st probe)	h	*16*	18.55 ± 2.07	0.47–23.98	*16*	23.57 ± 0.39	17.76–24	*16*	23.19 ± 0.68	13.03–24
Sequential	t_1E1_1E2	Time from the 1st E1 to 1st E2	h	*16*	15.05 ± 2.89	0.01–23.98	*16*	22.46 ± 1.50	0.01–24	*16*	22.38 ± 1.49	0.08–24
Sequential	t_1E1_1sE2	Time from the 1st E1 to 1st sustainable E2	h	*16*	19.86 ± 2.08	0.48–23.98	*16*	23.57 ± 0.39	17.77–24	*16*	23.2 ± 0.67	13.11–24
Sequential	t_1E12	Time to 1st E12	h	*16*	18.75 ± 2.10	0.47–23.98	*16*	23.57 ± 0.39	17.76–24	*16*	23.19 ± 0.68	13.03–24
Sequential	t_1E2	Time to 1st E2	h	*16*	18.8 ± 2.09	0.48–23.98	*16*	23.57 ± 0.39	17.77–24	*16*	23.2 ± 0.67	13.11–24
Sequential	t_1G	Time to the first G (after first penetration)	h	*16*	5.28 ± 1.94	0.16–23.98	*16*	10.01 ± 2.70	0–24	*16*	4.01 ± 1.84	0.06–23.98
Sequential	t_1pd	Time to 1st pd (from start of 1st probe)	min	*16*	12.6 ± 3.6	0.6–53.4	*13*	19.8 ± 9.6	0–154.8	*16*	42 ± 27.6	0–444
Sequential	t_1Pr	Time to 1st probe (in recording; = d_1Np)	min	*16*	15.6 ± 7.2	13.2–116.4	*16*	3 ± 1.2	0–12	*16*	7.2 ± 2.4	0–33
Sequential	d_1Pr	Duration of 1st probe	min	*16*	7.08 ± 2.11	0.06–23.91	*16*	11.23 ± 2.60	0.11–24	*16*	9.11 ± 2.42	0.13–24

C (intercellular activities during penetration i.e. pathway waveform); E1e (extracellular salivation); E1 (salivation in sieve element); E2 (phloem ingestion); F (derailed stylet mechanics); G (xylem ingestion); Np (non-probing); pd (potential drop; intracellular stylet tip puncture); s (sum); a (average); t (time); n (number); d (duration); Pr (probe); SE (Standard error); Range (minimum value–maximum value).

