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Behavior

Characterization of an EPG Waveform Library for Redbanded Stink Bug, *Piezodorus guildinii* (Hemiptera: Pentatomidae), on Soybean Plants

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Abstract

Feeding behaviors of the redbanded stink bug, *Piezodorus guildinii* (Westwood), on vegetative (stem and leaflet) and reproductive (pod) tissues of soybean, *Glycine max* (L.), were recorded using an AC-DC electropenetrograph (EPG) apparatus. Eight different probing waveforms were characterized and defined: Pg1a, Pg1b, Pg1c, Pg1d, Pg2, Pg3a, Pg3b, and Pg4 grouped into three different families, P, I, and N. Histological studies of intact stylets within salivary sheaths observed during Pg1b, Pg1c, Pg2, and Pg3 waveforms were correlated with the specific penetration sites. Waveforms Pg1a, Pg1b, Pg1c, and Pg1d (pathway—family P) occurred at the start of probing activities and represent stylet penetration deep into plant tissue. Waveforms Pg2, Pg3a, and Pg3b (family I) represent the food ingestion phase. Pg2 waveform represents xylem sap ingestion primarily on leaves and stems. During Pg3a, stylets were moving, lacerating deeply into pod tissue and partially retracting; during Pg3b, stylets were motionless inside the pod tissue. Pg3b occurred interspersed with waveform Pg3a. Waveform Pg4 (family N) represented short interruptions that occurred within waveform Pg2. The study demonstrated that *P. guildinii* uses the cell rupture strategy to ingest from endosperm in soybean pod, and the same insect could switch to salivary sheath feeding to ingest from xylem in soybean leaves and stems. This unusual behavior explains symptoms of stink bug damage to soybean. The *P. guildinii* waveforms defined herein will allow future EPG studies to aid in development of soybean varieties that resist the feeding and damage caused by this and other stink bug pests.

Key words: Heteroptera, Pentatomidae, electrical penetration graph, electropenetrography, electronic feeding monitor

The neotropical stink bug *Piezodorus guildinii* (Westwood) (common names: Neotropical green stink bug and small green stink bug in South America, and redbanded stink bug in the United States) is a major pest of soybean in the Americas (Panizzi and Slansky 1985a). In recent years it has increased in abundance in the southern United States (Baur and Baldwin 2006, Kamminga et al. 2012), and is reported as the predominant stink bug species pest of soybean in the Upper Gulf Coast of Texas (Vyavhare et al. 2014).

Piezodorus guildinii biology and feeding damage to soybean has been investigated for many years (e.g., Panizzi and Smith 1977, Panizzi and Slanksy 1985b, Corrêa-Ferreira and Azevedo 2002). More recently, it has been demonstrated that this species is the most damaging among the several species of stink bug pests of soybean, due to suspected maceration of seed tissues (seed endosperm) caused by the action of its saliva (Depieri and Panizzi 2011). That said, there has never been direct evidence of maceration (termed cell rupturing) feeding by *P. guildinii*.

Detailed studies to elucidate feeding behaviors of true bugs (Hemiptera: Heteroptera) are greatly needed, not only to understand the nature of crop damage by these pests, but also to aid in the development of resistant varieties of soybean and other crops. The most rigorous method to study hemipteran feeding is through the use of the electrical penetration graph technology (McLean and Kinsey 1964, Tjallingii 1978), also known as electropenetrography (both abbreviated EPG). The few EPG studies of heteropterans to date include species in the families Miridae (Cline and Backus 2002, Backus et al. 2007), Coreidae (Bonjour et al. 1991, Cook and Neal 1999), and Blissidae (Backus et al. 2013). For stink bugs (Pentatomidae), to date, the only published EPG paper monitoring feeding, characterizing waveforms, and correlating them to specific feeding sites was carried out with the neotropical stem feeder, *Edessa meditabunda* (F.) on soybean (Lucini and Panizzi 2016). However, that study used the DC (2nd generation) electropenetrograph (EPG), which has limitations for recording large insects (Backus and Bennett 2009).

In this study, we recorded feeding behaviors of stink bugs for the first time with the new AC-DC EPG technology, producing the first-ever waveform library to define stink bug waveforms at multiple input impedances (amplifier sensitivities). Feeding behaviors of *P. guildinii* were recorded on three different tissues of the soybean plant (i.e., leaflet, stem, and pod). The waveform library allowed us to hypothesize the biological meanings of the waveforms; those probably related to salivation, food ingestion, and interruptions are identified. Finally, we histologically correlated feeding in specific soybean cell types with ingestion of nutrients and/or water.

Materials and Methods

Stink Bug Rearing and Soybean Plants

Colonies of *P. guildinii* were established in the laboratory. Adults collected from the field at the Embrapa Wheat Experiment Station at Passo Fundo, RS, Brazil (28° 15′ S latitude; 52° 24′ W longitude) were taken to the laboratory and placed in clear plastic rearing boxes (25 by 20 by 20 cm), lined with filter paper and provided with pods of green bean, *Phaseolus vulgaris* L., raw shelled peanut, *Arachis hypogaea* L., mature seeds of soybean, *Glycine max* (L.), and fruits (berries) of privet, *Ligustrum lucidum* Ait.

From January 2015 to May 2015, boxes were kept in a walk-in chamber at 25 ± 1°C temperature, 65 ± 5% RH, and a photoperiod of 14:10 (L:D) h. Food was checked daily, and replaced when necessary. The colony was often re-invigorated with the addition of field-collected adults, and nymphs obtained were raised to adulthood.

Soybean seeds cv. 'Tordilha' were sown in small pots (100 ml) and big pots (5 liter) in a greenhouse. Plants grown in small pots with ca. 15 cm height at V2 stage were used for EPG studies on vegetative tissues (stem and leaflet); plants grown in bigger pots were used for EPG studies on reproductive tissue, i.e., pod (at R5 stage). Stems containing pods were excised (entire plants bearing pods were too big to fit into the Faraday cages) with a razor, and then put into a small glass vial filled with wet sand as substrate. The plant electrode was introduced into the wet sand.

Electropenetrography (EPG) Recordings

The current study used a new type of EPG monitor, the 3rd-generation, four-channel AC-DC monitor (Backus and Bennett 2009; EPG Technologies, Inc., Gainesville, FL) for a recording period of 8 h (9:00 AM–5:00 PM). Throughout the experiment, standard equipment settings consisted of 50 mV alternating current (AC), input impedance of 10^7 Ohms, and amplification (gain) setting on the control box of 450 × (actual gain 4500 because there is a fixed gain of 100 × in the head stage amplifier). Changes in voltage output during stylet probing were amplified and rectified. To prevent inadvertent rectifier fold-over, both the prerectification and postrectification output signals were simultaneously recorded in separate channels, then the offset knob was used to visually match the two signals so that native waveform fidelity was retained through rectification. Output signals were digitized at a rate of 100 samples per s per channel using a DI-710 (Dataq Instruments, Akron, OH) and recorded by using a HP Pentium notebook with WinDaq Lite software (Dataq). The recordings were processed with the head stage amplifiers placed inside a Faraday cage in a closed room kept at 25 ± 2°C with artificial light.

To analyze the feeding behavior of *P. guildinii*, females of a similar age (10–14 d old) were separated from the laboratory colony and were starved for 18 h prior to recording, and then, the bugs were immobilized using an adhesive tape placed on the posterior part of the abdomen (P.L. Mitchell, personal information) on the top of a Petri dish lid. After that, the stink bugs had their pronota sanded, using a piece of human dental sand paper (2.0 by 0.4 cm) (Metalúrgica FAVA Ind. & Com., model MF 435L, SP, Brazil) which was passed over the insect 10 times (ca. 10 s), to improve attachment success of the gold wire (Lucini and Panizzi 2016).

The gold wire (3 cm long and 100 µm [sold as 0.004 in] in diameter) (Sigmund Cohn Corporation, Mount Vernon, NY) was glued to a copper wire, which in turn was soldered to a brass nail (together termed a stub). At the tip of the gold wire, a small loop (0.5 mm) was made to expand the surface of gold that would contact the stink bug pronotum, for improved electrical conductivity. A drop of conductive silver glue (water: silver flakes [Sigma-Aldrich, Saint Louis, MO]: white glue [Elmer's GlueAll, Westerville, OH]) (1:1:1; v:w:w), was placed on the center of the sanded pronotum, and the tip of the wire holding the loop was sunk into the glue, and after, it was allowed to dry for 40 min before recording.

