Characterization and Correlation of New Electrical Penetration Graph Waveforms for the Corn Leafhopper (Hemiptera: Cicadellidae)

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ABSTRACT The corn leafhopper, Dalbulus maidis (DeLong & Wolcott) (Hemiptera: Cicadellidae), is a vector of three maize, Zea mays L., pathogens that have become limiting factors for maize production in some areas of the Americas. Insect feeding plays an important role in vector acquisition and inoculation of pathogens and hence the disease spread. Thus, we sought to understand the different probing and stylet penetration activities performed by this insect while feeding on maize plants, with the ultimate goal of characterizing potential sources of insect resistance in maize. Using electrical penetration graph technology, six distinct waveforms were characterized and correlated with major probing activities of D. maidis via transmission of corn stunt spiroplasma and excretion of honeydew as markers. Major waveforms comprise stylet pathway (waveform 1), active ingestion in nonsieve elements (waveform 2), nonvascular probing (waveform 3), phloem contact (waveform 4, the X wave), phloem ingestion (waveform 5), and oviposition (waveform 6). Our results support most previous findings with this species, and also indicate that some waveforms (2, 4, and 5) are related to biopotentials generated during probing, as was previously found for other hemipteran species. The most important finding from this work is that D. maidis ingests from phloem sieve elements more frequently and for longer durations than seen in previous research, probably due to longer observation periods used in this study. This work provides basic information relevant to the understanding of probing behavior of D. maidis and to the characterization of potential sources of insect-resistant maize.

KEY WORDS electrical penetration graph, electronic monitoring of insect feeding, maize pathogens, host plant resistance, vector

The corn leafhopper, Dalbulus maidis (DeLong & Wolcott) (Hemiptera: Cicadellidae), is a major pest of maize, Zea mays L., in the Americas. Its epidemiological importance is due to its prevalence from the southern United States to central areas of Argentina (Nault 1990) and to its high efficiency in transmitting three important maize pathogens: the mollicutes Spiroplasma kunkelii and maize bushy stunt phytoplasma (Nault 1980) and the maize rayado fino virus (Gamez 1973). These three pathogens, alone or in combination, cause “corn stunt,” a disease complex that has become a limiting factor for maize production in some areas of the United States and Latin America (Hruska et al. 1996, Summers et al. 2004). Changes in agricultural practices have played an important role in increasing the size of insect populations and hence the amount of disease. Indeed, although the host range of D. maidis includes only maize and teosinte (Pitre 1967), significant increases in the prevalence of corn stunt have developed in the past few years, due to the 1) abilities of all three pathogens to be transmitted in a persistent-propagative manner (Gamez 1973, Madden et al. 1984); 2) persistence of insects after maize plants are harvested (Larsen et al. 1992, Ebbert and Nault 1994); 3) ability of this leafhopper to move long distances (Nault 1990); and 4) increase in acreages planted in maize monocultures (Hruska et al. 1996, Summers et al. 2004), providing a consistent, year-round food source for the leafhopper vector.

The feeding behavior of D. maidis plays a central role in the transmission efficiency of corn stunt pathogens. Because the extent of insect–plant contact (Alivizatos and Markham 1986) relates directly to pathogen acquisition and inoculation efficiencies, and because such interaction lasts longer in plants suitable for insect feeding (Saxena 1969, 1985), preferred host plants are exposed to higher pathogen pressures than are plants less accepted by insects (Saxena 1969, 1985). Thus, control of leafhopper-transmitted pathogens, such as the rice tungro viruses, has relied mostly on insect-resistant cultivars that prevent successful insect feeding (Rezaul Karim and Saxena 1991, Azzam and Chancellor 2002).

Insect life-history parameters have been used extensively to identify sources of plant resistance (Sax-
ena and Khan 1989). However, they provide little information about the mechanism of resistance in different tissues. Electrical penetration graph (EPG) technology has been particularly helpful in efforts to overcome this difficulty, and to establish the mechanism and location, at the tissue level, of the traits conferring plant resistance. This approach can be effective for understanding the activities performed by hemipteran insects while probing (i.e., inserting or penetrating the styles into the plant tissues; synonymous with stylet penetration; Backus 2000). For example, extensive research characterized the probing behavior of several Nephotettix species (Cheng and Pathak 1972, Heinrichs and Rupasus 1984, Rupasus and Heinrichs 1990, Rezaul Karim and Saxena 1991) and Nilaparvata lugens (Stål) (Khan and Saxena 1988, Kimmins 1999, Alam and Cohen 1998, Hattori 2001), important pests of rice, Oryza sativa L. In turn, this information has allowed detection and characterization of rice genotypes resistant to tungro disease (Azam and Chancellor 2002).

The probing behavior of leafhoppers that serve as vectors of maize pathogens is not as well understood as that of other leafhoppers. Studies of the probing behavior of Cicadulina spp. (Kimmins and Bosque-Perez 1996, Lett et al. 2001), and of Graminella spp. and D. maidis (Wayadande and Nault 1996) have provided some information. The latter study identified the major stylet activities during probing behavior related to inoculation of the maize chlorotic dwarf virus (MCDV). Wayadande and Nault (1993) correlated inoculation of the phloem-restricted MCDV with production of X waves, which were correlated with stylet position in phloem cells (Wayadande and Nault 1996). However, because of the low resolution capabilities of the EPG equipment available for those studies (compared with present instruments), and the short EPG recording periods performed (only 3 h), the results of those studies do not completely explain the probing behavior of D. maidis as a vector of persistently transmitted pathogens.

Wayadande and Nault (1996) described five D. maidis waveforms and interpreted (named them to represent 1) salvation, 2) nonvascular ingestion, 3) nonvascular probing, 4) phloem contact and activities occurring therein (X wave), and 5) phloem ingestion. These interpretations were based upon salivary sheath termination points correlated with the waveforms. The two phloem-associated waveforms were always observed to occur in the same sequence: X wave followed by phloem ingestion. The phloem ingestion waveform was consistently associated with continuous excretion. Occasionally, X waves were followed by other waveforms besides phloem ingestion, but phloem ingestion was always preceded by the X wave pattern without exception. Because the three pathogens acquired and inoculated (i.e., transmitted) by D. maidis are all associated with phloem sieve elements, the stylet activities performed by this insect in phloem tissue will determine how efficiently they are acquired and inoculated.