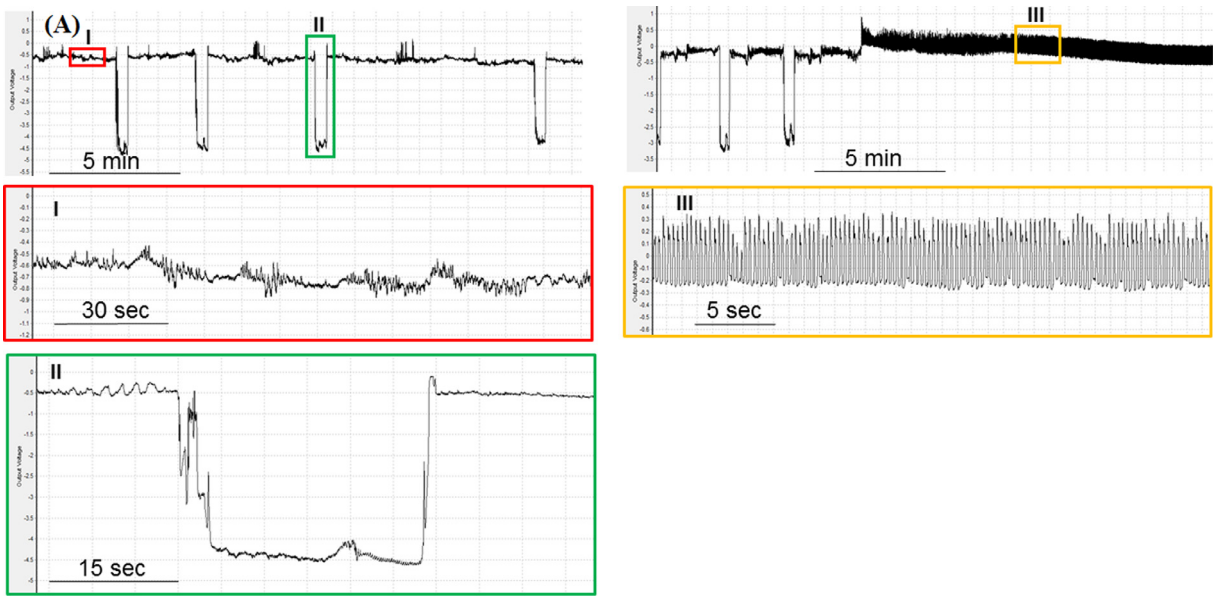

Fig. 2A. Waveforms C (I), pd (II) and G (III) generated by 24-h starved *Planococcus citri* feeding on an *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms C, pd and G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modified mouthparts of phloem-feeding hemipterans, especially in the process of both intracellular and intercellular navigations to locate feeding sites in sieve elements during pathogen acquisition and inoculation (Prado and Tjallingii, 1994; Fereres and Collar, 2001; Tjallingii and Prado, 2001; Sandanayaka et al., 2003; Morris and Foster, 2008; Garzo et al., 2016). These have been made possible because of EPG waveform correlation with real-time feeding activities of piercing-sucking hemipterans. It is relationship that exists between stylet puncture, penetration, positioning within plant cells (vascular tissues) and electrical charges across cell membranes (Walker, 2000). Posnette and Robertson (1950) earlier established that there was a relationship between stylet morphology of the cacao mealybug (*Formicococcus njalensis*) and the duration required for probing and feeding in comparison with leaf-hoppers and aphids. Data from the present study were comparable with published EPG data for mealybugs feeding on cassava (Calatayud et al., 1994) and citrus (Cid and Fereres, 2010). The dominant activity recorded for all species was C (Figs. 5A–5C) indicating the relatively protracted period spent by the mealybugs establishing stylet contact with vascular tissues in cacao leaves compared with, for example the relatively brief pathway phase shown by rapidly feeding aphids (Tjallingii, 1995; Stafford et al., 2012).

Waveform F has only previously been reported on cotton for cotton mealybug, and it has not been reported in any of the previous EPG recordings for other mealybug species (Calatayud et al., 1994; Cid and

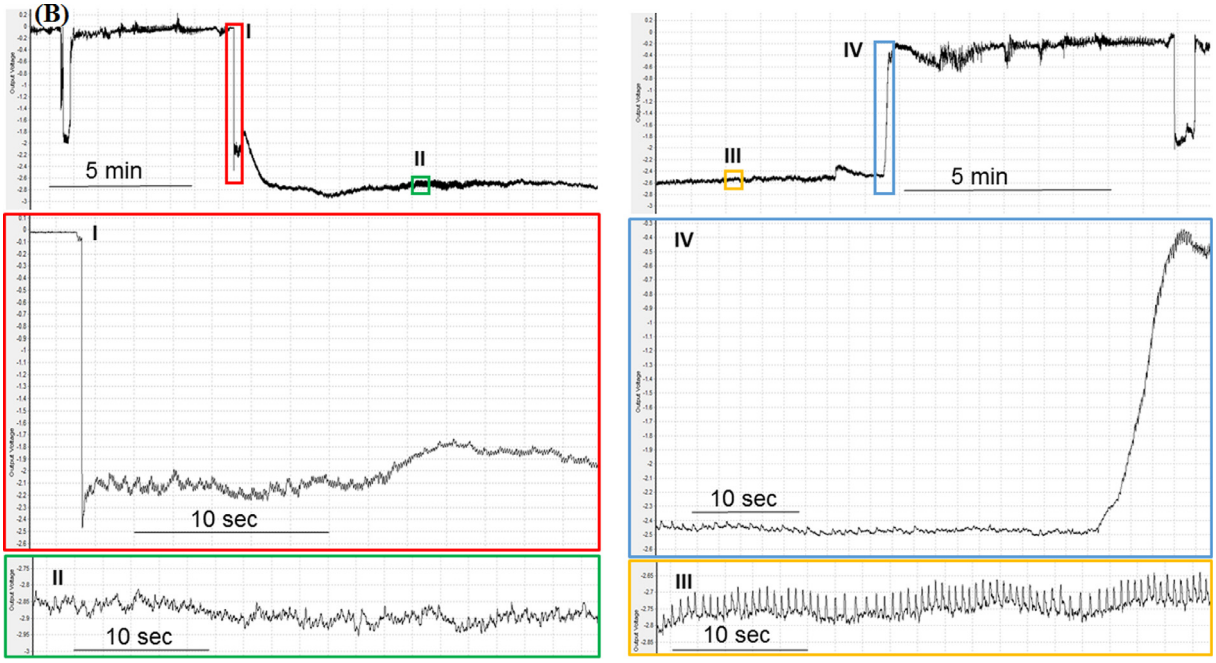

Fig. 2B. Initiation of waveform E1 (I) and its continuation (II). Waveform E2 (III) and its termination (IV) generated by 24-h starved *Planococcus citri* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1 and E2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