After that, the adhesive tape was carefully removed to avoid injury and the bug was allowed to dangle for 10 min before being used in the EPG recording, to ensure that the gold wire was firmly attached to the bug pronotum. The stub was connected to the EPG head stage amplifier and the copper plant electrode (3 cm long) was inserted into the substrate containing either the entire plant or stem piece containing pods, to close the circuit when the insect probed, allowing recordings. It successfully recorded 64 adult females, being 22 on leaflet, 25 on stem, and 17 on pod. The waveforms were characterized taking into consideration previous waveforms obtained for other species of sucking insects, considering shape, amplitude, frequency, electrical origin, i.e., resistance (R), and electromotive force or biopotential (emf). The minimum and maximum amplitude of each waveform were determined in comparison to the waveform with greater amplitude during each event, considering the lowest valley to the highest peak (providing a relative amplitude) according to Backus et al. (2013). Waveform amplitude and frequency were estimated based on the average of five observations for each bug, with variable number of observations for each waveform.

The primary component or electrical origin of waveforms, i.e., R and/or emf, can be determined by switching the input impedance (Ri) among different Ri levels during a particular waveform. According to Backus and Bennett (2009), at low Ri levels (10^6 to 10^7 Ohm), the R component is more emphasized, becoming mostly R at 10^6 Ohm (for the aphid model). In contrast, the emf component is more emphasized at high Ri levels (10^9 to 10^{13} Ohm), becoming entirely emf at 10^{13} Ohm (again, for the aphid model). Therefore, one can identify which is the main component, R, emf or mixture of both from changes in waveform amplitude and waveform appearance itself. All bug recordings began at Ri level of 10^7 and switches were made from 10^6 to 10^{13} Ohm during the occurrence of a specific waveform. For 10^{13} Ohm, the applied signal was changed to 0 mv before recording.

Plant Tissue Histology

We performed correlations via histology between the stylet position and/or salivary sheath tip in soybean stem and pod tissue and different waveforms observed during EPG-AC recordings. For this study, a second set of *P. guildinii* adult females was recorded using the same conditions as the previous EPG recordings. When the respective waveform of interest was observed, the EPG monitor was turned off and the stylets were carefully cut using an entomological micro-scissors. Next, a piece of soybean stem (~ 2 cm) or a single pod, containing the severed stylets, was excised. Then, this piece or

pod of tissue was carefully hand-cut into thin sections using a sharp razor blade (Wilkinson Sword, United Kingdom) under a stereomicroscope (Wild Heerbrugg, Model M5A, Switzerland); the processing steps were minimal and gentle to prevent the dislodgement of the stylets.

The stem and pod sections were placed separately in Petri dishes containing 1:1 V:V water: commercial sodium hypochlorite (10%) (bleach) solution to clear the tissues. After a few minutes, the sections were rinsed in distilled water, and subsequently stained in 1% toluidine blue solution for 5 min (Sigma Aldrich, St. Louis, USA), and then, semipermanent slides were prepared in glycerinated gelatin. The position of the stylet tips and/or salivary sheath in the soybean stem and pod were determined based on four specimens each for waveform Pg1b and Pg1c, six specimens for Pg2, and three specimens for Pg3. Digital images were captured, using Olympus BX50 (Shinjuku, Tokyo, Japan) microscope coupled with a Sony DXC 107 A video camera (Minato, Tokyo, Japan) linked to a computer.

Results

During the AC-DC EPG recordings on vegetative (stem and leaflet) and reproductive (pod) soybean developmental stages, adult females of *P. guildinii* performed 11 different waveforms. In the case of probing waveforms, types were denoted by Pg [for *P. guildinii*] followed by a number and subtypes by an additional, lower-case letter. The waveforms are named: Np, Z, R, Pg1a, Pg1b, Pg1c, Pg1d, Pg2, Pg3a, Pg3b, and Pg4 grouped into three different families labeled: P, I, and N. These waveforms are summarized in Table 1 and Figures 1–6. The 11 waveforms were described based on their electrical characteristics (i.e., frequency [Hz], relative amplitude [maximum and minimum], and electrical origin [resistance—R or electromotive force—emf]). Because the waveforms from all three plant structures evaluated presented similar characteristics, we grouped them in the same table.

As with other studies, waveforms were divided into nonprobing (Np, Z, and R), and probing waveform types (Pg1, Pg2, Pg3, and Pg4). Probing waveforms were composed of three main phases: 1) pathway, 2) ingestion, and 3) interruption. Pathway phase had only one family (P) that was divided into four different waveform subtypes (Pg1a, Pg1b, Pg1c, and Pg1d). Ingestion phase comprised two families: 1) I (waveforms Pg2, Pg3a, and Pg3b), which is hypothesized to represent ingestion behaviors, and 2) N (waveform Pg4), which represents interruption behaviors.

Nonprobing Waveforms
(Z, Np, and R)

During Z waveform, the bug ($n = 64$) was standing still, static on the plant surface. This behavior occurred during periods of low-amplitude, baseline recordings. These Z events were interspersed with events of a high-amplitude and irregular-frequency waveform with several peaks, termed Np ($n = 64$), which were visually correlated with the bug walking on the plant surface (Fig. 1A; Table 1). The waveform R ($n = 46$) was observed when the bug touched the plant surface with its rostrum (labium) before it started stylet penetration into plant tissue (waveform Pg1, see below). The R comprises high-amplitude peaks that can be distinguished from Np by its large, regular peaks, and from Pg1 by its large peaks that always return to baseline level before Pg1 starts (Fig. 1G; Table 1). However, this waveform was not clearly observed in all recordings,

Table 1. Summary of AC-DC EPG waveforms, their electrical characteristics, and proposed stylet activities for each waveform of *Piezodorus guildinii* on vegetative (leaf and stem—V2 stage) and reproductive (pod—R5 stage) development stages of soybean plants

Phase	Family	Type or subtype	Plant structure	Rel. amplitude (%)	Frequency (Hz)	Electrical origin	Proposed activities[a]
Nonprobing	–	Np	Leaflet/ stem/ pod	Low/high	Irregular	–	Walking on the plant surface
		Z	Leaflet/stem/ pod	Very low	Irregular	–	Standing still on the plant surface
		R	Leaflet/ stem/ pod	High	Irregular	–	Tapping plant surface with rostrum (labium)
Pathway	P	Pg1a	Leaflet/ stem/ pod	93 (45–100)	Irregular	R	Beginning of stylet penetration and secretion of thickest gelling saliva to form the trunk of the salivary sheath
		Pg1b	Leaflet/ stem/ pod	78 (31–100)	Irregular + burst regular spikes 3.6 Hz (2.3–4.7)	R	Stylet penetration and secretion of gelling saliva to form branches of a salivary sheath
		Pg1c	Leaflet/ stem/ pod	40 (15–89)	Irregular	R	Salivary of thinner gelling saliva and deeper penetration of stylets
		Pg1d	Stem/ pod	25 (12–46)	4.9 Hz (4.2–6.0)	–	Bug encountering a rigid cell layer requiring stylet protraction and retraction
Ingestion	I	Pg2	Leaflet/ stem/ pod	30 (13–61)	3.6 Hz (2.8–4.8)	Mixed; peak = R, wave = emf	Xylem sap ingestion
Salivation	I	Pg3a	Pod	29 (15–48)	Irregular	R/ emf	Watery salivation into seed endosperm
Ingestion		Pg3b	Pod	8 (5–12)	4.4 (4.3–4.6)	emf	Ingestion of macerated seed endosperm
Interruptions	N	Pg4	Leaflet/ stem/ pod	36 (14–66)	Irregular	R	Brief interludes of salivation during xylem ingestion

[a] Based on previous studies performed with piercing–sucking insects (see references in text).

