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"To Eat, or Not to Eat, That is the Question" - Answered by Real-Time Monitoring Techniques Combining with Computational Analysis for Feeding Behavior Study of Crapemyrtle Bark Scale (*Acanthococcus lagerstroemiae*)

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Summary

Crapemyrtle bark scale [(CMBS) *Acanthococcus lagerstroemiae*], an invasive and polyphagous sap feeder, has spread across 17 U.S. states. The infestation of CMBS negatively impacts the flowering and fruiting of various ornamental and fruit plants. Crapemyrtle bark scale host confirmation is critical to determine the insect's potential risks to the Green Industry and help develop strategic management of CMBS. Previously confirming CMBS hosts was time-consuming. We investigated the CMBS feeding activities using the electrical penetration graph (EPG) to monitor real-time stylet penetration to determine potential hosts

more efficiently. First, we characterized typical EPG waveforms (waveform C, waveform potential drop, waveform E and waveform G) of feeding activities for CMBS on a validated host, *Lagerstroemia limii*. We then tested the feeding behavior of CMBS using different species, including *L. speciosa*, *L. indica* × *speciosa* '18096', Mexican beautyberry (*Callicarpa acuminata*), three Ficus species (*F. pumila*, *F. tikoua*, and *F. auriculata*), and soybean (*Glycine max*), with the positive control (*L. limii*). Results showed that plant species significantly impacted phloem sap ingestion of CMBS, which could be used to rapidly confirm a potential CMBS host.

INTRODUCTION

Crapemyrtle bark scale (CMBS), *Acanthococcus lagerstroemiae* (Hemiptera: Eriococcidae), is an invasive polyphagous insect (Kozár et al., 2013) which has spread across 17 U.S. states since its initial report in Texas in 2004 (EDDMapS, 2021). The reduction in flowering or fruiting on ornamental plants and crops resulted from the infestation and the observation of CMBS found on native species sharpened the concern about this invasive insect's threat potential to the Green Industry and ecosystems (Gu et al., 2014; Merchant et al., 2018; Wu et al., 2021; Xie et al., 2020; Zhang and Shi, 1986). Therefore, it would be crucial to determine the host range of this relatively new invasive insect for better estimating its risks to the local economy and ecosystem.

The host range assessment involves accepting or rejecting plant species via insect feeding performance (Schoonhoven et al., 2005). However, measuring the feeding performance of sap-sucking insects typically needs time-consuming tests regarding biological traits (Herbert et al., 2009; Wang et al., 2019; Wu et al., 2021; Wu et al., 2010). Stylet penetration could be a vital parameter for sap feeders to rapidly assess the host range through a real-time feeding monitor technique and an electrical penetration graph [EPG (Prado and Tjallingii, 1997)]. The EPG technique can track the position of the hemipterans' stylet tips in different plant tissue via voltage fluctuations amplified as specific EPG waveforms (Tjallingii, 1985), and these EPG waveforms were associated with biological feeding activities through histology correlation work (Tjallingii and Esch, 1993). Applying the EPG monitoring techniques in the feeding behavior study of CMBS could confirm the host rapidly and improve the understanding of the CMBS-plant interaction, which would further assist in developing integrated management of CMBS.

To date, little is known about the feeding behavior of CMBS or the CMBS-plant interaction. This study aimed to characterize EPG waveforms related to the feeding activities of CMBS on a validated host plant (*Lagerstroemia limii*) and assess the plant host suitability for CMBS rapidly by comparing its feeding parameters among different plant species.

MATERIALS AND METHODS

Insects and Plants. Colonies of CMBS were established by attaching CMBS-infested branches to healthy *L. limii* plants and maintained in a handmade chiffon mesh-covered cage (58.0 cm long × 58.0 cm wide × 50.0 cm high) in a CONVIRON® (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) growth chamber [25 ± 1 ℃, 60±5 % relative humidity (RH), and a photoperiod of 16 h light

(L):8 h dark (D)] at the Department of Biology, Texas A&M University. All CMBS used for EPG recordings were female adults of CMBS (2.1 ± 0.7 mm long; 1.2 ± 0.5 mm wide) obtained from the colony.