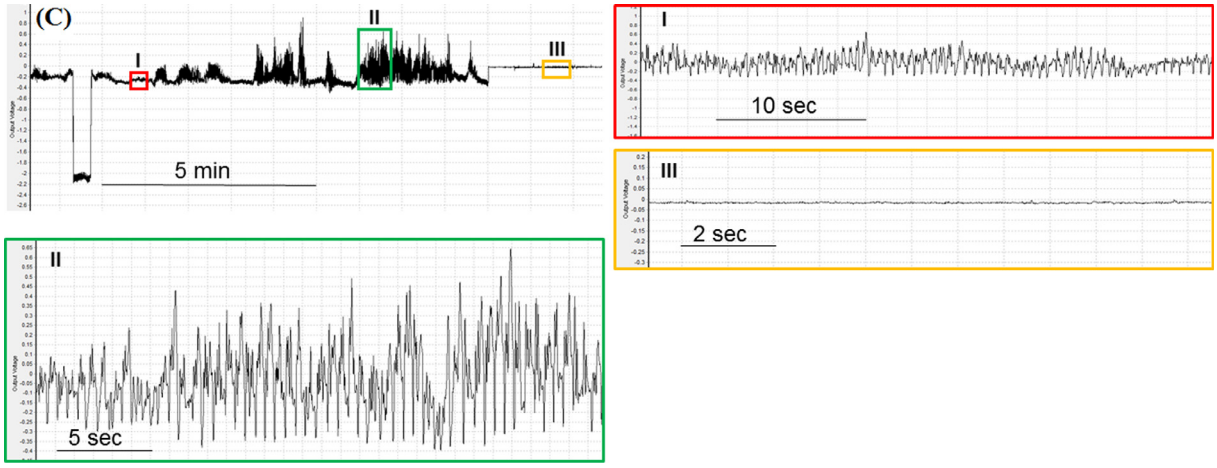

Fig. 2C. Waveform F (I), E1e (II) and np (III) generated by 24-h starved *Planococcus citri* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1e, F and np. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

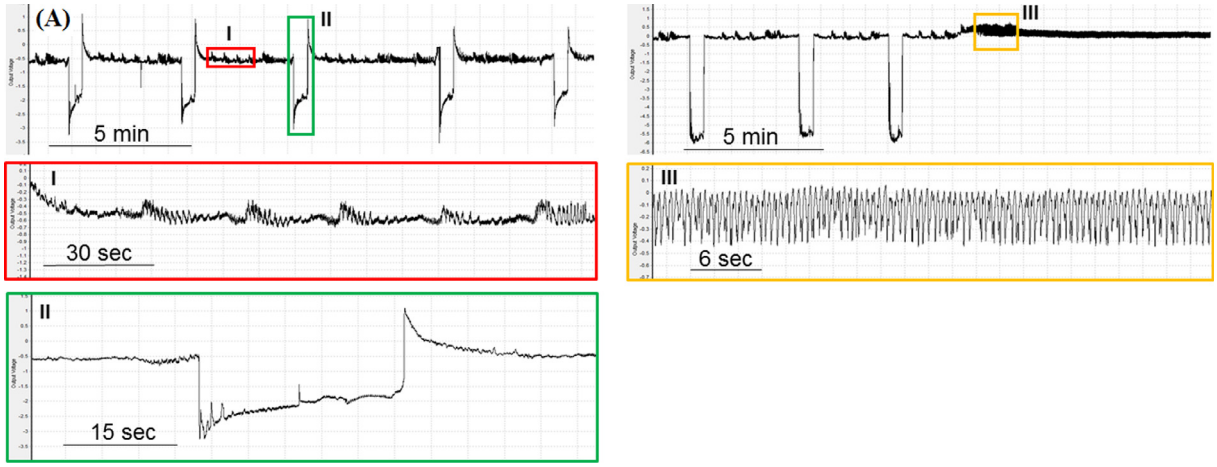

Fig. 3A. Waveforms C (I), pd (II) and G (III) generated by 24-h starved *Pseudococcus longispinus* feeding on an *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms C, pd and G.