The objectives of this work were to 1) characterize higher resolution EPG waveforms of D. maidis by using an electronically updated instrument, 2) correlate those waveforms with the major stylet activities performed during probing, and 3) determine their time of occurrence and sequence during probing by using more biologically relevant recording periods than in previous research. Our findings contribute to understanding of the behaviors related to transmission of persistently transmitted pathogens and to the identification of mechanisms of host plant resistance to D. maidis.

Materials and Methods

Insects, Plants, and Spiroplasmas. A colony of healthy D. maidis was initiated at Oklahoma State University in 1997 from insects provided by L. R. Nault (Ohio Agricultural Research Development Center, Wooster, OH). The Ohio State colony was established initially from insects collected in Mexico and was maintained on ‘Golden Bantam’ sweet corn. Insect and plant maintenance was carried out in aluminum-framed cages (Wayadande and Fletcher 1995) that were placed in a growth room at a constant temperature of 26°C, with a photoperiod of 16:8 (L:D) h (Nault 1980). Healthy insect colonies were kept separated from spiroplasma-exposed insects. All the experiments were performed using maize plants of the same cultivar, at the three-leaf stage. The youngest fully expanded leaf of each plant was folded over a horizontally placed plastic cylinder (10 cm in diameter) to expose the abaxial surface, preferred by D. maidis. Spiroplasma kunkelii Whitcomb strain CR2-3X (passage 9) was used for transmission tests. This strain was collected originally in Costa Rica (Gastro et al. 1992), and cultured in LDA3 medium (Lee and Davis 1989).

EPG Recordings. D. maidis females, 10–14 d old, were anesthetized by confining them singly in glass tubes (1.5 by 15 cm) that were chilled on ice for 2–3 min. Immobilized insects were placed, with the help of an “abdomen” type aspirator (sized to fit tightly around the abdomen) on a stage constructed under a stereomicroscope (model 530 220, Nikon, Melville, NY) and secured by application of a gentle vacuum. Insects were tethered to a 25.5-μm-diameter (sold as 0.001 in.), 2–3-cm-long gold wire (Sigmund Cohn, Mount Vernon, NY) by using silver conductive paint (Ladd Research Industries, Burlington, VT) to attach the wire to the pronotum. After tethering, insects were starved for 1 h before recordings were initiated.

EPG recordings were performed using a four-channel AC-DC monitor (designed and built by W. H. Bennett, University of Missouri, retired; Backus and Bennett 2009) for a period of 20 h. Throughout the experiment, standard equipment settings consisted of 100-mV alternate current (AC), 1,000-Hz substrate voltage, input impedance of 10^9 Ω, and amplification (gain) of 500×. Substrate voltage was applied through an electrode inserted into the soil, and the insect was connected to the electrical circuit by gluing the gold
wire to a copper wire (≈0.5 mm in diameter), which was soldered to a brass nail inserted into the input lead of the headstage amplifier. The insects and plants were placed inside a large Faraday cage (2 by 2 by 4 feet, constructed of an aluminum frame with a steel base), to reduce interference from external electrical noise. Output waveforms were converted to digital format at 100 samples per s by using a DI-720 analog-to-digital board, acquired with Windaq/Pro software (both from Dataq Instruments, Akron OH), and stored on a computer hard drive.

Correlation of EPG Parameters With S. kunkelii Transmission. Inoculative insects were obtained by feeding second to third instars of D. maidis on D10 medium (Alivizatos 1982) containing S. kunkelii at a titer of ≈10^8 cells/ml. Approximately 500 μl of this suspension was placed inside feeding sachets constructed of two layers of Paraflm membranes stretched over a 50-ml plastic dose cup, into which the test insects were introduced for an acquisition access period (AAP) of 24 h. After 28 d of incubation on healthy maize plants, insects eclosed to the adult stage and were used for inoculation tests.

To correlate waveform type with pathogen inoculation, wire-tethered inoculative insects were allowed to probe on spiroplasma-free plants while being recorded (first inoculation access period, IAP). Probing was interrupted by removing the insect from the plant immediately after the desired waveform or waveform combinations were observed and completed (described below). After recording this first IAP, the insects were chilled on ice (as described above) and gently detached from the gold wire. Insects were then placed singly on another maize plant for a second IAP of 3 d (Alivizatos and Markham 1986) to determine vector status. All leafhopper-exposed plants were kept for 60 d in a growth chamber (25°C and a photoperiod of 16:8 [L:D] h) for symptom expression and diagnosis.

Presence of S. kunkelii in the plants was confirmed by polymerase chain reaction (PCR) by using primers F2 and R6, which amplify a segment of the gene encoding spiralin (Barros et al. 2001), after extraction of nucleic acids using the CTAB method (Doyle and Doyle 1994). Leafhoppers were determined to be inoculative if at least one of the plants exposed to the insect in either IAP was PCR positive.

Diseased plants obtained from the inoculation experiments were used for acquisition tests. To correlate waveform type with acquisition, spiroplasma-free insects had access to S. kunkelii-infected plants while being recorded, until the probing was interrupted as described for inoculation. The insects were then detached from the gold wire and caged singly for a period of 2 wk, to allow multiplication of spiroplasmas. After this period, the insects were tested singly for S. kunkelii by using PCR as described above.

For both inoculation and acquisition tests, insects were sorted into one of three groups, depending on the types of waveforms (see Results for waveform descriptions) that they had displayed by the time the probes were interrupted: group 1, with waveforms 1 + 2 (no probing in phloem); group 2, waveforms 1 + 2 + 4 (phloem contact includes X wave only); and group 3, waveforms 1 + 2 + 4 + 5 (phloem contact plus phloem ingestion). Grouping was done in this way because of the sequential appearance of different waveforms: i.e., waveform 5 was always preceded by waveform 4, which in turn was always preceded by waveforms 1 and 2 (see Results). The results were expressed as percentage of test plants that became infected (inoculation test), or percentage of insects that acquired S. kunkelii (acquisition test).