Plants used for characterizing the feeding behavior of CMBS were the confirmed host plant *L. limii* (n = 20). Plants used for comparing the feeding parameters by plant species were *L. limii* (n = 25), *Lagerstroemia speciosa* (n = 25), *Lagerstroemia indica* × *speciosa* '18096' (n = 20), *Callicarpa acuminata* (n = 20), *F. auriculata* (n = 20), *Ficus pumila* (n = 20), *Ficus tikoua* (n = 20), and *Glycine max* (n = 25). The Arabic number in the paratheses represented how many plants were tested for each species. The crapemyrtle plants (*L. limii* and *L. speciosa*) were initially provided by North Florida Research and Education Center (Quincy, FL). The crapemyrtle hybrid '18096' was selected from our crapemyrtle breeding program at the Department of Horticultural Sciences (College Station, TX). The *Ficus* species and Mexican beautyberry (*C. acuminata*) were initially provided by John Fairey Garden Conservation Foundation (Hempstead, TX). All these test plants were propagated via cuttings. They were maintained in 1 qt plastic pots (The HC Companies, Twinsburg, OH) filled with Jolly Gardener Pro-Line C/25 growing mixture (Oldcastle Lawn and Garden Inc, Poland Spring, ME) in the greenhouse at 25 ± 5 °C, 50 ± 10% RH, and a photoperiod of 10.5:13.5 (L:D) h.

Electrical penetration graph recordings of CMBS feeding on different plant species

The CMBS penetration activities were monitored by the EPG devices on different plant species, using individual CMBS female adults in a Faraday cage to characterize the feeding behavior of CMBS and test if plant species affect the feeding behavior. The EPG experiment was conducted in a climate-controlled room (25 ± 1 °C, 60±5 % RH, and a 16 h: 8h photoperiod) at the Department of Biology. The feeding behavior was monitored and recorded for 24 hours, and the recording was replicated using a new insect and a new plant for each species per time.

All typical EPG waveforms in the recordings were labeled manually. After comparing EPG waveforms of other sap-sucking insects (Prado and Tjallingii, 1994; Tjallingii, 1985; Tjallingii and Esch, 1993; Tjallingii, 2006), typical feeding waveforms of CMBS on the host were characterized with the help of EPGminer, a semiautomatic analysis application. Based on the biological feeding activities, EPG parameters about the feeding activities of CMBS on each plant species were compared, including the total duration of stylet pathway phase (waveform C and potential drop), total duration of phloem salivation (E1), total duration of phloem ingestion (E2), and total duration of xylem ingestion (G).

Data processing and statistical analysis

The ggplot2 (Hadley, 2016) and the plotly (Sievert, 2020) were used to generate visuals from R. The EPGminer package (supplementary) was newly developed to extract and analyze the EPG data. The values (mean with standard deviation) for the frequency and voltage (relative amplitude) were calculated by using functions, wave_topfreq and wave_volts, respectively.

Data analysis was performed using JMP® 16 (SAS Institute, Cary, NC). The parameters listed in Table 1 were analyzed using the one-way analysis of variance (ANOVA) to test the effect of plant species on the total duration of each feeding waveform. Tukey's Honestly Significant Difference (HSD) test (α =0.05) was used to compare the difference in each mean value.

Table 1. Characteristics of the EPG waveforms recorded during CMBS feeding on *Lagerstroemia limii*

EPG waveform		Waveform characteristics				Correlations
		Voltage level	Frequency (Hz)		Relative amplitude (%) [z]	Activities assigned for similar waveforms in other hemipterans
			Min-Max	Medium ± SE		
C		Extracellular	0.59-1.61	0.98 ± 0.10	11.81± 1.00	Sheath salivation and other intercellular stylet pathways
pd	pd1	Intracellular	0.42-6.10	4.35 ± 0.71	20.20 ± 2.20	Short cell punctures
	pd2	Intracellular	1.25-3.71	3.07 ± 0.28	23.38 ± 2.70	
E1		Intracellular	0.49-2.05	1.08 ± 0.24	32.43 ± 1.80	Phloem salivation
E2		Intracellular	0.49-2.05	0.78 ± 0.20	34.53 ± 2.90	Phloem sap ingestion
G		Extracellular	1.37-3.00	1.86 ± 0.20	11.72 ± 0.30	Xylem sap ingestion

[z] Relative amplitude (%) = (mean of amplitude for each waveform - mean of amplitude for non-probing)/ 5 ×100%

RESULTS AND DISCUSSION

Characterization of typical EPG waveforms for CMBS feeding behavior

EPG waveforms were characterized for CMBS when feeding on a host plant *L. limii* (Table 1), according to their shape, voltage level (extra- or intracellular), relative amplitude and frequency. These waveforms were labeled as C, pd1, pd2, E1, E2, and G.

Waveform C (Fig. 1A), correlating to gel salivation and other stylet pathway activities, was detected whenever CMBS started penetration and intercellular stylet pathway.