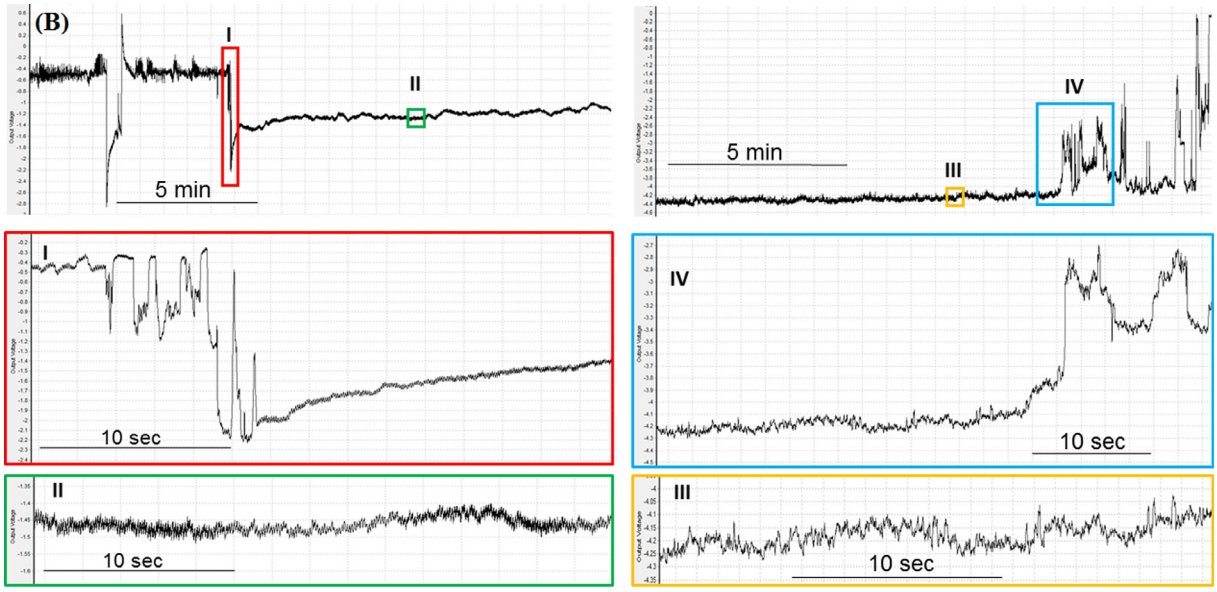

Fig. 3B. Initiation of waveform E1 (I) and its continuation (II). Waveform E2 (III) and its termination (IV) generated by 24-h starved *Pseudococcus longispinus* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1 and E2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

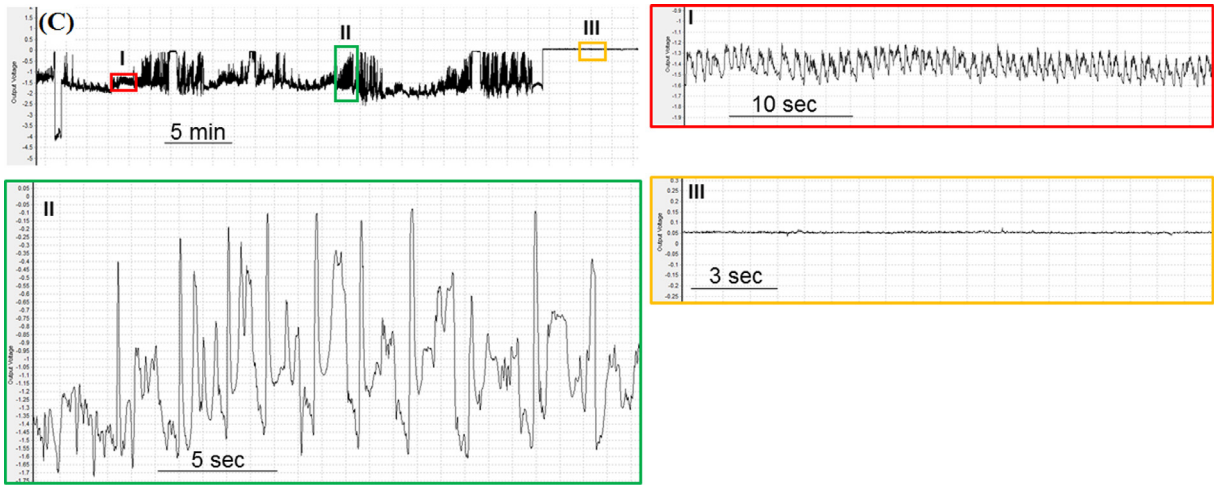

Fig. 3C. Waveform F (I), E1e (II) and np (III) generated by 24-h starved *Pseudococcus longispinus* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1e, F and np. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