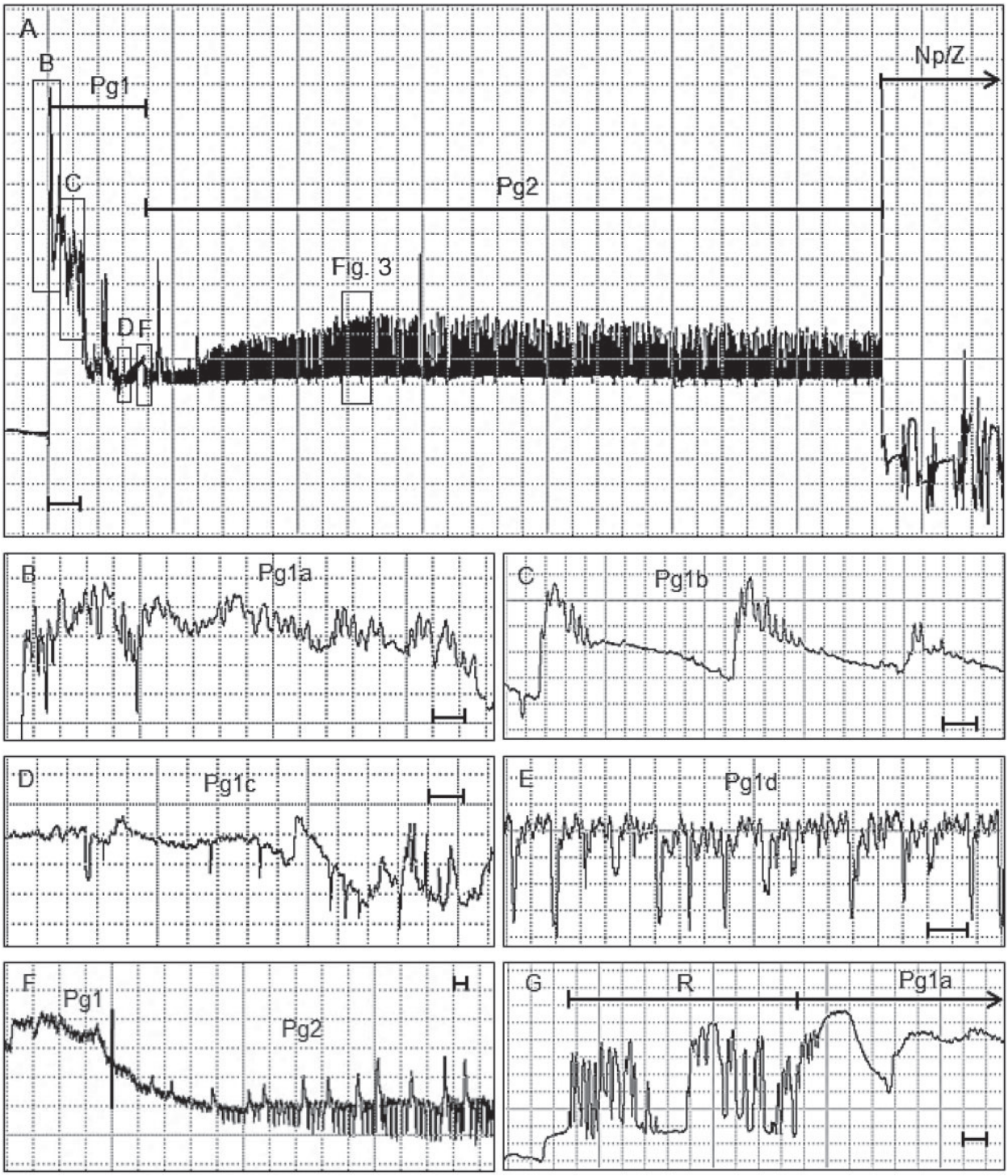

Fig. 1. Waveforms generated using EPG, AC applied voltage, and 10^7 Ohms input impedance, for *Piezodorus guildinii* adult on leaflet, stem, and pod of soybean. (**A**) Compressed overview of feeding behavior on soybean leaflet (~53 min, Windaq compression 400 [80 s/vertical div.], gain 16×); Scale bar is 100 s. (**B–E**) Detail of the waveforms Pg1a, Pg1b, Pg1c, and Pg1d, respectively. (**F**) Transition between waveform Pg1 and Pg2. (**G**) Detail of waveform R showing the moment that the bug touched the plant surface before starting stylet insertion (waveform Pg1a). (B has Windaq compression 4 [0.8 s/vertical div.], gain 8×, 14.5 s; C has compression 3 [0.6 s/vertical div.], gain 16×, 15 s; D has compression 4 [0.8 s/vertical div.], gain 64×, 13 s; E has compression 3 [0.6 s/vertical div.], gain 64×, 12 s; F has compression 10 [2 s/vertical div.], gain 32×, ~37 s); G has compression 5 [1 s/vertical div.], gain 16×, ~22 s). Scale bars are 1 s.

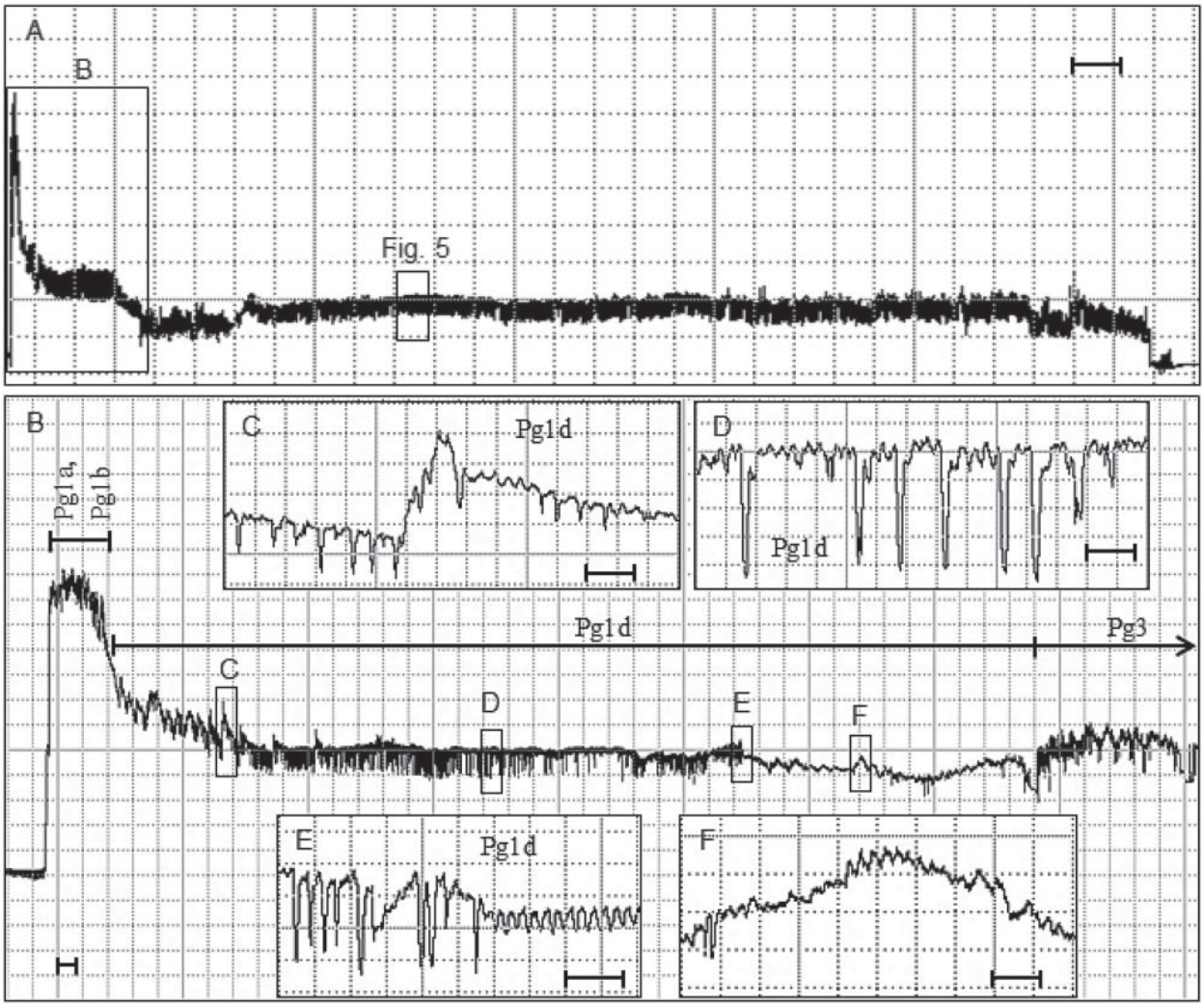

Fig. 2. Waveforms generated using EPG, AC applied voltage, and 10^7 Ohms input impedance, for *Piezodorus guildinii* on soybean pod (seed endosperm). (**A**) Compressed overview during feeding behavior of one probe (Windaq compression 800 [160 s/vertical div.], gain 4×, ~ 80 min), scale bar is 200 s. (**B**) Expanded view showing the sequence of events, waveforms Pg1a → Pag1b → Pg1d → Pg3, and details of the waveform Pg1d. (**C**) Detail of spike during early stages of waveform Pg1d; (**D**) middle stage of the waveform Pg1d; (**E-F**) transitional pattern before to begin the waveform Pg3. (B has Windaq compression 70 [14 s/vertical div.], gain 16×, ~ 11 min, scale bar is 10 s; C has compression 3 [0.6 s/vertical div.], gain 32×, 9 s; D has compression 3 [0.6 s/vertical div.], gain 64×, 9 s; E has compression 3 [0.6 s/vertical div.], gain 64×, 6 s; F has compression 4 [0.8 s/vertical div.], gain 64×, 8 s. Scale bars are 1 s.

because usually the bug touched the surface only briefly and once before beginning stylet insertion.