Honeydew Excretion. Rate of excretion and pH of honeydew also were used to correlate waveforms with insect activities. The rate of honeydew excretion was obtained by observing the insect probing through a stereomicroscope set inside the Faraday cage and counting for at least 10 min the number of honeydew droplets released after a particular waveform had started. Ten insects were observed for each of waveforms 2 and 5. The results were expressed as number of droplets per minute. The pH was determined by spotting pH indicator strips (Whatman, Maidstone, United Kingdom; range, pH 4 – 8) with individual honeydew droplets that were aspirated after deposition on the leaf surface. Buffers of known pH (range, pH 4 – 8) were used as standards, spotting 2 μl on the pH indicator tape. The pH of the honeydew was assigned to the closest color in the pH standards.

Statistical Analysis. Outputs of EPG recordings were inspected visually, and notes were made on the types of waveforms and the time of appearance of each waveform. These data were stored in an Excel (Microsoft, Redmond, WA) spreadsheet for the analysis and interpretation of results. Probing behavior and its association with either inoculation or acquisition of spiroplasmas was analyzed using chi-square contingency tests (Prado and Tjallingii 1994). For inoculation tests, only those insects that inoculated S. kunkelii either to the plant in which recording took place (first IAP) or to the test plant (second IAP) were considered inoculative and were included in the statistical analysis.

Both nonsequential (duration of probes and waveform events) and sequential parameters (information inherent in the sequential order of waveforms in a probe) (Helden and Tjallingii 2000, Backus et al. 2007, Sarria et al. 2009) used to characterize probing behavior of D. maidis.

Results

Characterization of D. maidis Waveforms. When females of D. maidis probed on maize plants, six different waveform types (Table 1; Figs. 1 and 2) were identified in the EPG output; these types were designated 1 through 6 according to their time of first detection in the behavioral sequence of a typical probe. The recordings showed a flat baseline when insects were not probing, whereas the output voltage of all the waveforms was higher than that of the baseline.

Waveforms 1–3 occurred early in the insect-plant interaction (Table 1). All the probes started with
waveform 1 and then proceeded into other waveforms, typically waveform 2, 3, or 4.

**Waveform 1.** The first waveform (Fig. 1D, section 1) in a probe is characterized by a sudden increase in voltage, a high waveform amplitude (distance between peaks and valleys within the same waveform, expressed as a percentage of the distance between baseline and highest peak voltage), and variable frequency, making an overall irregular waveform of short duration (Fig. 1). Average duration per event of waveform 1 is highly variable (Table 1), because many short-duration test probes consisting primarily of waveform 1 were <1 min in duration, whereas in other, multievent probes, the duration of waveform 1 could last several minutes before transition to a different waveform.

**Waveform 2.** The waveform that typically follows waveform 1 is highly regular, with low amplitude and voltage level, as well as a constant frequency of 5 Hz (Fig. 1D, section 2, and F). The time of first occurrence and average duration per event of this waveform can be highly variable (Table 1), from short events lasting a few seconds, up to 20 min without interruption.

**Waveform 3.** This regular waveform is similar to waveform 2 in amplitude and voltage level, but its frequency is much lower and more variable (Fig. 2A). It was detected in few insects and for brief time periods, shifting from and to waveform 2 within the same

| Waveform | Time to first event (minutes) | Amplitude (%) | Voltage level (%) | Mean duration (minutes) |
|----------|-------------------------------|---------------|-------------------|------------------------|
| 1        | 2.9 ± 2.1                     | 75.7 ± 10.1   | 76.2 ± 10.7       | 0.9 ± 0.1              |
| 2        | 45.9 ± 7.4                    | 42.7 ± 8.0    | 6.7 ± 3.4         | 6.8 ± 0.7              |
| 3        | 102.5 ± 141.3                 | 53.9 ± 14.6   | 10.4 ± 5.2        | 5.8 ± 7.2              |
| 4*       | 83.7 ± 66.4                   | 54.9 ± 10.0   | 5.9 ± 5.2         | 36.9 ± 12.5            |
| 5        | 138.1 ± 94.7                  | 63.1 ± 14.9   | 4.5 ± 2.3         | 208.2 ± 123.7          |
| 6        | 217.6 ± 136.1                 | 52.1 ± 8.7    | 95.8 ± 6.8        | 1.3 ± 0.1              |

*Only naturally terminated (i.e. followed by waveform 5) waveforms were considered in the estimation, because others were artificially terminated at different stages.*

![Fig. 1. Samples of major waveforms displayed during probing of D. maidis females. Overview of six probes, five short test probes (from the first of which is taken the expanded and amplified views in inset boxes D and F), followed by a much longer ingestion probe (from which expanded and amplified views are taken for inset boxes A, B, C, E, and G (horizontal bar, 100 s; vertical bar, 1,000 mV). Inset A, spike during early stages of waveform 4 (horizontal bar, 10 s; vertical bar, 1,000 mV). Inset B, middle stage of waveform 4 (horizontal bar, 10 s; vertical bar, 100 mV). Inset C, transition from waveform 4 to waveform 5 (horizontal bar, 10 s; vertical bar, 100 mV). Inset D, expansion of first probe containing an event of waveform 2 (horizontal bar, 10 s; vertical bar, 1,000 mV). Inset E, waveform 5 (horizontal bar, 10 s; vertical bar, 100 mV). Inset F, expansion of waveform 2 (horizontal bar, 1 s; vertical bar, 50 mV). Inset G, expansion of waveform 5 (horizontal bar, 1 s; vertical bar, 20 mV).
Both its mean duration per event and first occurrence were highly variable (Table 1).

Waveform 4. Waveform 4 (Fig. 1A-C) lasted longer and occurred later in a probe than the shorter, earlier waveforms 1–3 (Table 1). The frequency of waveform 4 increased from 0.2 to 0.3 Hz at the beginning to 5 Hz (when it merged into waveform 5; see below). There also was a gradual increase in voltage level toward the later part of the waveform 4, reaching the same voltage level as waveform 5. During early stages of waveform 4, there usually were two to five “spikes” (sudden increase in voltage with an irregular form), as shown in Fig. 1A. However, approximately half of the probes of D. maidis females were naturally terminated at different stages of waveform 4 before reaching the characteristic 5-Hz frequency, when waveform 5 would begin in a nonterminated probe.

Waveform 5. This waveform (Fig. 1E and G) had a high voltage level, steady frequency of 5 Hz, and two different, alternating and repeating episodes: a short, leading section (0.4–2.0 s) with low amplitude and a following episode with higher amplitude lasting 1.5–4.0 s. Mean duration per episode of both types varied not only among insects but also among probes and even within the same waveform event.