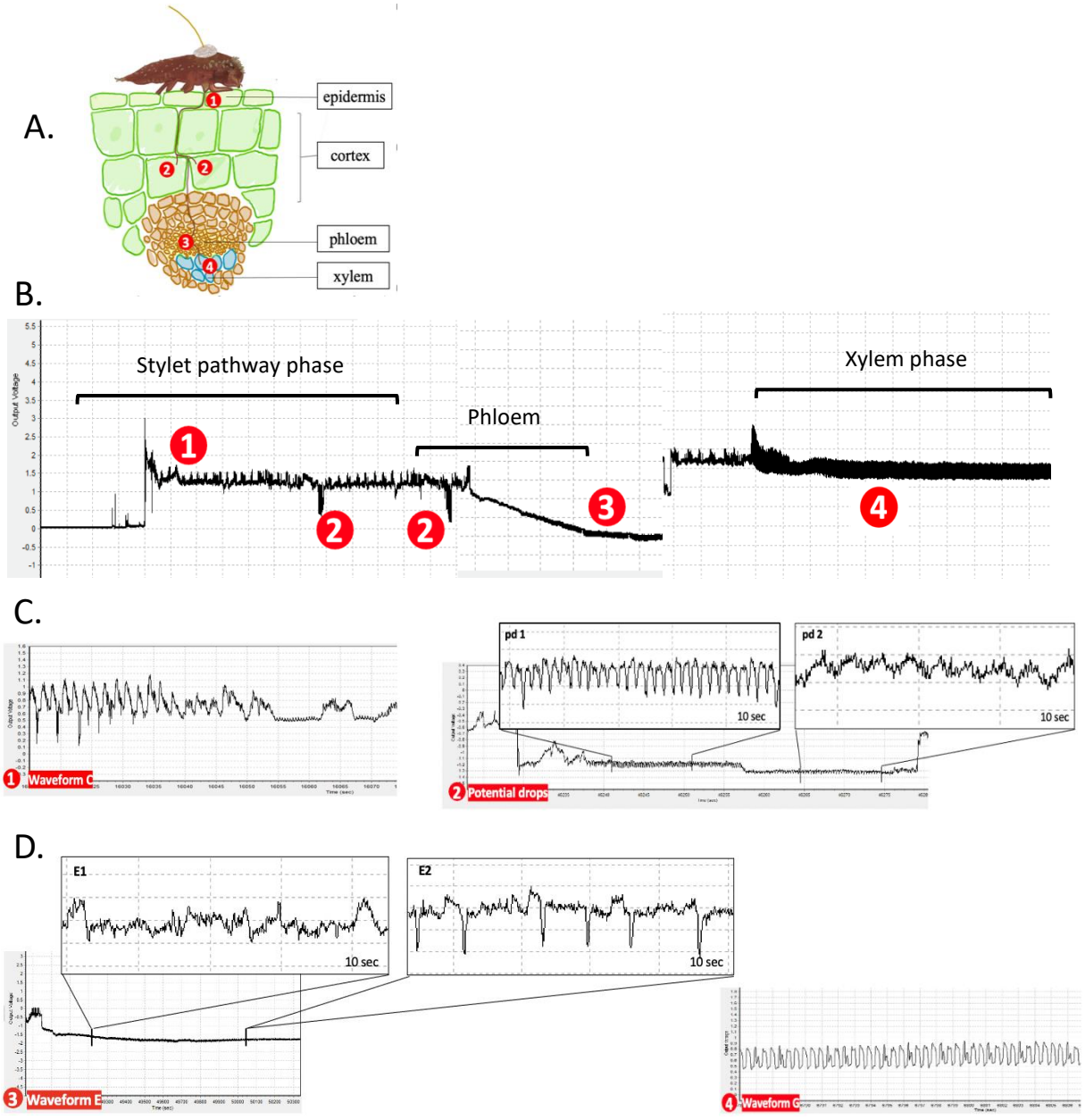

Figure 1 General scheme of characteristic feeding behavior of CMBS on *Lagerstroemia limii.* A: A diagram shows CMBS's stylet tip positions in a plant's stem when feeding. B: General scheme of characteristic EPG waveforms. C: ① Waveform C was detected when CMBS was probing intercellular part; ② Waveform potential drop (pd) was detected when the stylet tip punctured plant cells; ③ Waveform E1 was detected when intracellular stylet activity in mesophyll and phloem salivation occurred; Waveform E2, characterized by negative peaks, was detected when phloem sap ingestion occurred; ④ Waveform G was detected when xylem sap ingestion occurred.

Potential drops (Fig. 1B) were frequently observed during the stylet pathway phase. At the start, the voltage suddenly dropped when the stylet was supposed to puncture cells; during the low intracellular voltage level, the potential drops were often clearly divided into potential drop 1 (pd 1) and potential drop 2 (pd 2) periods. Waveform E complex (Fig. 1B), consisting of E1 and E2 phases during phloem phase, often sequentially followed the stylet pathway phase. The voltage level of the complex gradually and dramatically dropped below zero, which was much lower than other waveforms. Waveform E1, correlated to watery salivation, had positive peaks. Waveform G (Fig. 1C), correlated to xylem sap ingestion during the xylem phase, had a higher voltage level (extracellular) than other waveforms.