Pathway Phase

Family P (Pg1a, Pg1b, Pg1c, and Pg1d)

Histological correlations demonstrated that a salivary sheath was secreted during at least the beginning of each probe in all plant tissues. However, the salivary sheaths were easily observed under the microscope but digital images did not show the sheathes well in some sections, because histological views were of thick, free-hand sections, thus, salivary sheaths were often obscured by overlying tissues. We named the waveform phase at the beginning of each stylet penetration/probe pathway phase. This phase represents penetration deep into plant tissue, on leaflet, stem, or pod. The single family, P, consisting of one waveform type, Pg1, was divided into four different subtypes, named Pg1a, Pg1b, Pg1c, and Pg1d (Figs. 1B–E and 2B–F). These waveforms were observed in all three plant tissues, except the waveform Pg1d, which was only observed occasionally on stem and more frequently on soybean pod, but never on leaves. Moreover, pathway phase showed high voltage level at the beginning after stylet insertion, which decreased gradually over time (Figs. 1A and 2B).

Waveform Pg1a

This waveform ($n = 64$) occurred immediately after the stylet insertion into plant tissue in every probe. The voltage level rose suddenly from baseline (waveform Z) as a straight-vertical increase (Figs. 1B and 2B). Therefore, this waveform represents the first electrical contact between stylets and the plant tissue, and thus the first one associated with probing events. Pg1a was very brief (duration a few seconds) with R-dominated origin and high-amplitude with irregular-peaks; in addition, it had, most of the time, the highest relative amplitude among all the waveforms registered (Table 1).

Waveform Pg1b

Always recorded after waveform Pg1a, this waveform ($n = 60$) was a highly stereotypical series of repeating episodes. Each episode was characterized by a rapid increase of the voltage level followed by a gradual decrease (Fig. 1C). Often it started with high-amplitude peaks comprised of burst spikes (2.3–4.7 Hz-frequency) on top of a plateau. Pg1b had an R-dominated origin (Table 1).

Waveform Pg1c

This waveform ($n = 42$) followed the Pg1b waveform, but was not visible in all recordings on each of the three plant structures. Pg1c was characterized by a brief potential drop relative to Pg1b, which marked its beginning. Pg1c was primarily an underlying wave with medium-to-high amplitude, irregular-frequency, and R-dominated origin (Fig. 1D; Table 1).

Waveform Pg1d

Often following waveform Pg1b, Pg1d ($n = 16$) was only found during bug feeding on stem and pod. Spikes at its beginning were common, but spikes disappeared once the waveform became stable in appearance (Fig. 2C). This stereotypical waveform occurred before waveforms Pg2 in stem and pod, and Pg3 in pod. It often had low-to-medium amplitude (12–46%), high regular-frequency ranging from 4.2 to 6.0 Hz, with downward peaks (Figs. 1E and 2C–E; Table 1). At the end of the Pg1d and just before beginning waveform Pg3, and sometimes Pg2, a very short transitional pattern was observed, which was characterized by a mixture of regular-frequency (4.8 Hz) and irregular-frequency plateaus (Fig. 2E, F).

Ingestion Phase

Family I (Pg2, Pg3a, and Pg3b)

The family I was composed of three different waveforms, named Pg2, Pg3a, and Pg3b. Waveform Pg2 ($n = 62$) occurred in the following sequences: either Pg1a → Pg1b → Pg2 or Pg1a → Pg1b → Pg1c →Pg2 (the latter more often), while the sequence Pg1a → Pg1b → Pg1d → Pg2 (recorded only in stem and pod) was rarely observed. At the beginning of Pg2, a decrease of voltage level from Pg1b or Pg1c was often observed (Fig. 1F). Pg2 was recorded in all plant structures tested (stem, leaflet, and pod), and (in general) it presented similar electrical characteristics, sometimes with slight differences only in appearance of the waveform (Fig. 3A–F). Pg2 showed medium-amplitude (mean of 30%), and regular frequency ranging from 2.8 to 4.8 Hz (mean of 3.6 Hz) for all three plant structures assessed (Table 1). Moreover, Pg2 was always composed of repetitive waves interspersed with upward peaks, which occurred at regular intervals over the entire waveform (Fig. 3B).

Regarding the electrical origin (Fig. 4A–H), the present study demonstrated that waveform Pg2 has a mix of R and emf components. At low Ri levels (10^6 to 10^8 Ohm), peaks were more emphasized than waves, which were tiny at 10^6 Ohm (Fig. 4A, B); in contrast, the wave portion was more strongly emphasized at Ri 10^9 or higher inputs (Fig. 4G, H), where peaks almost completely disappeared into the wave portion. This change supports that the peak was essentially R component, whereas the wave portion was mostly emf-dominated.

Waveform Pg3 was observed only during feeding activities of *P. guildinii* on soybean pod and was divided into two different subtypes: Pg3a and Pg3b (Fig. 5A, B). In general, Pg3 was always preceded by Pg1d, occurring in the sequence Pg1a → Pg1b → Pg1d → Pg3 (Fig. 2B), and it was not as regularly repetitive as waveform Pg2. The waveform Pg3a was only observed in 35% of the bugs recorded on pods ($n = 6$), and presented low-to-medium amplitude, ca. 30% (ranging from 15 and 48%), irregular frequency with several large downward peaks (Fig. 5A, B; Table 1). Waveform Pg3b ($n = 6$) was often characterized by short durations, always interspersed with waveform Pg3a (Fig. 5A, B). It was also low-amplitude with relatively flat-line appearance (mean of 8%) but with a high-regular frequency, ranging from 4.3 to 4.6 Hz (Table 1).

Pg3a demonstrated a mixed of R and emf origin. At low Ri levels (10^6 to 10^7 Ohm), Pg3a showed downward peaks, whereas at high Ri levels (10^9 Ohm or higher) most peaks inverted to upward (Fig. 6). This inversion was not due to rectifier fold-over (see Methods). Waveform Pg3b was tiny at 10^6 Ohm compared with higher Ri levels, and its appearance was more clearly viewed at 10^7 to 10^9 ohm (Fig. 6). These results support that Pg3b was mostly composed of emf component (Table 1).

Interruption Phase

Family N (Pg4)

This family comprised short interruptions (≈ 4 s), that occurred within waveform Pg2, and presented one waveform type, named Pg4. Waveform Pg4 ($n = 23$) showed irregular form, medium-to-high amplitude (14–66%) and a flat-spiky plateau. Pg4 occurred mainly at the beginning of the waveform Pg2; it was observed in 36% of the bugs at a mean repetition rate of 3 events/bug in the three plant structures evaluated (Fig. 3G, H; Table1).

Correlations Between Specific Waveforms and Position of Stylets or Salivary Sheath Tips in Plant Tissues

During stylet penetration of *P. guildinii*, a salivary sheath always surrounded the stylets the entire length of the stylet progress (Fig. 7A–D) on soybean stem. For waveforms Pg1b and Pg1c, all tips of severed stylets and salivary sheaths ($n = 4$) were observed in parenchyma tissue on soybean stem and pod. These findings support that Pg1 represents stylet pathway through this tissue (Fig. 7A, B). The salivary sheath is sometimes not clearly observed in the figures, as shown in Fig. 7B, E (however, when viewed directly under a microscope, the sheath is clear in this image). However, on some rare occasions, it appears that no sheath is secreted by the stink bug (Fig. 7C). During Pg2, all sections on stem and pod ($n = 6$) revealed that both stylets and salivary sheath tips (when created) were in xylem cells on soybean stem (Fig. 7C, D) and pod (Fig. 7E). For Pg3, we were unable to obtain visible sections to take images; however, cutting the pods ($n = 3$) during this waveform revealed an opaque-white, damaged area in seed endosperm near the severed stylet bundle, which likely was caused by stink bug feeding (Fig. 7F).

Discussion

The current study is the second paper to study the feeding behavior of a stink bug (Pentatomidae), in this case, *P. guildinii*, an important pest of soybean crops in South America and in the United States. We have described 11 waveforms obtained using an AC-DC EPG, associated with the probing behavior of the *P. guildinii* on three different structures of the soybean plant. In addition, we herein produce the first waveform library (collection of waveform appearances at multiple input impedance [Ri] levels) for any pentatomid bug.