Waveform 6 (Fig. 2B). This waveform consisted of a low-amplitude, relatively flat line at very high, steady voltage level. Waveform 6 corresponded to oviposition, and each single waveform was visually correlated to insertion of the ovipositor and deposition of a single egg. Thus, it was not correlated with either spiroplasma transmission or honeydew excretion.

Comparison of Waveforms 2, 4, and 5. Although waveforms 2 and 5 shared similar amplitude and a 5-Hz frequency, they differed in some other characteristics: 1) waveform 5 had a higher voltage level than waveform 2; 2) waveform 5 had a regularly varying amplitude, i.e., a short episode of low amplitude followed by a longer episode of high amplitude, whereas waveform 2 lacked a period of low amplitude; and 3) waveform 5 was always preceded by waveform 4, whereas waveform 2 was always preceded by waveforms 1 or 3. In additional experiments to characterize these two waveforms, the recording conditions were adjusted to detect primarily emf component of the EPG output, by using an input impedance of $10^{13}$ Ohms, 0 V in the input voltage and a DC (direct current) output (Walker 2000). Using these emf-only monitor settings, the frequency and shapes of waveforms 2, 4, and 5 remained unaltered (data not shown), indicating that all three waveforms had a significant emf component (Walker 2000).

Correlation of EPG Waveforms With Stylet Activities. Table 2 shows the efficiency of inoculation and acquisition of the pathogen S. kunkelii by insects whose probes were artificially terminated at different stages during probing. No spiroplasmas were inoculated when probing was interrupted after only waveforms 1 or 2 were displayed. The chi-square analysis indicated that inoculation of spiroplasmas always was correlated with waveform 4, with no further increase in inoculation efficiency when probing continued to include waveform 5 ($P = 0.418$). Although inoculation of S. kunkelii correlated only to waveform 4, with no further increase in inoculation efficiency when probing continued to include waveform 5 ($P = 0.418$), it was not correlated with either spiroplasma transmission or honeydew excretion.

Fig. 2. Sample of less-common waveforms displayed during probing of D. maidis females. Box A, waveform three (horizontal bar, 10 s; vertical bar, 100 mV). Box B, waveform 6 (horizontal bar, 10 s; vertical bar, 1,000 mV). Arrows indicate the moment of ovipositor insertion (A) and removal (B).
to phloem sieve elements, our results strongly support that waveforms 4 and 5 represent stylet activities in phloem sieve elements. Characteristics of excrated honeydew varied during different waveform events of *D. maidis* probing behavior (Table 3). *D. maidis* produced honeydew only infrequently when waveforms 1, 3, 4, or 6 were displayed, but steady production of honeydew was correlated consistently with waveforms 2 and 5. Honeydew was excreted at a lower rate and was of lower pH during waveform 2 than during waveform 5; however, measurement of the excretion rate during the former waveform was difficult due to its short average duration (7 min) and occasional shifting from and to waveform 3. Honeydew excretion and pH suggests that waveforms 2 and 5 represent ingestion from non-phloem cells and phloem sieve elements, respectively.

Table 4 shows data from waveforms produced by *D. maidis* females having access to maize plants. All insects displayed all waveforms except waveform 3; that waveform occurred in only 5% of the insects (data not shown). Insects spent most (93%) of their probing duration with their stylets inserted into plant tissues. The waveforms recorded for the longest mean duration per insect (waveform duration per insect, WDI) were waveform 5 (62% of the total time) and waveform 4 (15% of the total time). These phloem-related waveforms together comprised up to 15.2 h (77% of the total probing duration). The insects also spent an average of 2.2 h (9% of the total probing duration) in other stylet activities, including waveforms 2 and 3.

*D. maidis* probed actively into plant tissues, at a mean rate of 50.1 probes per insect (number of waveform events per insect; NWEI) in 20 h. Probes were separated by short nonprobing events (≈1 min each). The average duration of those short probes (waveform duration per event per insect; WDEI) that lacked waveform 4 (or the combination of waveforms 4 + 5) was 1.5 min. In contrast, although far fewer probes included waveforms 4 or 5 (8.2 probes per insect for waveform 4, of which 4.2 included waveform 5), the mean durations of these probes were significantly longer than those that did not include waveforms 4 and 5. The average duration of each waveform event 4 was 21 min, whereas each waveform event 5 lasted an average of 178 min.

**Discussion**

**Biological Meanings of *D. maidis* Waveforms.** Interpretation of the probing activities associated with different EPG waveforms is based on previous *D. maidis* correlations (Wayadande and Nault, 1993, 1996), similarities of *D. maidis* waveforms with those of other leafhopper species, acquisition and inoculation of the phloem-associated pathogen *S. kunkelii*, and characteristics of honeydew excretion. EPG monitoring of probing behavior of *D. maidis* females reveals EPG waveforms similar to those previously described for other leafhoppers, including those reported in an earlier characterization by Wayadande and Nault (1996) for this same species. However, because of improved electronics of the present EPG monitor, the information obtained in this work provides a higher level of detail than was possible to gather during most of the previous research in leafhoppers and therefore can better explain the probing behaviors related to pathogen transmission.

**Waveform 1.** This waveform resembles the formerly described “salivation” waveform of several leafhopper species, including *D. maidis* (Wayadande and

### Table 2. Inoculation and acquisition efficiency of the pathogen *S. kunkelii* by insects which probing was interrupted in different waveforms

| Waveform | Inoculation | Acquisition |
|----------|-------------|-------------|
|          | No. total plants | No. infected plants | % infected plants | No. total insects | No. infected insects | % infected insects |
| 1 + 2    | 15          | 0            | 0.0a          | 15            | 2            | 11.1a         |
| 1 + 2 + 4| 31          | 26           | 83.9b         | 47            | 26           | 55.3b        |
| 1 + 2 + 4 + 5 | 26       | 22           | 84.0b         | 40            | 37           | 92.5c        |

Different letters indicate significant differences by Fisher’s LSD test (P < 0.05) for each parameter.

### Table 3. Characteristics of honeydew excreted by *D. maidis* females displaying putative ingestion waveforms (n = 10)

| Waveform | pH            | Excretion rate |
|----------|---------------|---------------|
|          | 4.96 ± 0.67a  | 0.34 ± 0.13a  |
| 5        | 7.12 ± 0.38b  | 0.65 ± 0.15b  |

Different letters indicate significant differences by Fisher’s LSD test (P < 0.05) for each parameter.