Comparison of feeding parameters of CMBS among different plant species

Even though plant species did not affect the total duration of waveform E1 (F = 1.9326; df = 7, 71; P = 0.0769), it affected the total duration of waveform C (F = 6.8815; df = 7, 71; P < 0.0001) and the total duration of waveform E2 (F = 8.2204; df = 7, 71; P < 0.0001) [Table 2]. After reaching the sieve elements, the insect spent the longest time in phloem sap ingestion on *L. limii* (234.78 ± 60.16 min) and the crapemrytle hybrid '18096' (286.43 ± 136.38 min), which was at least twice longer duration on *L. speciosa* (85.49 ± 38.84 min) and *C. acuminata* (19.84 ± 6.48 min). No individuals had phloem salivation or phloem ingestion on *F. pumila*, *F. auriculata*, or *G. max*.

Comparing with *F. auriculata*, even though the total duration of waveform G was multiple times greater on other species where the xylem ingestion occurred (varying from 90.27 ± 57.43 min to 423.54 ± 88.01 min), no significance was shown among the species [*L. limii*, *L. speciosa*, the hybrid '18096', *C. acuminata*, *F. tikuoa*, *F. pumila*, and *G. max* (F = 1.8371; df = 7, 71; P = 0.0934)].

From the perspective of stylet penetration activities, our study is the first report to elucidate the occurrences of phloem and xylem ingestion by CMBS on its host plant through the EPG techniques. We developed an R programming-based application to help identify and characterize the EPG waveforms with less human input. The comparison results of feeding parameters among different species indicated that CMBS accomplished the ingestion of phloem sap and xylem sap on the confirmed host plants (*C. acuminata*, *F. tikoua*, *L. limii*, *L. speciosa*) and the crapemyrtle hybrid '18096'. But CMBS did not intake phloem sap on *F. pumila*, *F. auriculata*, and *G. max*. With that, the "to eat, or not to eat" question was answered by applying the EPG techniques combined with computational analysis in the feeding behavior study of CMBS.

Supplementary

1) algorithm:
https://github.com/LylChun/epgminer

2) EPGminer in website version:
https://epgdata.shinyapps.io/epg-miner_app/

3) software version:
https://github.com/LylChun/epg-miner/tree/master/inst/epg-miner_app/rsconnect/shinyapps.io/epgdata

Table 2. Electrical penetration graph parameters of CMBS feeding on different plant species. C represents the stylet pathway phase; E1 represents phloem salivation; E2 represents phloem ingestion; G represents xylem ingestion.

Electrical penetration graph parameter	Plant Type			
	Lagerstroemia limii	***Lagerstroemia speciosa***	***Lagerstroemia indica* × *speciosa* '18096'**	***Callicarpa acuminata***
Total duration of C (min)	488.65 ± 84.53 bc	428.32 ± 76.55 bc	671.19 ± 216.54 abc	549.78 ± 86.02 bc
Total duration of pd (min)	14.76 ± 3.25 ab	24.34 ± 7.00 ab	19.87 ± 4.54 ab	26.20 ± 9.25 ab
Total duration of E1 (min)	63.33 ± 32.46 a	35.37 ± 11.37 a	51.36 ± 19.15 a	47.90 ± 11.77 a
Total duration of E2 (min)	234.78 ± 60.16 a	85.49 ± 38.84 bc	286.43 ± 136.38 ab	19.84 ± 6.48 c
Total duration of G	288.52 ± 54.60 a	239.28 ± 86.96 a	90.27 ± 57.43 a	289.15 ± 64.38 a
Electrical penetration graph parameter	**Plant Type**			
	Ficus tikoua	***Ficus pumila***	***Ficus auriculata***	***Glycine max***
Total duration of C (min)	768.57 ± 54.24 ab	477.63 ±108.22 bc	1183.10 ± 153.45 a	262.20 ± 57.31 c
Total duration of pd (min)	43.90 ± 12.82 a	20.87 ± 7.23 ab	14.77 ± 11.76 ab	3.17 ± 1.74 b
Total duration of E1 (min)	39.64 ± 9.75 a	0.00 a	0.00 a	0.00 a
Total duration of E2 (min)	99.66 ± 26.79 abc	0.00 c	0.00 c	0.00 c
Total duration of G	317.46 ± 39.78 a	423.54 ± 88.01 a	0.00 a	190.79 ± 79.39 a

Means (± SE) followed by different letters within a row for each electrical penetration parameters were different by Tukey's Honestly Significant Difference (HSD) test (α =0.05).

The p-value for the comparison difference in the total duration of a certain waveform: C < 0.0001, pd = 0.0128, E1 = 0.0769, E2 <0.0001, and G = 0.0934.

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