Probing behavior of the brown planthopper, Nilaparvata lugens Stål (Homoptera: Delphacidae) on a non-host barnyard grass, and resistant and susceptible varieties of rice

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(Received 31 July 2000; Accepted 27 October 2000)

Abstract
The feeding activity of Nilaparvata lugens (Stål) (Homoptera: Delphacidae) was compared on the barnyard grass, Echinochloa Crus-galli var. oryzicola, which contains an antifeedant, (E)-aconitic acid, and on resistant (bph4) and susceptible rice varieties using an AC electronic monitoring system (EMS). N. lugens made more frequent probes on the barnyard grass and the resistant rice than on the susceptible rice. Mean duration of probes tended to be the shortest and the percentage of probes that consisted of the primarily salivation phase only was the highest on the barnyard grass, followed by the resistant rice. The most distinct difference in N. lugens feeding activities between the barnyard grass and the resistant rice was the frequency of the primarily ingestion phase. On the barnyard grass, the insect was rarely successful in achieving the primarily ingestion phase, indicating that probing was interrupted before the arrival of the stylets at the sieve elements. On the other hand, on the resistant rice the primarily ingestion phase was observed as frequently as it was on the susceptible rice. However, it stopped within 5 min on the resistant rice in most cases. Probing activities were strongly inhibited by 1% (E)-aconitic acid diluted with distilled water. The inhibitory effect was considerably mitigated when diluted with 15% sucrose. These results suggest that on the barnyard grass, interruption of probing activity by (E)-aconitic acid can occur in stylet pathway tissues rather than in ingestion tissues (e.g. the phloem where sucrose is located).

Key words: Nilaparvata lugens, EMS, barnyard grass, (E)-aconitic acid, plant resistance

INTRODUCTION
The brown planthopper, Nilaparvata lugens (Stål) is one of the most serious pests of rice in Japan and Tropical Asia (Sogawa, 1982). It was observed that the barnyard grass, Echinochloa Crus-galli (Linn.) Beauv. var. oryzicola (Vasing.), a gramineous weed in paddy fields, did not suffer damage from N. lugens even when severe population outbreaks occurred in the field (Kim et al., 1975). This insect cannot survive on the barnyard grass due to reduced feeding (Kim et al., 1975; Saxena and Pathak, 1979). Also, (E)-aconitic acid (hereafter referred to as “aconitic acid”) was identified as an antifeedant from this plant (Kim et al., 1976). On the other hand, Katsuahara et al. (1994) reported that aconitic acid was not detected in the phloem sap of barnyard grass, collected by laser stylectomy using Laodelphax striatellus. Thus, it is likely that N. lugens feeding activity on the barnyard grass is interrupted by aconitic acid located in non-phloem tissues such as the parenchyma. Nevertheless, there are two possibilities in the mode of feeding interruption by aconitic acid in the plant. One is that arrival of the stylets at the phloem is disturbed from its contact with aconitic acid in non-phloem tissues. The other is that ingestion of phloem sap is inhibited in the sieve element by aconitic acid flowing from surrounding tissues ruptured by probing, after the stylets had reached it.

In this paper, feeding interruption of N. lugens on a barnyard grass that contains aconitic acid as an antifeedant in non-phloem tissue was examined using an AC electronic monitoring system (EMS). The results are also compared with those obtained on the resistant rice variety ‘Babawee,’ with the bph4 gene (Lakshminaraya and Khush, 1977). The resistance mechanism of this variety has not been studied, although little honeydew excretion has been observed when N. lugens fed on it (Ito et al., 1994).
MATERIALS AND METHODS

Plants. Barnyard grass, Echinochloa Cruss-galli var. oryzicolor and two varieties of rice, Oryza sativa L., ‘Toyoshikish’ (susceptible) and ‘Babawee’ (resistant, bph4) were grown in a green-house under natural light. All plants were used at the 10–12 leaf stage.