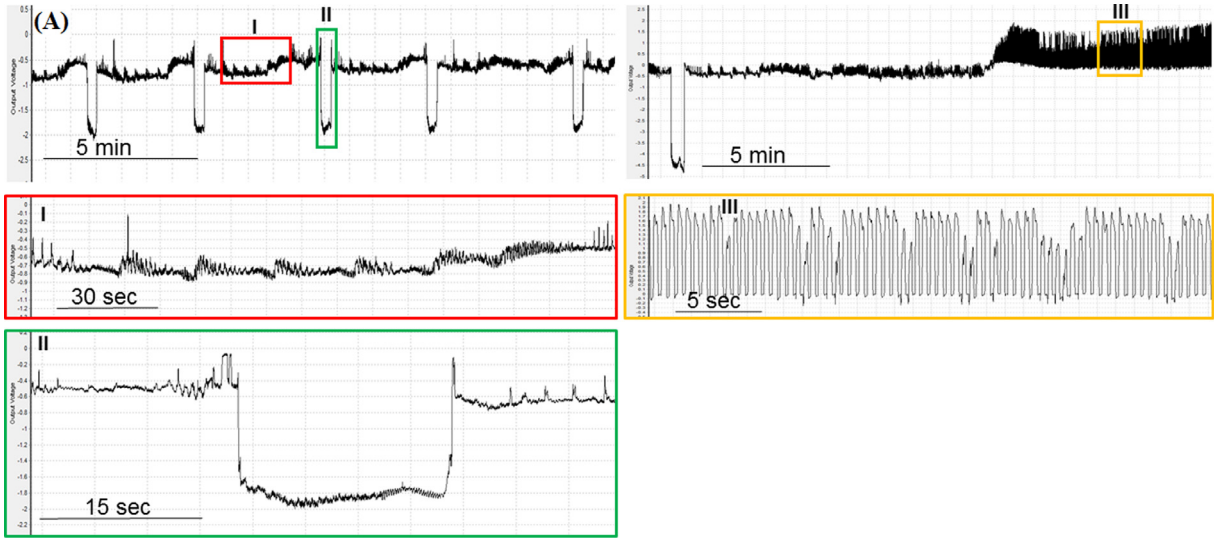

Fig. 4A. Waveforms C (I), pd (II) and G (III) generated by 24-h starved *Pseudococcus viburni* feeding on an *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms C, pd and G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fereres, 2010). In the present study, waveform F has its second report and the first for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* on *T. cacao*. In starved hemipterans, an increase in the activity of waveform G is an indication of dehydration and stress during EPG monitoring (Ramírez and Niemeyer, 2000; Pompon et al., 2010, 2011). Starved hemipterans would perform this activity in keeping with the need to maintain their internal homeostasis prior to feeding. The xylem ingestion waveform, G, was apparently an indication of how 'thirsty' the starved mealybugs were as they settled to feed on the cacao leaves. The generation of the G waveform shows that the stylets have penetrated the parenchyma through to the xylem tissues, rich in water and soluble mineral nutrients (Lucas et al., 2013). In this study, the contribution of G to the 24 h feeding duration of *Pl. citri* on cacao ranged from 0 to 95.35%. Waveform G was the next most sustained activity (Figs. 5A–5C). The G waveforms observed in this study closely matched those described for *Pl. citri* and *Ps. longispinus* on grapevines (Cid and Fereres, 2010; Sandanayaka et al., 2013b) and *Ph. solenopsis* on shoeblack plant (Wu et al., 2013). The occurrence of waveform G is likely to be influenced by the water stressed nature of the individuals following the 24 h starvation period. This pre-treatment was imposed to establish a common baseline for all individuals at the beginning of the EPG recording, but it is also likely to be representative of the status of windblown/dislodged juveniles arriving on CSSV-free cacao plants in the field (i.e. the individuals of most importance in terms of either long distance or jump spread of the virus) (Thresh, 1958; Thresh et al., 1988).

The positioning of the stylet by feeding piercing-sucking hemipterans within the sieve elements of host plant tissues is associated with E1 and E2 waveforms. These two waveforms are significant in the transmission of phloem-limited viruses via upload and download by mealybugs feeding activities. The first E1/E2 were recorded for *Pl. citri* individuals at 18.55 ± 2.07 h after the first probe, which was 12 h later than the commencement of E1/E2 observed for *Pl. citri* on grapevine (6.39 ± 1.5 h) (Cid and Fereres, 2010). Cid and Fereres (2010) also reported a mean E1 duration of 45.7 ± 9.78 s for *Pl. citri,* n = 11 of 20, fed on grapevine; nine of those resulted in E2, which lasted for over 4 h. In the present study on cacao, 6/16 of *Pl. citri* could perform E1 for a duration of 3.28 ± 2.01 min and five of those individuals proceeded to the sustained phloem ingestion phase (E2) which lasted between 6.82 s and 6.73 h (Fig. 5A). This fell between the duration of E2 reported for *Pl. citri* on grapevine (Cid and Fereres, 2010) and *Ph. manihoti* on cassava (Calatayud

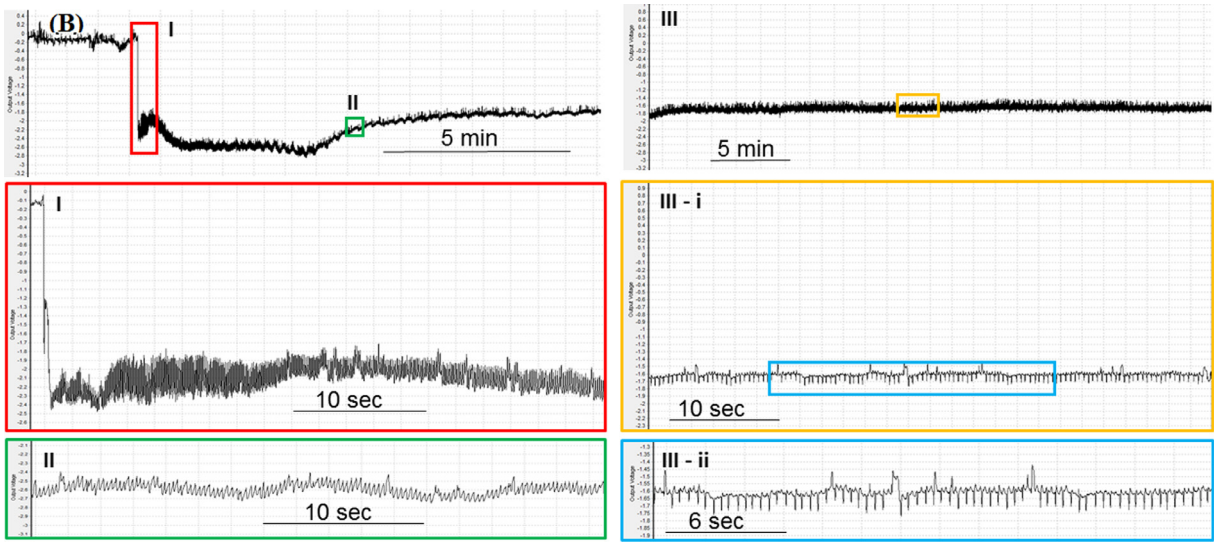

Fig. 4B. Initiation of waveform E1 (I) and its continuation (II). Waveform E2 (III) generated by 24-h starved *Pseudococcus viburni* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1 and E2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

