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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.