Insects. The brown planthopper, N. lugens which is avirulent to ‘Babawee,’ was taken from stock colonies reared successively at our institute on rice seedlings (‘Koshihikari’) at 25±2°C, 60±10% r.h. and L16:8D photoperiod. N. lugens was collected in paddy fields in Izumo, Shimane in 1988. In all experiments, 3–7 d old adult brachypterous females were used.

Electronic monitoring of feeding. Feeding activities of N. lugens on each plant were recorded using an AC electronic monitoring system (EMS) adapted from Kawabe (1994). A gold wire (20 µm diameter, 5 cm length) was attached to the dorsalum of a carbon dioxide-anesthetized planthopper with conductive silver paint, while the opposite end of the wire was connected via a larger wire (1 mm, 6 cm) to the amplifier input. The insects were given access to a plant in water in a 50 ml-Erlenmeyer flask, that was connected to an oscillator (0.5 V, 500 Hz AC), after being starved but provided with water immersed in filter paper for 1–2 h. The set-up was enclosed by an earthed Faraday cage. The signal output from the amplifier was acquired using TRS software (Tsukuba Rica Seiki Inc.) and analogue-to-digital hardware and a computer (NEC 9821V10, CPU: Pentium 100 MHz). All recordings were acquired for about 300 min. Then probing behavior of each insect for 240 min from the start time of the first probe was analyzed for waveforms. Each variety or plant was tested 15 times using fresh insects and fresh plants. Data were analyzed by Tukey’s test.

Honeydew test. Honeydew droplets produced during EMS recording were collected on a 185 mm diameter of filter paper (Advantec Toyo No. 1) to confirm the correlation between the quality of honeydew and waveforms. The paper was mounted on a 215 mm diameter plastic disc which was attached to the hour hand of an electric clock. The disc rotated in a horizontal plane at one revolution per 12 h. Leaf sheaths of rice were horizontally set 1 cm from the paper surface. A planthopper wired for EMS recording was placed on the leaf sheath to collect honeydew on the peripheral area of the paper disc. The paper was treated with 0.1% nyn-hydren solution in acetone and heated to 100°C for 2 min to visualize amino acids after 10–12 h-recording.

Feeding activity in relation to (E)-aconitic acid. Diet solutions of 1% (w/v) aconitic acid dissolved in distilled water or in 15% (w/v) sucrose solution were produced after pH was adjusted to 7.0 with 2 N NaOH. Feeding activities of each of 15 insects on aconitic acid or other solutions were monitored with the same apparatus as previously reported by Hattori (1997), except that the Teflon ring (3.0 mm diameter, ca. 1 mm height) was larger and was filled with 20 µl of samples. The effects of aconitic acid and sucrose on the duration of ingestion and the percentage of salivation duration per probe (after being transformed from arcsine square root) were analyzed by two-way ANOVA.

RESULTS

Waveforms recorded during stylet probing of N. lugens

As reported by Velosum and Heinrichs (1986), three distinctive waveforms were observed during stylet probing; S, A, and I (Figs. 1a, b and c; 2a, b). As in another published work (Kimmins, 1989), S waveform appeared to consist of several waveforms. This waveform may represent not only salivation of the stylet sheath but also movement of the stylets and sampling of plant fluid. Because it is likely that sheath salivation represents the major biological meaning of S, this waveform is designated “primarily salivation phase.” Likewise, the I waveform contains rapidly alternating, moderately high and low amplitude components (Fig. 2b), which may indicate some salivation into, though ingestion from, sieve elements, and thus can be termed “primarily ingestion phase.” These waveforms are thought to be analogous to the stylet pathway phase and sieve element phase of aphids, respectively, according to the terminology suggested by Reese et al. (2000). The biological meaning of the A waveform is not known, although Velosum and Heinrichs (1986) hypothesized that it was related to sensory testing of phloem sieve elements. The I waveform always followed the A waveform.
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Fig. 1. An example of waveforms recorded during feeding activity by *N. lugens* on (a) barnyard grass, (b) resistant (*bph4*, Babawee, middle) and (c) susceptible (Toyonishiki, bottom) varieties of rice. (a) also shows an example of probes consisting solely of salivation. P: probe (duration from stylet insertion to stylet withdrawal); S: primarily salivation phase; A: A waveform (related to sensory testing of phloem); I: primarily ingestion phase. Charts are to be read from left to right.