According to the literature, hemipterans use one of two feeding strategies, known as salivary sheath feeding and cell rupture feeding (Backus et al. 2005b, previously known as lacerate-and-flush feeding [Miles 1972]). Using the first strategy, an insect creates a salivary sheath, by secretion of solidifying (gelling) saliva, which surrounds

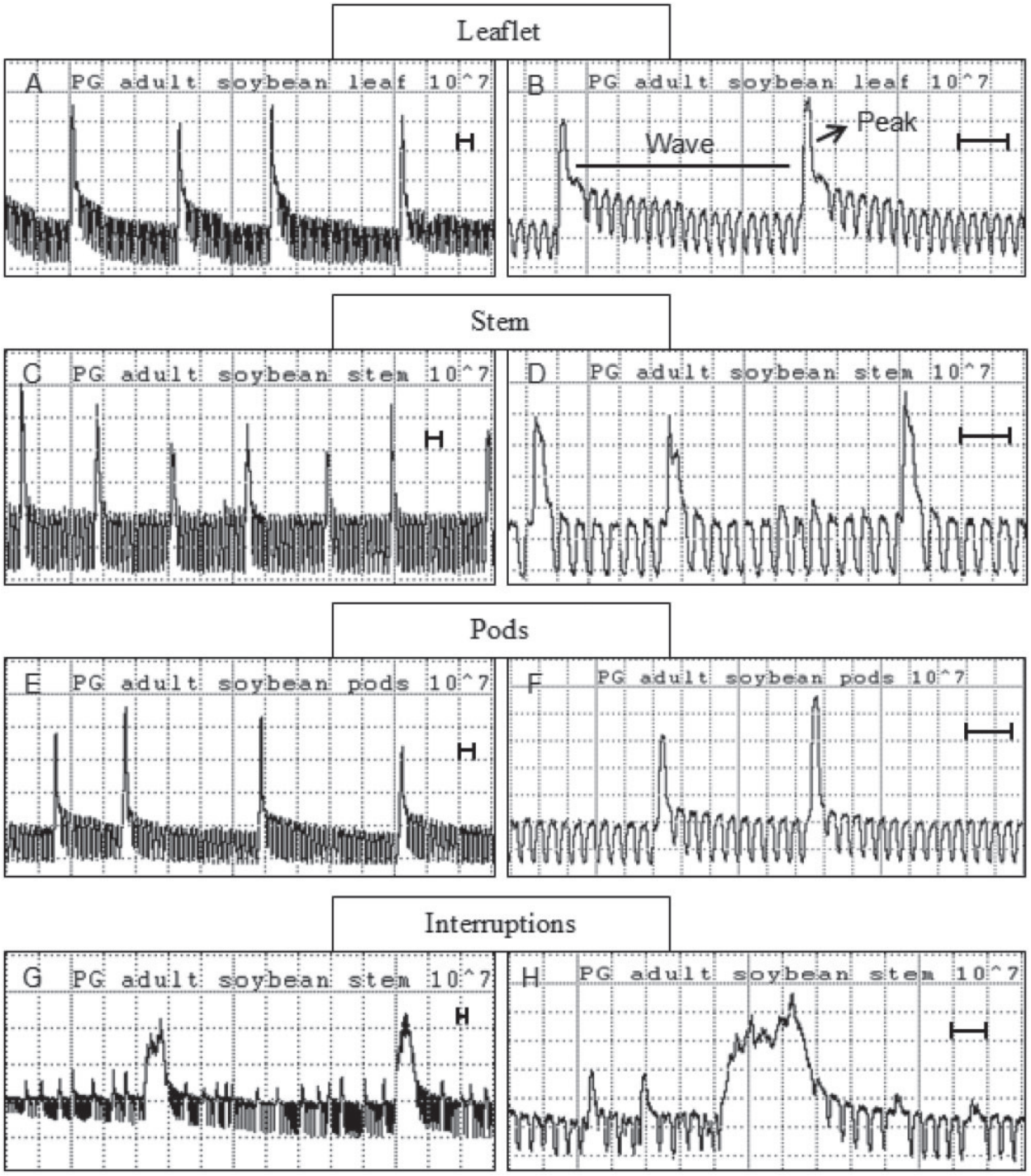

Fig. 3. Detail of the waveform Pg2 recorded during feeding behavior of *Piezodorus guildinii* on soybean leaflet (**A**, **B**); stem (**C**, **D**); and pod (**E**, **F**) (AC applied voltage, 10^7 Ohms input impedance). (B) Definition of wave versus peak. (**G-H**) Detail of the interruptions (waveform Pg4) observed during waveform Pg2. (A has Windaq compression 10 [2 s/vertical div.], gain 32×, 30 s; B has compression 3 [0.6 s/vertical div.], gain 32×, 10 s; C has compression 10 [2 s/vertical div.], gain 64×, 30 s; D has compression 3 [0.6 s/vertical div.], gain 64×, 10 s; E has compression 10 [2 s/vertical div.], gain 32×, 30 s; F has compression 3 [0.6 s/vertical div.], gain 32×, 10.8 s; G has compression 20 [4 s/vertical div.], gain 16×, 60 s; H has compression 5 [1 s/vertical div.], gain 32×, 15 s). Scale bars are 1 s.

the stylets during its entire stylet progress toward to its preferred feeding site. Waveforms during sheath formation are termed pathway phase. On the other hand, in the second strategy, cell rupture feeding, the insect does not make a complete salivary sheath. Instead, watery saliva is secreted simultaneously with continuous movement of the stylets deep into the plant tissue, which causes cell laceration and enzymatic maceration of tissues; the insect then ingests the slurry of cell contents (Miles 1972).

We hypothesize that *P. guildinii* could use both strategies when feeding on soybean plants; i.e., on soybean stem, leaf, and pod

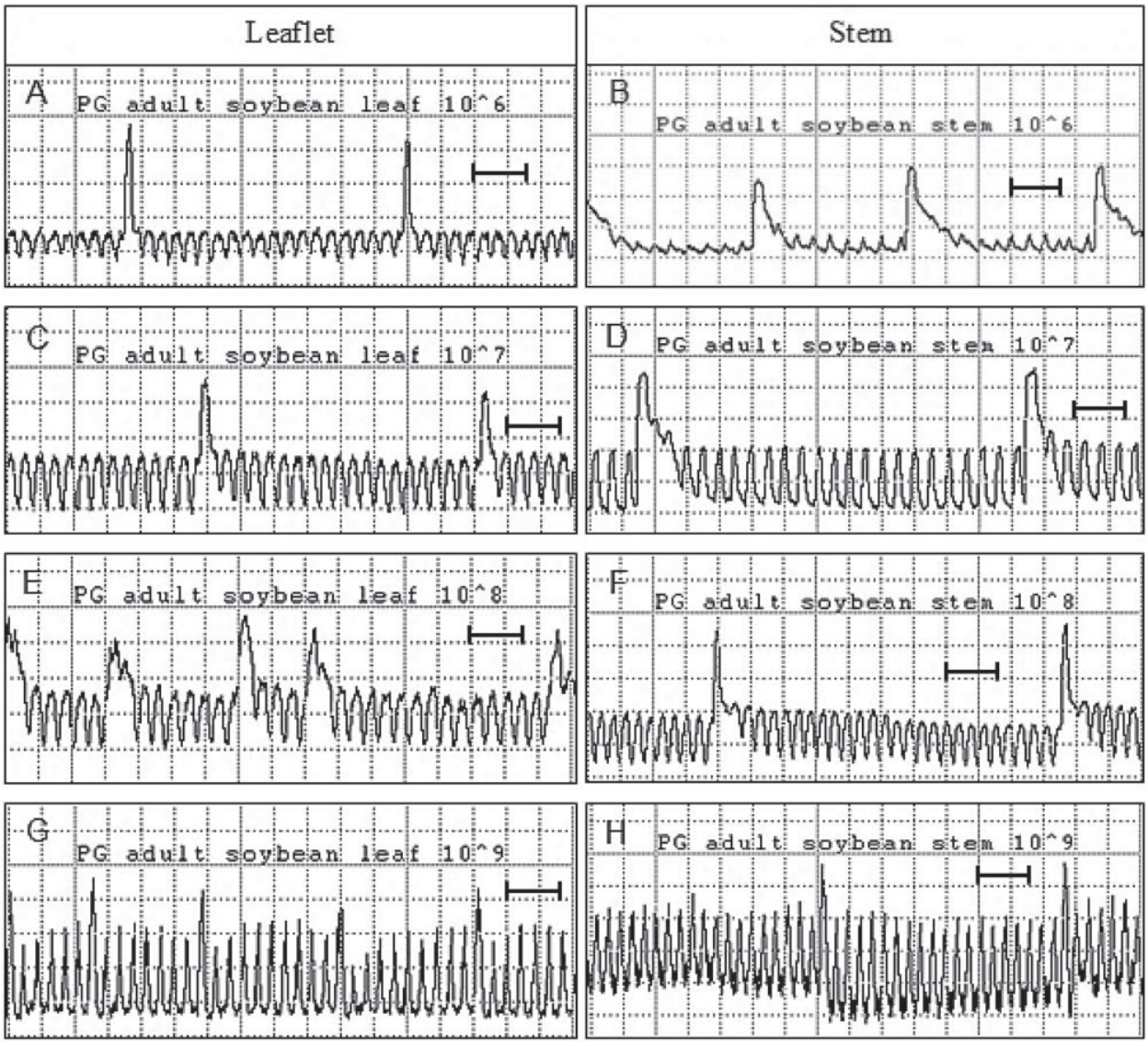

Fig. 4. Comparison of stable Pg2 waveform of adults of *Piezodorus guildinii* recorded at four Ri levels (10^6, 10^7, 10^8, and 10^9 Ohms) using AC applied signal on leaflet (**A**, **C**, **E**, **G**) and stem (**B**, **D**, **F**, **H**). Leaflet has Windaq compression 3 [0.60 s/vertical div.], gains $10^6 = 128\times$; 10^7–$10^9 = 16\times$; Stem has compression 3 [0.60 s/vertical div.], gains 10^6–$10^7 = 64\times$; 10^8–$10^9 = 32\times$. Scale bars are 1 s.