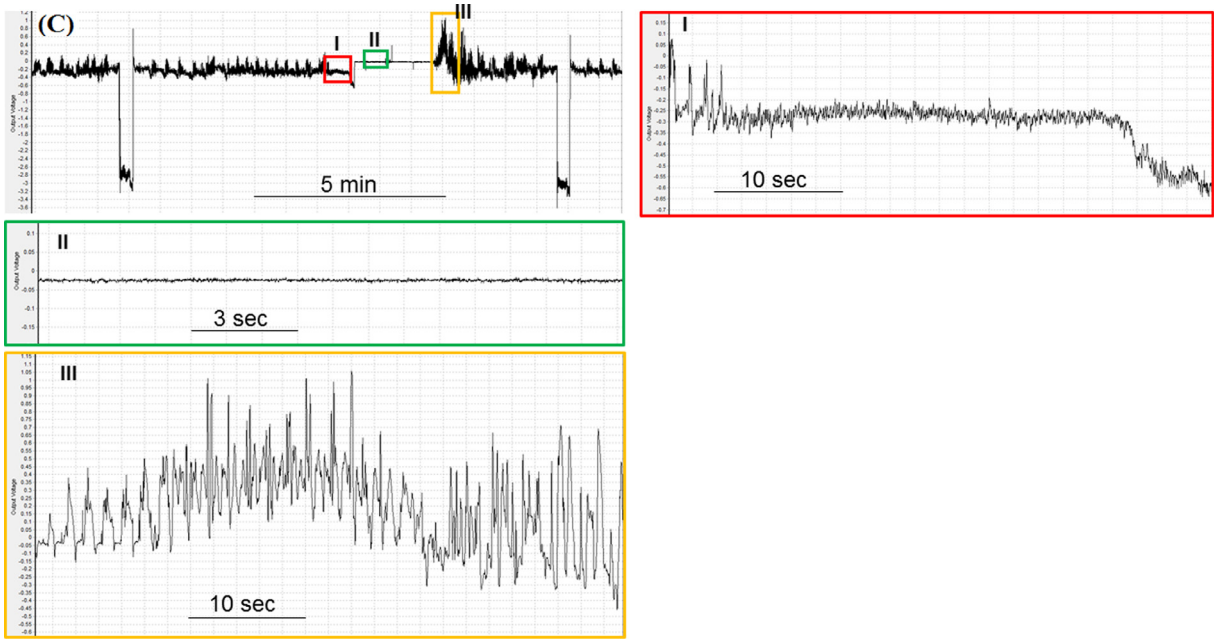

Fig. 4C. Waveform F (I), np (II) and E1e (III) generated by 24-h starved *Pseudococcus viburni* feeding on *in situ* CSSV-free *T. cacao* leaf (var. Amelonado). Matching colour boxes are magnifications of waveforms E1e, F and np. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 1994). E1/E2 were also comparable with those of *Ps. longispinus* on grapevine (Sandanayaka et al., 2013a). Sandanayaka et al. (2013a) tested GLRaV-3 free and GLRaV-3 infected grape with *Ps. longispinus* adults. Ten of the 24 GLRaV-3-free *Ps. longispinus* individuals reached E1 and three proceeded to E2. Similarly, in the present study involving 16 24-h starved *Ps. longispinus* individuals, the proportion of probing was 89.9 ± 2.42% with a single individual exhibiting E1 and E2 (Fig. 5B).

Significant differences ($p \leq 0.05$) were apparent for the duration of the intra- and extracellular salivation events (waveforms E1 and E1e, respectively) and xylem ingestion (waveform G) (Table 2) for the three mealybug species. The intracellular salivation event is the stylet activity predominantly associated with virus transmission by hemipterans (Stafford et al., 2011). Results from this study could aid in understanding the relative efficiency of CSSV inoculation by its mealybug vector species. Future work utilizing this EPG approach could address the potential for CSSV transmission during stylet activity phases. For example, EPG monitoring of viruliferous mealybugs probing virus-free plants and interrupting that feeding prior to phloem access could allow for the determination of the susceptibility of cacao to CSSV infection during the early probing events by mealybug vectors. Putative CSSV resistant cacao genotypes could be assessed to determine if they possess characteristics that significantly alter the feeding behaviour of mealybug vectors.