Fig. 2. Major waveforms recorded during feeding activity of *N. lugens*: (a) S: primarily salivation phase; (b) A: A waveform (related to sensory testing of phloem), I: primarily ingestion phase; (c) waveforms recorded with *N. lugens* feeding on 1% (E)-aconitic acid solution in distilled water. Isl: ingestion of solution, Sal: salivation in solution. Charts are to be read from left to right.

**Frequency and duration of probes on different plants and rice varieties**

A probe in the present study is defined as the duration of time from stylet insertion to stylet withdrawal. The number of individual probes per insect was significantly higher on both the barnyard grass and the resistant rice variety, Babawee (*bph4*) than on the susceptible rice. This is complementary to the mean duration of individual probes, which was the shortest on the barnyard grass, intermediate on the resistant rice, and longest on the susceptible rice. The probing duration per insect showed a similar tendency (Table 1).

<table>
<thead>
<tr>
<th>Plant</th>
<th>No. of probes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Probing duration (min)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnyard grass</td>
<td>8.4±1.0 a</td>
<td>16.7±2.0 a</td>
</tr>
<tr>
<td>Babawee (<em>bph4</em>)</td>
<td>8.9±1.4 a</td>
<td>20.8±6.5 a</td>
</tr>
<tr>
<td>Toyonishiki (S)</td>
<td>4.7±0.9 b</td>
<td>48.2±7.4 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>R (*bph4*) and S in parentheses show resistant and susceptible varieties of rice, respectively.

<sup>b</sup>Values (mean±SE) followed by the same letter within a column are not significantly different by Tukey's test (*p<0.05*).
Table 2. Duration of S waveform (primarily salivation phase) and the percentage of probes consisting of only S waveform by N. lugens on the barnyard grass, resistant and susceptible rice varieties

<table>
<thead>
<tr>
<th>Plant</th>
<th>S waveform duration (min)</th>
<th>% of probes with waveform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per probe</td>
<td>per insect</td>
</tr>
<tr>
<td>Barnyard grass</td>
<td>4.9±0.4 a</td>
<td>41.2±7.3 ab</td>
</tr>
<tr>
<td>Babawee (R)</td>
<td>5.9±1.3 a</td>
<td>52.4±7.7 a</td>
</tr>
<tr>
<td>Toyonishiki (S)</td>
<td>5.8±0.5 a</td>
<td>27.6±4.2 b</td>
</tr>
</tbody>
</table>

a R and S in parentheses show resistant and susceptible varieties of rice, respectively.

b Values (mean±SE or %) followed by the same letter within a column are not significantly different by Tukey’s test (p<0.05).

c Percentages of salivation in a probe were analyzed after being transformed from arcsine.

Duration of S waveform and percentage of probes consisting of S only

The mean duration of the S waveform per probe did not differ significantly among the three plants. However, the duration per insect was significantly longer on the barnyard grass and the resistant rice than on the susceptible rice (Table 2). Probes that consisted of only S (Fig. 1a) were observed during recording on all three plants, but the percentage of such probes differed significantly among the three plants, being the highest in the barnyard grass (Table 2).

Frequencies and duration of I waveform and quality of honeydew produced during I waveform

Frequencies of the I waveform per probe and per insect were quite low on the barnyard grass. In contrast, frequencies both per probe and per insect were higher on rice, and not significantly different between the resistant and susceptible rice. However, mean duration of I per insect was significantly different among the 3 plants, being much shorter on the barnyard grass and the resistant rice than on the susceptible rice (Table 3). The frequency distribution of the I waveform events showed that very few events of long duration (>10 min) were made on the barnyard grass and resistant rice in most cases (Fig. 3). On the other hand, on the susceptible rice, about 20% (15/73) of the I waveform events recorded were sustained for over 60 min (Fig. 3). Insects continued this waveform for an average of 349.6±92.4 min (mean±SE, N=7) when allowed to feed on the plant over the 240 min standard recording time.