(pod-wall) the stink bug uses salivary sheath feeding while on soybean pod (seed locus) it may use cell rupture feeding. The pathway phase waveforms (Pg1) characterized herein seems to occur during both sheath feeding and cell rupture feeding.

Pathway Phase (Pg1a, Pg1b, Pg1c, and Pg1d)

Pg1a occurred during short events (few seconds), and undoubtedly represents initial stylet probing of the plant. This waveform always occurred after a non-probing phase (waves Z, Np and R) and it was performed for all bugs recorded. In the stereotypical sequence, the next waveforms recorded were usually Pg1b and Pg1c. These three waveforms were recorded on all three plant structures assessed and probably represent secretion of gelling saliva, formation of the salivary sheath, and stylet penetration deeper into plant tissue toward the preferred ingestion site. This interpretation was confirmed in all histological sections correlated with Pg1b and Pg1c, as recorded in other salivary sheath feeders (e.g., Bonani et al. 2010, Miranda et al. 2009, Seo et al. 2009, Lucini and Panizzi 2016). These hypotheses are supported by electrical origins, which were strongly R-dominated for all three waveform subtypes, since the saliva is highly electrical conductive. In addition, Pg1b resembles parts of the putative waveform G2 of chinch bugs, *Blissus insularis* Barber and *Blissus occiduus* Barber (Backus et al. 2013), which was composed of high-amplitude with plateaus (with spikes on the top) and valleys; G2 was correlated with putative secretion of gelling saliva and formation of salivary sheath.

Sometimes, the salivary sheath was not clearly observed, or may not have been created by the stink bug. Creation of the sheath appeared to vary by plant tissue. On stem and pod, five out of six evaluated samples showed an evident salivary sheath. However, on soybean pod, the salivary sheath may not have been created during stylet movements (although our image is out of focus and does not definitively show absence of sheath saliva). Nonetheless, we hypothesize that *P. guildinii* may secrete only watery saliva when feeding on pod. In histological images, a slight layer of sheath saliva

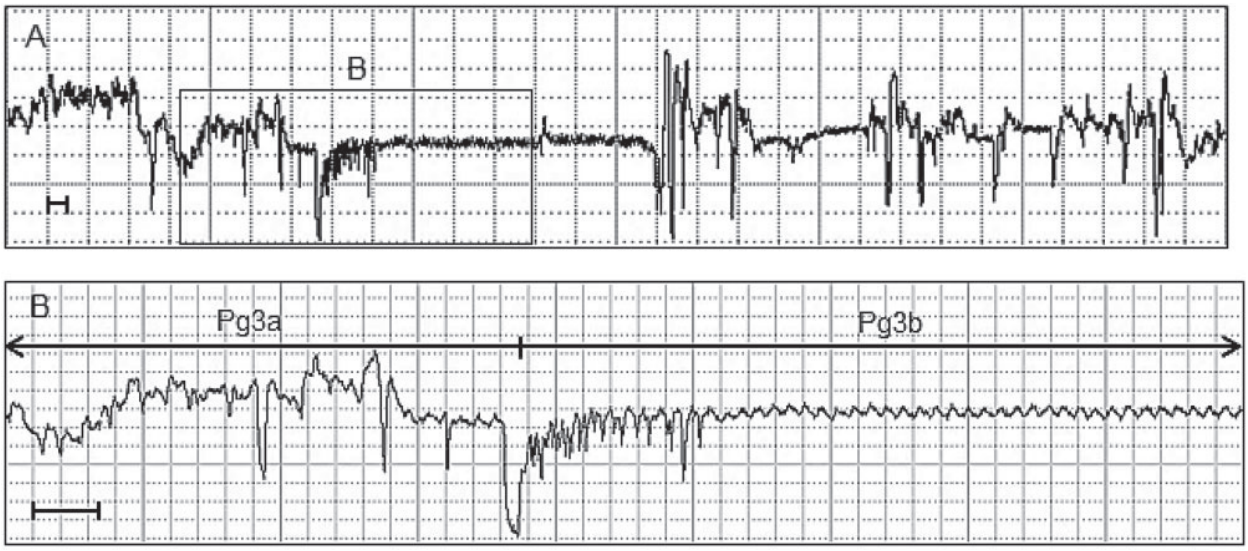

Fig. 5. Detail of the waveforms Pg3a and Pg3b recorded during feeding behavior of *Piezodorus guildinii* on soybean pod (seed endosperm) using EPG, AC applied voltage, and 10^7 Ohms input impedance. (**A**) Detail of the waveform Pg3. (**B**) Waveforms Pg3a and Pg3b, which may represent the cell-rupturing and saliva secretion (wave Pg3a), and ingestion of the cell content (Pg3b) of seed endosperm. (A has Windaq compression 10 [2 s/vertical div.], gain 64×, 60 s; B has compression 2 [0.4 s/vertical div.], gain 128×, 18 s). Scale bars are 1 s.

may surround the stylets at the beginning of stylet insertion, and again later, near xylem tissue, there may be gelling saliva to create salivary sheath. Nonetheless, this hypothesis can only be definitively supported via thin sections of probed plant tissue.

Waveform Pg1c is similar to putative waveform H of chinch bugs (Backus et al. 2013) and waveform B1 of the sharpshooter *Homalodisca coagulata* (Say) (Backus et al. 2005a), which have been correlated with protracting (extending inward) and retracting (withdrawal outward) the stylets for deep penetration into the vascular cells (in these cases xylem); such activities were previously correlated with the sharpshooter waveform B1 via histological studies. All of the previously published waveforms, B1, G2 and H co-occur with salivary sheath formation and are strongly R-dominated, like at Pg1b and Pg1c of *P. guildinii*.

The waveform Pg1d was recorded only during feeding behavior of *P. guildinii* on either stem or (more often) pod. Pg1d occurred either before Pg2 in stem and pod or Pg3 in pod. On the pod, we observed the occurrence of this waveform when the stink bug fed on any part of the pod, i.e., seed locus, pod-wall joint, or pedicel; in the latter (observed three times), however, the bug did not reach the ingestion site. We hypothesized that this waveform might be an X-wave (a stereotypical transition pattern, correlated with stylet penetration and subsequent activities inside the preferred ingestion tissue, that is performed 100% of the time before stylets of salivary sheath feeders enter such cells) recorded in other sheath feeders (Wayadande and Nault 1993, Backus et al. 2013, Lucini and Panizzi 2016). However, we now suspect that Pg1d is not an X-wave, because it was observed in only a few recordings before Pg2 on stem and pod, and it was not observed on leaflet. Moreover, Pg1d was also recorded in pods preceding waveform Pg3.

When Pg1d occurred on soybean pod, it was visually correlated with *P. guildinii* pushing its head and forcing down its stylets into the plant tissue, and subsequently retracting the head and stylets upward. Therefore, it seems plausible that Pg1d represents lacerating or sawing stylet movements. Perhaps Pg1d could be related to the bug encountering a rigid (lignified) cell layer that would make stylet penetration more difficult. Such a cell layer was observed in histological analyses of the soybean pod (sclerenchyma), which the bug needs to overcome to reach the seed endosperm. To our knowledge, this is the first time that this waveform with regular downward peaks has been recorded during feeding behavior studies using EPG.

Ingestion Phase: Xylem Sap Ingestion (Pg2)

The waveform Pg2 was recorded in *P. guildinii* that fed on leaflet, stem, or pod. It is probably associated with stylet activities in xylem vessels, more specifically, sap ingestion. Pg2 strongly resembles the putative waveform H-I2 recorded to chinch bugs, *B. insularis* and *B. occiduus* (Backus et al. 2013) and waveform Em2 of the pentatomid *E. meditabunda* (Lucini and Panizzi 2016) which are characterized by regular waves interspersed with occasional peaks of larger amplitude. In addition, Pg2 shares some electrical characteristics with the previous, published waveforms such as: 1) high relative amplitude, 2) mixture of electrical origins, with peaks R-dominated, and waves emf-dominated, and 3) not being preceded by an X wave. Putative H-I2 and Em2 have been correlated with ingestion from xylem vessels (Backus et al. 2013, Lucini and Panizzi 2016). Furthermore, Em2 was strongly correlated with salivary sheath termini in xylem vessels via histological studies (Lucini and Panizzi 2016). In addition, our histological studies with *P. guildinii* demonstrated that both stylet tips and salivary sheaths terminate in xylem vessels during Pg2 recorded on soybean stem and pod.