It has been demonstrated that sustained feeding (salivation and ingestion) within the phloem tissues (indicated by EPG waveforms E1 and E2) of *S. tuberosum* by viruliferous psyllid, *Bactericera cockerelli* (Šulc), did not usually result in the transmission of the pathogen, '*Candidatus* Liberibacter solanacearum', which causes the zebra chip

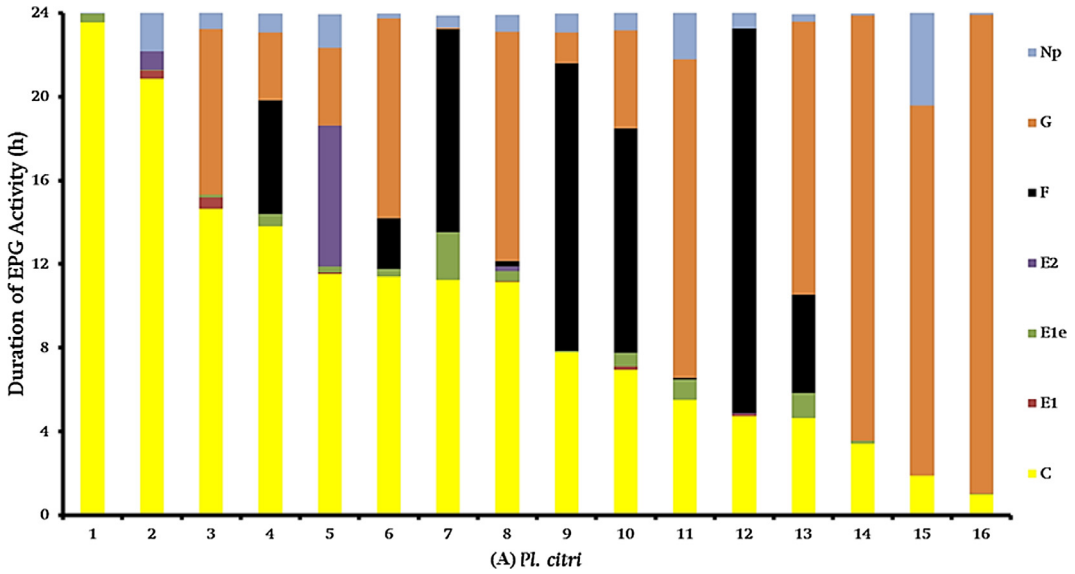

Fig. 5A. Duration of EPG activities for 16 individual mealybugs species of *Planococcus citri.*

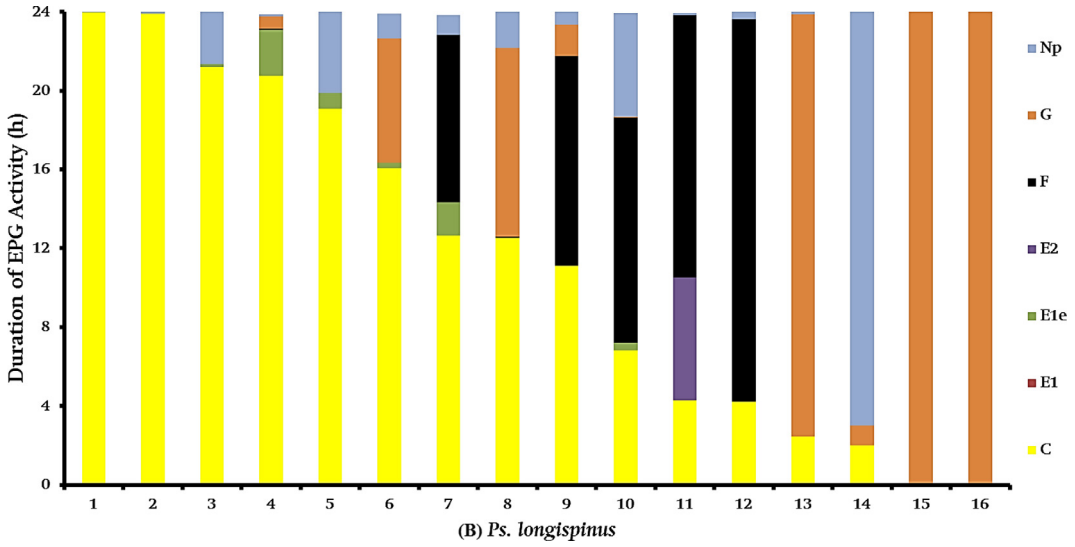

Fig. 5B. Duration of EPG activities for 16 individual mealybugs species of *Pseudococcus longispinus.*