### Table 4. Nonsequential parameters of different waveforms of *D. maidis* females having access to maize plants of the susceptible Golden Bantam

| Waveform | WDI (h) | NWEI (probes per insect) | WDEI (min) |
|----------|---------|--------------------------|------------|
| Baseline | 82.4 ± 30.8 | 80.1 ± 41.6 | 1.03 ± 0.08 |
| 1        | 82.9 ± 7.8  | 81.0 ± 41.4 | 1.02 ± 0.08 |
| 2        | 90.6 ± 15.5 | 11.3 ± 5.8  | 8.1 ± 0.94  |
| 3        | 35.6 ± 8.8  | 3.0 ± 2.1   | 11.9 ± 1.7  |
| 4        | 175.1 ± 12.8| 8.2 ± 4.7   | 21.7 ± 2.3  |
| 5        | 730.6 ± 31.8| 4.2 ± 1.6   | 174.0 ± 27.0|

WDI waveform duration per insect (total time an average insect spent in each activity, in minutes); NWEI, number of waveform events per insect (number of times that each waveform was recorded for an average insect (only insects that performed the indicated waveform were included in the analysis); WDEI, waveform duration per event per insect (average duration of each waveform event, in minutes (only insects that performed the indicated waveform were included in the analysis).
Waveform 2. This waveform is similar to waveforms related to either “nonsieve element ingestion” in *D. maidis* (Wayadande and Nault 1996), “xylem ingestion” in *N. cincticeps* (Kawabe and McLean 1980, Kawabe 1985) and *N. virescens* (Rapusas and Heinrichs 1990), and “active ingestion” either from xylem or mesophyll cells by *C. mbila* (Lett et al. 2001). Waveform 2 is similar to those above mentioned waveforms in its waveform sequence after stylet pathway, its regular shape, and its low amplitude. Also, *D. maidis* waveform 2 shares a 5-Hz frequency and an emf origin with waveform 2 of *C. mbila* (Lett et al. 2001).

Despite the good correlations we obtained for the other waveforms, a full definition of the biological activity of waveform 2 remains inconclusive. Data on honeydew pH and excretion rate agreed with those of Wayadande and Nault (1996), who related this activity to “nonsieve element ingestion” because salivary sheath tips were found mostly in mesophyll or bundle sheath cells. Similar results in *C. mbila* (Lett et al. 2001) and in *Rhopalostiphum padi* (L.) (Prado and Tjallingii 1994) were interpreted as active ingestion, either from mesophyll, bundle sheath, or xylem cells. However, in *N. cincticeps* (Kawabe and McLean 1980), a waveform similar to our waveform 2 was correlated with position of salivary sheath termini in xylem cells and excretion of honeydew at a rate higher than that seen for phloemi ingestion (≈7 drops per min), similar to our findings. Hence, our results of honeydew excretion could be due to probing in mesophyll cells or in xylem cells for the brief periods observed for *D. maidis* (Table 1), probably until insects gained water lost during pre-experiment handling. Consequently, we suggest that waveform 2 represents active ingestion, either from xylem or parenchyma cells.

Waveform 3. A firm definition of this waveform cannot be determined from the present work. It occurred rarely, and its characteristics included medium amplitude and voltage level, a sudden increase followed by a gradual reduction in voltage, and low and variable frequency (Fig. 2B). Waveform 3 is associated with waveform 2; the EPG recording moved back and forth between these two waveforms. In *N. virescens* (Rapusas and Heinrichs 1990) and *N. cincticeps* (Kawabe and McLean 1980, Kawabe 1985), a similar waveform was designated the “resting” waveform and in *D. maidis* (Wayadande and Nault 1996) it was called “nonvascular probing” because both specimens the stylet tips were located in mesophyll cells and no ingestion, as indicated by excreted honeydew, was detected. A similar waveform (waveform “D”) was associated with possible mesophyll ingestion in the xylem-ingesting *Graphocephala atropunctata* (Signoret) (Almeida and Backus 2004), and in the mesophyll-ingesting *Empoasca krasleri* Ross & Moore and *Empoasca fabae* (Harris) (waveform Ib in Calderon and Backus 1992). Because mesophyll lacks a strong positive turgor pressure, ingestion of this tissue would require active suction of cell contents by the cibarial pump. Hence, confirmation of the activity related to this waveform would require correlation between the EPG output and cibarial pump pulses, as was carried out for *R. padi* for waveform G (Tjallingii 1978) and for *H. vitripennis* for waveform C (Dugravot et al. 2008). However, this waveform was correlated visually in our tests with the insect moving actively while keeping the stylets inserted, so it could be related to waveform 3 of *C. mbila* (Lett et al. 2001), a similar-appearing waveform (although of inverted shape; probably an artifact due to rectification; E.B., personal communication) that was interpreted as “stylet work.”
plants and also for a high proportion of acquisition by insects when they probe in infected plants. These results agree with previous findings of inoculation of phloem-associated viruses by *Gra mineillla nigrifrons* (Forbes) (Wayadande and Nault 1993), *Gic dulilina stor eyn* China (Kimmins and Bosque-Perez 1996), *R. padi* (Prado and Tjallingii 1994), and *Sitobion avenae* (F.) (Scheller and Shukle 1986). Indeed, it is known that spiroplasmas traverse the gut wall, multiply in the insect body, and reach the salivary ducts after crossing the cells of the salivary glands (Wayadande and Fletcher 1995). These findings strongly indicate that these pathogens are inoculated during salivation into host tissues. Also, because spiroplasmas colonize sieve tube members and the termination points of salivary sheaths also were traced to these cells (Wayadande and Nault 1996), our data strongly suggest that waveform 4 therefore represents salivation in phloem sieve elements.

**Waveform 5.** After phloem salivation, insects in this study performed a highly regular waveform that is higher-resolution, more detailed version of the “sieve element ingestion” waveform, recorded previously from *D. maidis* (Wayadande and Nault 1993). Thus, waveform 5 represents phloem ingestion, based on its correlation with salivary sheath tips terminating in the phloem (Wayadande and Nault 1996), the acquisition of phloem-restricted spiroplasmas, and the excretion of neutral pH honeydew.