Most of the bugs performed waveform Pg2 on all three plant structures; however, during the recording time about half of them ingested xylem sap once and no more than twice. This sap ingestion is likely to be related to the 18-h starvation period before EPG recording; ingestion of liquid from xylem tissue may be a strategy used to avoid dehydration and to maintain water balance, as observed in aphids and psyllids (Spiller et al. 1990, Bonani et al. 2010, Pompon et al. 2010), and stink bugs (Lucini and Panizzi 2016). In the same way, we propose that *P. guildinii* also may be using this adaptation to overcome stress; it is known that this stink bug feeds preferentially on soybean seed endosperm, concentrated in

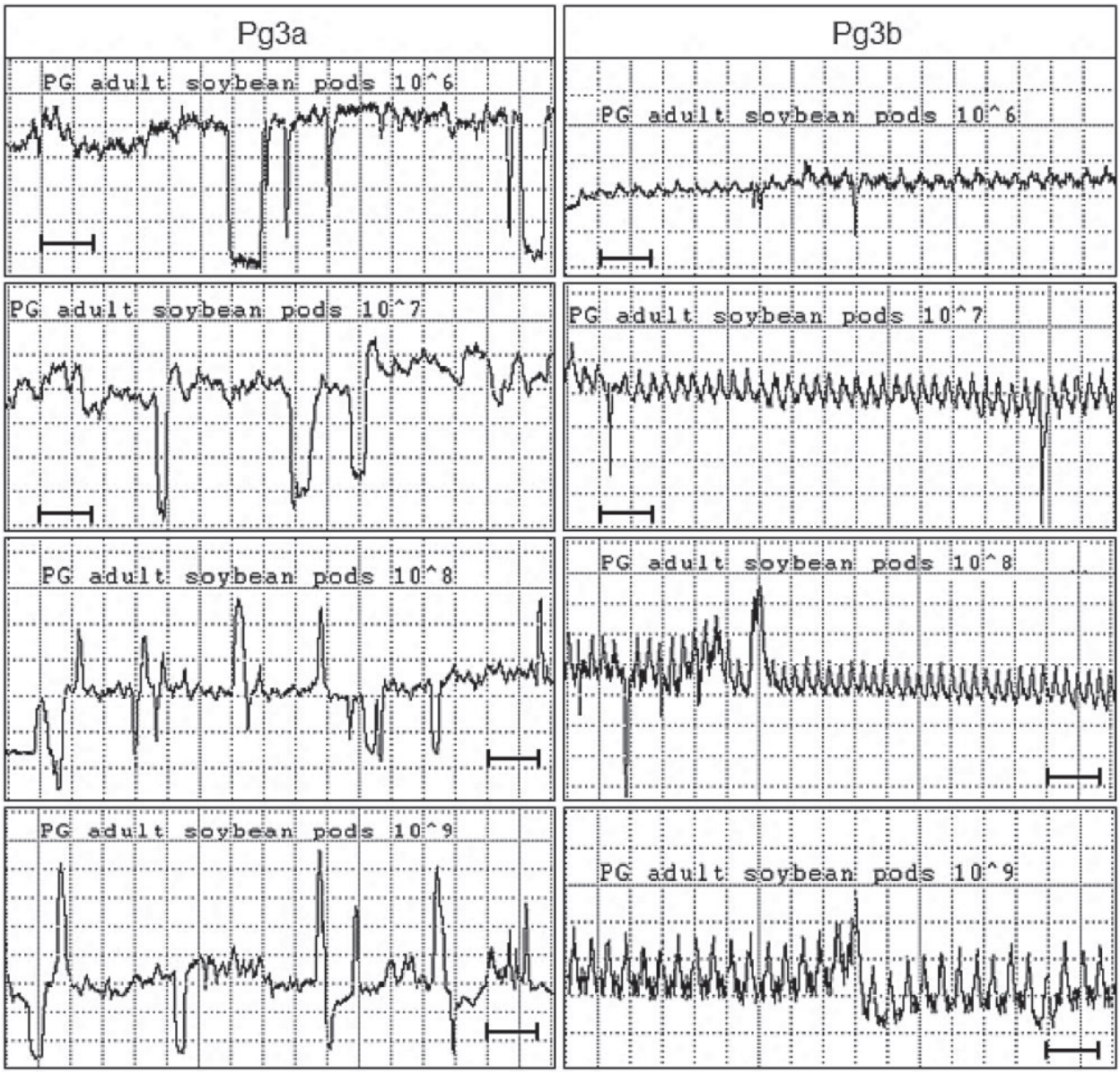

Fig. 6. Comparison of stable Pg3a and Pg3b waveform of adults of *Piezodorus guildinii* recorded at four different input impedance (Ri) levels (10^6, 10^7, 10^8, and 10^9 Ohms) using AC applied voltage. (Pg3a has Windaq compression 3 [0.60 s/vertical div.], gains: $10^6 = 128\times$; $10^7 = 64\times$; 10^8, $10^9 = 16\times$; Pg3b has compression 3 [0.60 s/vertical div.], gains: 10^6, $10^7 = 128\times$; 10^8, $10^9 = 32\times$). Scale bars are 1 s.

nutrients (Panizzi and Slansky 1985b), which demand ingestion of a more watery food to balance the nutrient concentration.

Ingestion Phase: Seed Endosperm Activities (Pg3)

Waveform Pg3 was found only during feeding of the stink bug on soybean pod, and does not resemble the waveform Pg2, which was correlated with xylem vessels. This suggests that Pg3 probably is correlated with salivation and ingestion of seed endosperm. The resulting damage (putatively from the effects of watery saliva) was visually observed by cutting the seeds with a razor blade.

During the waveform Pg3a we observed that the stylets were rapidly and repeatedly protracted deeply into the pod tissue and partially retracted. This behavior, similar to the one observed during Pg1d was performed continuously during this waveform; this is also is similar to waveform Ia recorded for the leafhoppers *Empoasca fabae* (Harris), *Empoasca kraemeri* (Ross & Moore) (Calderon and Backus 1992) and waveform B for the bug *Lygus hesperus* Knight (Cline and Backus 2002) (both of these studies used an AC monitor with fixed Ri level of 10^6 Ohm), as well as waveforms E1-B and E1-C for *Empoasca vitis* Göthe (Jin et al. 2012) (which used a DC monitor with fixed Ri level of 10^9 Ohm). All of these published waveforms have been correlated with different stylet activities that occur simultaneously, such as intracellular penetration of several cells, salivation, and activities of stylet protraction and retraction into plant tissue. However, no ingestion (or very rare ingestion) is thought to occur during these waveforms, i.e., the main function of these activities is likely to be cell rupturing, i.e., preparing the area by mechanical and chemical mechanisms for future ingestion events. In a similar way, *P. guildinii* could be using this strategy to dissolve the