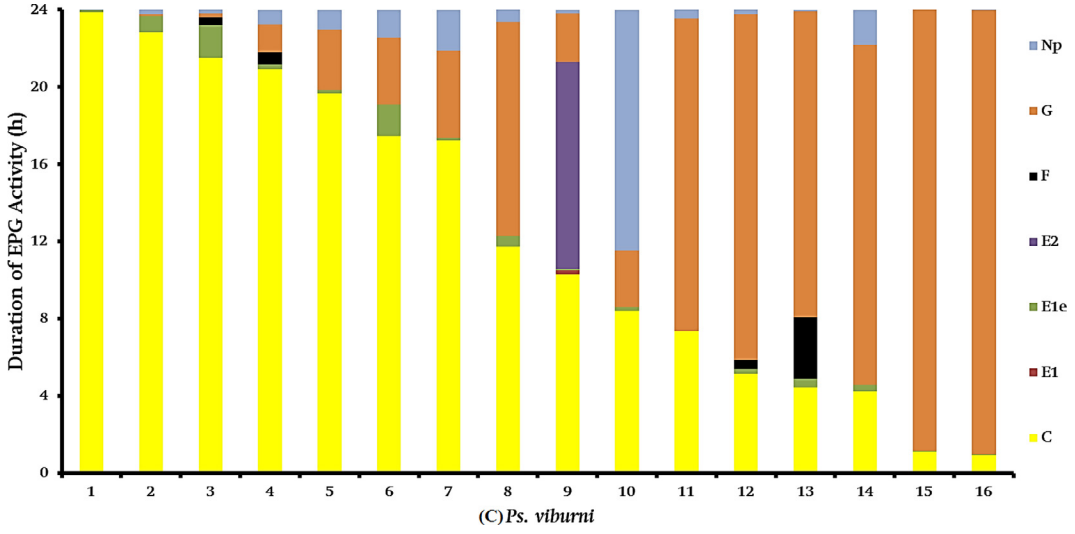

Fig. 5C. Duration of EPG activities for 16 individual mealybugs species of *Pseudococcus viburni.*

Table 2
Analysis of variance for electropenetrography of *Planococcus citri* (Risso), *Pseudococcus longispinus* (Targioni Tozzetti) and *Ps. viburni* (Signoret) on *Theobroma cacao* L.

Mealybug species	EPG waveform (log_e)						
	C	E1e	E1	E2	F	G	Np
Planococcus citri	6.09	2.53	0.83	0.83	2.99	4.77	3.58
Pseudococcus longispinus	5.65	1.35	0.02	0.37	2.27	2.98	3.30
Ps. viburni	6.26	2.61	0.16	0.40	0.97	5.23	3.08
F-probability	*0.46*	*0.05*	*0.02*	*0.68*	*0.11*	*0.05*	*0.72*
LSD ($p \leq 0.05$)	*NS*	*1.12*	*0.59*	*NS*	*NS*	*1.90*	*NS*

C (intercellular activities during penetration i.e. pathway waveform); E1e (extracellular salivation); E1 (salivation in sieve element); E2 (phloem ingestion); F (derailed stylet mechanics); G (xylem ingestion); Np (non-probing). LSD = Least Significant Difference at ≤95% confidence level; NS = not significantly different at ≤95% confidence level.

disease of potato (Mustafa et al., 2015). In aphids, it has also been demonstrated that the inoculation of the phloem-limited pathogen, *Cauliflower mosaic virus* (CaMV), by *Brevicoryne brassicae* (L.) to turnip (*Brassica rapa* L. (var. Just-Right)) by *Myzus persicae* (Sulzer) was possible after the initial intracellular stylet penetration of the epidermis and mesophyll (Moreno et al., 2005). It is therefore indicative from this study that there are promising applications in the use of EPG technology in disease resistance breeding studies on *T. cacao* genotypes and the possibility of using EPG as a rapid means of screening *T. cacao* genotypes for CSSV and its vector resistance, respectively.

Interestingly, the feeding behaviour of *Ps. viburni* on any of its hosts has never been monitored with EPG prior to this study, its EPG waveforms comparison were directly interpreted and discussed in the light of those of *Pl. citri* and *Ps. longispinus*. The EPG data demonstrating cacao phloem access (E1 and E2) by *Ps. viburni* (Fig. 5C) supports the finding in this study of the species' capacity for CSSV transmission despite its apparent absence from *T. cacao* in the field. This species is however present on the African continent (Daane et al., 2012) and its range is expanding illustrating the potential for changes to the current CSSV vector fauna that could be investigated with the aid of EPG-based approaches. The host-range for these three mealybug species vary in geographic distribution and taxonomic classifications. *Pl. citri*, *Ps, longispinus* and *Ps. viburni* forage on 82 families (191 genera), 82 families (157 genera), 89 families (236 genera) (García Morales et al., 2016) of which the family Malvaceae (with the genus *Theobroma*) is common to all. There is an evidence that scale insects in the tropics do have broader host ranges (Hardy et al., 2015), thus the long-term expected invasiveness in the tropics where cacao is grown cannot be ruled out for *Ps. viburni*.

5. Conclusion

The first molecular evidence of CSSV acquisition and transmission by two tropical mealybug species and one temperate origin mealybug species have been reported in this study. Also, the first use of EPG to study mealybug feeding behaviour on *T. cacao* has been reported in this study. CSSV is characterised as a phloem-limited mealybug-transmitted pathogen (Jacquot et al., 1999), but its mode of transmission by mealybug vectors still requires detailed studies to test whether prolonged stylet penetration in vascular tissues (phloem phase) by viruliferous mealybugs on its host, *T. cacao*, always results in CSSV transmission and if shorter duration of tissue probes without access to the phloem by viruliferous mealybugs could cause the transmission of CSSV. In this way, such studies could inform about the threshold size of viruliferous mealybug populations necessary for the effective transmission of CSSV and in turn help to elucidate the likely effectiveness of mealybug control regimes, be they pesticide, parasitoid or pheromone based, for the prevention of CSSV and its vector spread.

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