**Waveforms 4 and 5: Phloem-Contact Waveforms.** The facts that waveforms 4 and 5 occur without changes in the voltage level and that waveform 5 is always preceded by waveform 4 strongly support that events related to both waveforms take place at the same cell type (Backus et al. 2009). This supports that salivation into the sieve tubes is necessary to allow the initiation of waveform 5 (Wayadande and Nault 1993, 1996).

The biological activity associated with waveforms similar to waveform 4 of *D. maidis*, defined usually as the X wave, has been a topic of debate since its first detection in the pea aphid, *Acyrtosiphon pisum* (Harris) (McLean and Kinsey 1967), and its subsequent finding in leafhoppers (Chang 1978, Kawabe and McLean 1980, Wayadande and Nault 1993). In the seminal work of McLean and Kinsey (1967), it was suggested that both salivation (to block the plant defense response) and uptake of small quantities of phloem sap would occur during this activity. Recent evidence (Tjallingii 2006, Will et al. 2007) supports the hypothesis of McLean and Kinsey (1967) that phloem salivation would prevent the accumulation and polymerization of phloem contents leading to blockage around stylet tips, impairing insect ingestion. In addition, it is likely that phloem sap is passively taken up, regulated by timely opening and closing of the precibarial valve (McLean and Kinsey 1967, 1984; Lett et al. 2001). Finally, very recent findings (Backus et al. 2009) on sharpshooter-leafhopper X waves support that they represent a complex combination of salivation plus fluid uptake and expulsion. Backus et al. (2009) also updated the definition of an X wave, describing it as a transitional waveform that represents stylet penetration of the preferred ingestion cell type for any vascular-specializing species. The complex mixture of behaviors and physiologies in the X wave together represent ingestion-cell acceptance and preparation.

When waveform 4 from *D. maidis* increases in frequency to the same frequency as that of waveform 5, it could indicate that salivation events are “conditioning” the phloem until passive, regulated ingestion begins. The increasing flow of phloem sap into the insect would make it necessary to regulate incoming sap flow more frequently via the precibarial valve; as its speed increases, it would produce a higher frequency in the EPG waveform. Thus, we agree with Backus et al. (2009) that the term “phloem salivation” does not completely describe the events taking place in waveform 4. Hence, we propose the term “phloem conditioning” for the probing activities correlated with the *D. maidis* X wave. Phloem conditioning reflects both salivation (proposed to inhibit host defenses) and fluid uptake (probably as a consequence of passive influx of phloem contents) occurring in phloem cells, as demonstrated by significant spiroplasma inoculation and acquisition, respectively, during this waveform.

Inoculation of phloem-inhabiting pathogens has been well documented to occur during the X wave (waveform 4) as described above. However, information about acquisition of phloem-associated pathogens was reported only for *R. padi* acquiring barley yellow dwarf virus (family *Luteoviridae*, genus *Luteovirus*, BYDV) (Prado and Tjallingii 1994) and *Myzus persicae* (Sulzer) acquiring cauliflower mosaic virus (Palacios et al. 2002). In the first case, acquisition of the phloem-associated BYDV was highly correlated with phloem ingestion, but low levels of BYDV acquisition were obtained from *R. padi* females that did not produce a phloem-related waveform, probably as a result of the puncture of sieve tube members without continuing the probe with a phloem salivation waveform. Our results agree with those of Prado and Tjallingii (1994), in that *S. kunkeli* also can be acquired by individuals that do not display phloem-related waveforms. In addition, unlike what was found by Prado and Tjallingii (1994) for *R. padi*, acquisition of spiroplasmas increased significantly in those insects that made stylet contact with a phloem sieve element (waveform 4) without reaching ingestion (waveform 5) from this cell type. This could be due to passive ingestion of phloem content between bouts of salivation into phloem cells, as hypothesized by McLean and Kinsey (1967).

The information obtained in the previous correlations builds upon our knowledge of *D. maidis* probing behavior. This is because the 3-h recording period used in previous work on this species (Wayadande and Nault 1996) might not have been long enough to completely characterize a pathosystem in which highly efficient transmission of spiroplasmas requires longer periods of insect–plant contact. Also, the lower waveform resolution of the EPG equipment available at the time of the 1996 study was less optimal for study
of *D. maidis* probing behavior. The most noticeable difference between the results obtained in this study and in that of Wayadande and Nault (1996) is the proportion of insects achieving phloem contact and phloem ingestion. In this work, 80–90% of the insects made phloem contact, and 80% ingested from phloem within 3 h, and all the insects ultimately reached phloem ingestion. In Wayadande and Nault (1996), in contrast, only 31% of the insects reached phloem, and 26% had ingested by the end of the 3-h period. More insects might have reached phloem ingestion in the earlier study if the recording had been extended for longer time periods. Also, the differences between our results and those of Wayadande and Nault (1996) might relate to our use of intact plants to monitor the probing behavior. The excised leaves used in the previous study might have had altered chemical or physical properties of phloem sieve tube members, making this tissue less suitable for insect feeding.

Our results indicate that insects spend 78% of their probing time in phloem-related activities (waveforms 4 + 5), 8% in active (nonphloem) ingestion (waveform 2), and 2% in nonvascular probing (waveform 3). In the work of Wayadande and Nault (1996), phloem-related activities covered 36% of the time, whereas 26% of the time was spent in nonvascular probing and 30% in nonsieve element ingestion. Also, our results indicate that insects spend a high proportion (around 80%) of the time in phloem after they establish the first contact with that tissue, as described for other phloem-ingesting hemipterans (Helden and Tjallingii 1993, Helden and Tjallingii 2000). These results indicate clearly the preference of *D. maidis* to seek out and ingest from phloem over other tissues. However, like Wayadande and Nault (1996), we did observe *D. maidis* probing on nonphloem tissues early in the access period, as has been found in other leafhopper species (Kimmins 1989, Saxena and Khan 1989, Spiller et al. 1990).

In this work, major biological activities related to the probing behavior of *D. maidis* were correlated with different electrical waveforms, by using an EPG approach. These results reinforce the conclusions made previously by Wayadande and Nault (1996) for this species but provide additional detail and understanding of the biological activities that accompany plant tissue probing, by using a more modern instrument. Therefore, this article updates findings in Wayadande and Nault (1996). As has been noted for other insect species (Walker 2000), this work provides a basis from which to understand the dynamics of pathogen transmission and the mechanisms of plant resistance, and it is a valuable tool in the management of diseases whose pathogens are transmitted by *D. maidis*.