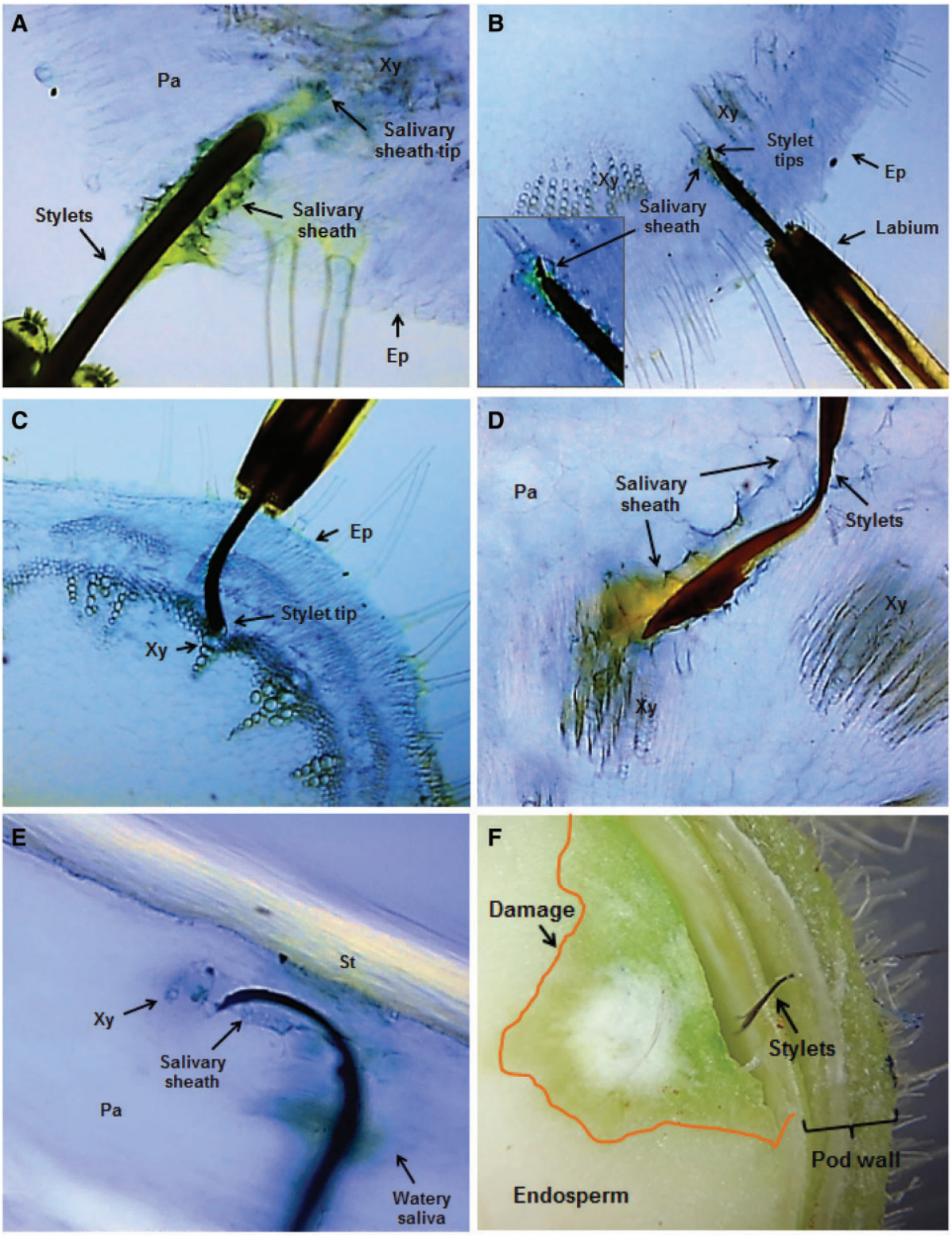

Fig. 7. Cross-sections of soybean plant containing stylets and salivary sheaths of *Piezodorus guildinii* on soybean stem and pod. (**A**) Salivary sheath ending in parenchyma tissue of soybean stem during waveform Pg1b (deeper pathway portion of probe) (10×). (**B**) Stylet and salivary sheath tips in parenchyma tissue of soybean stem, near the likely ingestion cell type (xylem) during waveform Pg1c (5×). (**C, D**) Stylet tips and salivary sheath ending in xylem vessels (longitudinal cut in D) during waveform Pg2 recorded on soybean stem (5× and 10×, respectively). (**E**) Stylet tips in xylem vessels during waveform Pg2, recorded on soybean pod-wall (10×). Note the small salivary sheath near xylem vessel. The proximal portion of the stylet bundle (out of focus) is surrounded by a fuzzy deposit of putative saliva that may be watery saliva or out-of-focus sheath saliva. (**F**) Cross-section of soybean pod showing damage near the insertion point of the stylets of the stink bug. Recording was terminated in waveform Pg3 after previous pathway (Pg1 waveforms). An orange line was drawn around the damaged area. Ep, stem and pod epidermis; Pa, parenchyma; St, Sclerenchyma; Xy, xylem.

endosperm cells. Depieri and Panizzi (2011) observed that *P. guildinii* feeding on soybean seeds caused the complete dissolution of the protein bodies through chemical action, i.e., salivary enzymes secreted during salivation periods in feeding. In *L. hesperus*, Backus et al. (2007) observed the occurrence of cell laceration by stylets; in addition, the secretion of enzymes in the watery saliva, e.g., polygalacturonases (Shackel et al. 2005), enhance the action of mechanical cell rupture.

During waveform Pg3b, *P. guildinii* stylets were motionless inside the pod tissue for a brief time, and after this brief period, the stylets again moved vigorously (waveform Pg3a). Pg3b occurred interspersed with waveform Pg3a; however, event durations of Pg3b were very short compared with events of Pg3a. Thus, waveform Pg3b probably represents fluid ingestion from the cells previously macerated by saliva. Similarly, the waveform Ic, recorded with *E. fabae* and *E. kraemeri*, is associated with ingestion of fluids originated by cell rupturing (Calderon and Backus 1992), which was also observed during waveform C1 of *L. hesperus* (Cline and Backus 2002). In those recordings at Ri 10^6 Ohm using old-fashioned strip chart recorders, Ic as well as C1 appeared as flat lines, and, according to these last authors, this suggests that little or no salivation occurs at this time. Our recordings of Pg3b at Ri 10^6 Ohm were also very flat, but showed slightly more detail probably due to high-quality, instrumentation amplifiers and more advanced, computerized recording. The flat line could also be due to lack of detection of emf at low Ri level, for this emf-dominated waveform.

It is known from the literature that *E. fabae* and *E. kraemeri* use the cell rupture strategy of hemipteran feeding (Backus et al. 2005b), originally termed lacerate-and-flush (Miles 1972, Calderon and Backus 1992). Backus et al. (2005b) describe four different "stylet penetration tactics" within the cell rupture strategy. For *L. hesperus*, Backus et al. (2007), described its tactic as lacerate/macerate-and-flush. So, *P. guildinii* could be using a similar stylet penetration tactic to rupture soybean endosperm cells. However, it would differ from the tactics used by other cell rupture feeders in having a salivary sheath present in the early part of the probe.

Interruption Phase: Saliva Injections Into Xylem (Pg4)

We also observed brief interruptions (Pg4) during xylem vessel ingestion (Pg2) in 36% of the recorded bugs. Waveform Pg4 is similar to the other interruption waveforms discussed in the literature (named family N) during xylem ingestion, such as, those for the sharpshooters *H. coagulata* (Backus et al. 2005a) (now *H. vitripennis*) and *Bucephalogonia xanthophis* (Berg) (Miranda et al. 2009), for the chinch bugs *B. insularis* and *B. occiduus* (Backus et al. 2013), and for the pentatomid *E. meditabunda* (Lucini and Panizzi 2016). It is possible that the interruptions represent watery salivation and putative tasting and testing of xylem cells (Backus et al. 2005a, 2013). Thus, the xylem ingestion interruptions performed by *P. guildinii* also may be related to salivary secretion and testing of xylem cells, because Pg4 (when observed) occurred mainly at the beginning of xylem ingestion.

In conclusion, our results showed that *P. guildinii* ingests cell contents of the vascular bundle, similar to other salivary sheath feeders. Furthermore, it feeds on vascular cells during both vegetative (leaflet and stem) and reproductive stages (pod), since leaflet, stem, and pod share the same plant tissues, i.e., parenchyma, and vascular bundle. The only difference is that pod contains endosperm (seed reserve tissue, related to mesophyll and parenchyma), which is not present in leaflet and stem.

P. guildinii ingesting exclusively in xylem vessels and not in phloem sieve elements differed from aphids (Tjallingii 2006), whiteflies (Janssen et al. 1989), mealybugs (Cid and Fereres 2010), planthoppers (Seo et al. 2009, Ghaffar et al. 2011), psyllids (Bonani et al. 2010, Civolani et al. 2011), chinch bugs (Backus et al. 2013), and the pentatomid *Edessa meditabunda* (Lucini and Panizzi 2016), in which the ingestion sites are both types of vascular cells. In addition, *P. guildinii* has long been known to be an important seed feeder, mostly of soybean (Panizzi and Smith 1977); we observed and summarized two ingestion waveform types during *P. guildinii* probing activities on soybean pod, i.e., seed endosperm. To our knowledge, this is the first time that the feeding behavior of a seed-feeder has been studied via EPG.

This study used the new AC-DC EPG technology to generate a waveform library for the first time for any stink bug, laying the groundwork for future EPG studies of pentatomid feeding. We demonstrated that the feeding process of the redbanded stink bug *P. guildinii* on three different structures of the soybean plant involves 11 waveforms with variable characteristics. Of these, two waveforms (Pg2 and Pg3b) are correlated with ingestion. The study also elucidates that beyond ingesting nutrients from the seed endosperm (during Pg3b), which is its preferred feeding site, *P. guildinii* also ingests from xylem vessels (during Pg2) on soybean stems and leaves.

Results from this study demonstrated that *P. guildinii* probably uses the cell rupture strategy without production of salivary sheaths to ingest from endosperm in soybean pod; thus, providing support for the concept that saliva-derived maceration is the cause of damage. Interestingly, the same insect could switch to salivary sheath feeding to ingest from xylem in soybean leaves and stems, perhaps for hydration. Accordingly, stink bugs probably can shift their feeding strategies to maintain a proper nutritional balance during food uptake. This switching of feeding strategy has not been previously documented via EPG for stink bugs, and will be helpful in understanding the cause of damage and development of resistant soybean varieties, in future studies.

Acknowledgments

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