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References Cited

Alam, S. N., and M. B. Cohen. 1998. Durability of brown planthopper, *Nilaparvata lugens*, resistance in rice variety IR64 in greenhouse selection studies. Entomol. Exp. Appl. 89: 71–78.

Alivizatos, A. S. 1982. Feeding behaviour of the spiroplasma vectors *Dalbulus maidis* and *Eucaecillius variegatus* in *cito* and in *crito*. Ann. Ben. Phytopath. Inst. 13: 128–144.

Alivizatos, A. S., and P. C. Markham. 1986. Acquisition and transmission of corn stunt spiroplasma by its leafhopper vector *Dalbulus maidis*. Ann. Appl. Biol. 105: 535–544.

Almeida, R. P. P., and E. A. Backus. 2004. Stylet penetration behaviors of *Graphocephala atropunctata* (Signoret) (Hemiptera, Cicadellidae): EPG waveforms characterization and quantification. Ann. Entomol. Soc. Am. 97: 838–851.

Azzam, O., and T. C. B. Chancellor. 2002. The biology, epidemiology, and management of rice tungro disease in Asia. Plant Dis. 86: 88–100.

Backus, E. A. 2000. Our own jabberwocky: clarifying the terminology of certain piercing-sucking behaviors of homopterans, pp. 1–13. In C. P. Walker and E. A. Backus (eds.), Principles and applications of electronic monitoring and other techniques in the study of homopteran feeding behavior. Thomas Say Publications in Entomology, Entomological Society of America, Lanham, MD.

Backus, E. A., and W. H. Bennett. 2009. The AC-DC Correlation Monitor: new EPG design with flexible input resistors to detect both R and emf components for any piercing-sucking hemipteran. J. Insect Physiol. 55: 869–884.

Backus, E. A., J. Habihi, F. Yan, and M. Ellersieck. 2005. Stylet penetration by adult *Homalodisca coagulata* on grape: electrical penetration graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*. Ann. Entomol. Soc. Am. 98: 787–813.

Backus, E. A., A. R. Cline, M. Ellerseiek, and M. S. Serrano. 2007. *Lygus hesperus* (Hemiptera: Miridae) feeding in cotton: new methods and parameters for analysis of nonsequential electrical penetration graph data. Ann. Entomol. Soc. Am. 100: 296–310.

Backus, E. A., W. J. Holmes, F. Schreiber, B. J. Beardon, and G. P. Walker. 2009. Sharpshooter X wave: correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. Ann. Entomol. Soc. Am. 102: 847–867.

Barros, T. S. L., R. E. Davis, R. O. Resende, and E. L. Dally. 2007. Design of a polymerase chain reaction for specific detection of corn stunt spiroplasma. Plant Dis. 85: 475–480.

Calderon, J. D., and E. A. Backus. 1992. Comparison of the probing behaviors of *Emoepoa fabae* and *E. kraemeri* (Homoptera: Cicadellidae) on resistant and susceptible cultivars of common beans. J. Econ. Entomol. 85: 88–99.

Castro, V., C. Rivera, S. A. Isard, R. Gamez, J. Fletcher, and M. E. Irwin. 1992. The influence of weather and microclimate on *Dalbulus maidis* (Homoptera: Cicadellidae) flight activity and the incidence of diseases within maize and bean monocultures and bicultures in tropical America. Ann. Appl. Biol. 121: 469–492.

Chang, V. C. 1978. Feeding activities of the sugarcane leafhopper: identification of electronically recorded waveforms. Ann. Entomol. Soc. Am. 71: 31–36.

Cheng, C. H., and M. D. Pathak. 1972. Resistance to *Nephotettix virescens* in rice varieties. J. Econ. Entomol. 65: 1148–1153.
Doyle, J., and M. Doyle. 1994. Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Dugravot, S., E. A. Backus, R. Reardon, and T. A. Miller. 2008. Correlations of cibarial muscle activities of *Homalodisca* spp. sharpshooters (Hemiptera: Cicadellidae) with EPG waveforms and excretion. J. Insect Physiol. 54: 1467–1478.

Ebbert, M. A., and L. R. Nault. 1994. Improved overwintering ability in *Dalbulus maidis* (Homoptera: Cicadellidae) vectors infected with *Spiroplasma kunkelii* (Mycoplasmatales: Spiroplasmataceae). Environ. Entomol. 23: 634–644.

Gamez, R. 1973. Transmission of rayado fino virus of maize (*Zea mays*) by *Dalbulus maidis*. Ann. Appl. Biol. 73: 285–292.

Hattori, M. 2001. *Isolation of plant DNA from Spiroplasma kunkelii* (Homoptera: Cicadellidae) on a non-host barnyard grass, and resistant and susceptible varieties of rice. Appl. Entomol. Zool. 36: 83–89.

Heinrichs, E. A., and H. R. Rapusas. 1984. Feeding, development, and tungro virus transmission by the green leafhopper, *Nephotettix virescens* (Distant) (Homoptera: Cicadellidae) after selection on resistant rice cultivars. Environ. Entomol. 13: 1074–1078.

Helden, M., and F. Tjallingii. 1993. Tissue localization of lettuce resistance to the aphid, *Nasonovia ribisnigri*, using electrical penetration graphs. Entomol. Exp. Appl. 65: 269–278.

Heldén, M. V., and F. Tjallingii. 2000. Experimental design and analysis in EPG experiments with emphasis on plant resistance research, pp. 144–171. In Principles and applications of electronic monitoring and other techniques in the study of homopteran feeding behavior. Thomas Say Publications in Entomology, Entomological Society of America, Lanham, MD.

Hruska, A. J., S. M. Gladstone, and R. Obando. 1996. Epidemic roller coaster: maize stunt disease in Nicaragua. Am. Entomol. 42: 248–252.

Kawabe, S. 1985. Mechanism of varietal resistance to the rice green leafhopper (*Nephotettix cincticeps* Uhler). JARQ 19: 115–124.

Kawabe, S., and D. L. McLean. 1980. Electronic measurement of probing activities of the green leafhopper of rice. Entomol. Exp. Appl. 27: 77–82.

Khan, Z. R., and R. C. Saxena. 1988. Probing behavior of three biotypes of *Nilaparvata lugens* (Homoptera: Delphacidae) on different resistant and susceptible rice varieties. J. Econ. Entomol. 81: 1338–1345.

Kimmins, F. M. 1989. Electrical penetration graphs from *Nilaparvata lugens* on resistant and susceptible rice varieties. Entomol. Exp. Appl. 50: 69–79.

Kimmins, F. M., and N. A. Bosque-Perez. 1996. Electrical penetration graphs from *Cicadulina spp.* and the inoculation of a persistent virus into maize. Entomol. Exp. Appl. 50: 46–49.

Larsen, K. J., L. R. Nault, and G. Moya-Raygoza. 1992. Overwintering biology of *Dalbulus leafhoppers* (Homoptera: Cicadellidae): adult populations and drought hardness. Environ. Entomol. 21: 506–577.

Lee, I. M., and R. E. Davis. 1989. Serum-free media for cultivation of spiroplasmas. Can. J. Microbiol. 35: 1092–1099.

Lett, J. M., M. Granier, M. Grondin, P. Turpin, F. Molinaro, F. Chiroleu, M. Peterschmitt, and B. Reynaud. 2001. Electrical penetration graphs from *Cicadulina mbila* on maize, the fine structure of its stylet pathways and consequences for virus transmission efficiency. Entomol. Exp. Appl. 101: 93–109.

Madden, L. V., L. R. Nault, S. E. Heady, and W. E. Styer. 1984. Effect of maize stunting mollicutes on survival and fecundity of *Dalbulus* leafhopper vectors. Ann. Appl. Biol. 105: 431–441.

McLean, D. L., and M. G. Kinsey. 1967. Probing behavior of the pea aphid, *Acyrthosiphon pisum*. I. Definitive correlation of electronically recorded waveforms with aphid probing activities. Ann. Entomol. Soc. Am. 60: 400–406.

McLean, D. L., and M. G. Kinsey. 1984. The precibarial valve and its role in the feeding behavior of the pea aphid, *Acyrthosiphon pisum*. Bull. Entomol. Soc. Am. 30: 26–31.

Nault, L. R. 1980. Maize bushy stunt and corn stunt: a comparison of disease symptoms, pathogen host ranges, and vectors. Phytopathology 70: 659–662.

Nault, L. R. 1990. Evolution of an insect pest: maize and the corn leafhopper, a case study. Maydica 35: 165–175.

Palacios, I. M., D. Drucker, S. Blanc, S. Leite, A. Moreno, and A. Fereres. 2002. Cauliflower mosaic virus is preferentially acquired from the phloem by its aphid vectors. J. Gen. Virol. 83: 3163–3171.

Pitre, H. N. 1967. Greenhouse studies of the host range of *Dalbulus maidis*, a vector of the corn stunt virus. J. Econ. Entomol. 60: 417–421.

Prado, E., and F. Tjallingii. 1994. Aphid activities during sieve element punctures. Entomol. Exp. Appl. 72: 157–165.

Rapusas, H. R., and E. A. Heinrichs. 1990. Feeding behavior of *Nephotettix virescens* (Homoptera: Cicadellidae) on rice varieties with different levels of resistance. Environ. Entomol. 19: 594–602.

Rezaul Karim, A., and R. C. Saxena. 1991. Feeding behavior of three *Nephotettix species* (Homoptera: Cicadellidae) on selected resistant and susceptible rice cultivars, wild rice, and graminaceous weeds. J. Econ. Entomol. 84: 1208–1215.

Sarria, E., M. Cid, E. Garzo, and A. Fereres. 2009. Excel workbook for automatic parameter calculation of EPG data. Comput. Electron. Agric. 67: 35–42.

Saxena, K. N. 1985. Patterns of insect-plant relationships determining susceptibility or resistance of different plants to an insect. Entomol. Exp. Appl. 12: 751–766.

Saxena, K. N. 1985. Behavioural basis of plant resistance or susceptibility to insects. Insect Sci. Appl. 6: 303–313.

Saxena, R. C., and Z. R. Khan. 1989. Factors affecting resistance of rice varieties to planthopper and leafhopper pests. Agric. Zool. Rev. 97–132.

Scheller, H. V., and R. H. Shukle. 1956. Feeding behavior and transmission of barley yellow dwarf virus by *Sitobion avenae* on oats. Entomol. Exp. Appl. 40: 189–195.

Spiller, N. J., L. Koenders, and W. F. Tjallingii. 1990. Xylem ingestion by aphids—a strategy for maintaining water balance. Entomol. Exp. Appl. 55: 101–104.

Summers, C. G., A. S. Newton, Jr., and D. C. Oppeynoon. 2004. Overwintering of corn leafhopper, *Dalbulus maidis* (Homoptera: Cicadellidae), and *Spiroplasma kunkelii* (Mycoplasmatales: Spiroplasmataceae) in California’s San Joaquin Valley. Environ. Entomol. 33: 1644–1651.

Tjallingii, W. F. 1978. *Overwintering of corn leafhopper, A. maidis* (Homoptera: Cicadellidae): adult populations and drought hardness. Environ. Entomol. 7: 506–577.

Walker, G. P. 2000. A beginner’s guide to electronic monitoring of homopteran probing behavior, pp. 14–40. In G. P. Walker and E. A. Backus [eds.]. Principles and applications of electronic monitoring and other techniques in the study of homopteran feeding behavior.
Wayadande, A. C., and J. Fletcher. 1995. Transmission of Spiroplasma citri lines and their ability to cross gut and salivary gland barriers within the leafhopper vector Circulifer tenellus. Phytopathology 85: 1256–1259.

Wayadande, A. C., and L. R. Nault. 1993. Leafhopper probing behavior associated with maize chlorotic dwarf virus transmission to maize. Phytopathology 83: 522–526.

Wayadande, A. C., and L. R. Nault. 1996. Leafhoppers on leaves: an analysis of feeding behavior using conditional probabilities. J. Insect Behav. 9: 3–22.

Will, T., A.J.E. van Bel, A. Thonnessen, and W. F. Tjallingii. 2007. Molecular sabotage of plant defense by aphid saliva. Proc. Natl. Acad. Sci. U.S.A. 104: 10536–10541.

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