Sequential spots of honeydew droplets (ca. 25/h) produced during sustained I waveform (over 30 min) always showed high ninhydrin positivity, indicating they were amino acid-rich.

Effect of (E)-aconitic acid on the duration of Isl and Ssl waveforms

To study planthopper probing on a diet solution, Isl waveform (ingestion of solution, Fig. 2c) and Ssl waveform (salivation in solution) were recorded. During Isl waveform over 20 min, honeydew excretion was often observed. The duration of the Isl waveform on distilled water and aconitic acid solution averaged 31.2 min and 1.8 min, respectively (Fig. 4, two-way ANOVA, $F=10.96, p<0.01$). Addition of sucrose to distilled water and
aconitic acid solution significantly increased the Isi duration to 109.4 min and 17.6 min, respectively (two-way ANOVA, $F=19.08$, $p<0.001$). There was a significant difference in the effect of the interaction between aconitic acid and sucrose ($F=5.41$, $p<0.05$), indicating that sucrose considerably mitigated the inhibitory effect of aconitic acid on ingestion. On the other hand, the percentage of Ssl waveform duration in a probe significantly increased with addition of aconitic acid, from 16.0 to 45.5% in distilled water and from 6.6 to 40.9% in sucrose solution (two-way ANOVA, $F=22.37$, $p<0.001$).

**DISCUSSION**

Waveforms from *N. lugens* probing activities on rice have been recorded with all types of electronic monitors. Velusamy and Heinrichs (1986) used an AC monitor similar to the one used in the present study, Kimmins (1989) and Losel and Goodman (1993) used a Tjallingii (1988)-type DC monitor, and Khan and Saxena (1988) used a DC battery. The AC monitor’s S waveforms in this study appears to be a sequential composite corresponding to patterns 1 and 2 of Kimmins (1989). Likewise, the AC A waveform is virtually identical in appearance to, and has a similar peak frequency of 12–15 peaks per minute as, pattern 3 of Kimmins (1989). Further, the A waveform always precedes the 1 waveform, as pattern 3 always precedes pattern 4, which was shown to be associated with ingestion of phloem by honeydew analysis (Kimmins, 1989). Khan and Saxena (1988) reported that when the insect produced the A waveform, the terminal location of the stylet sheath ended in the phloem, although the specific phloem cell-type was not determined.

Velusamy and Heinrichs (1986) and Khan and Saxena (1988) ascribe solely phloem sap ingestion to their 1 waveform, the latter study by light histological correlation of salivary sheaths. On the other hand, the two DC-system studies found two types of ingestion waveforms, which were shown by honeydew analysis to be associated with phloem and xylem ingestion (patterns 4 and 5, respectively, of Kimmins (1989) and types III and II, respectively, of Losel and Goodman (1993)). Patterns 4 and 5 are distinctly different in appearance, and only pattern 4 is always accompanied with pattern 3. Durations of xylem ingestion were always shorter than those of phloem ingestion in these studies, as Sogawa (1980) reported that *N. lugens* ingests primarily from phloem on rice. It was confirmed that honeydew droplets produced during the 1 waveform contained high levels of amino acids, as did those produced during the pattern 4 obtained by Kimmins (1989). Thus, the 1 waveform in this study, which always followed the A waveform may solely represent phloem ingestion, although a waveform related to xylem ingestion was not determined.

The feeding process of *N. lugens* can be divided into two main behavioral phases: 1) exploratory probing with the secretion of coagulable sheath material (sheath salivation), along with stylet movement and breakage of cell walls and membranes, and 2) sucking (ingestion) (Sogawa, 1973). The analysis in this study emphasized these two phases, and the waveforms representing them.

*N. lugens* responded to a resistant rice, Babawee (bph4) with increased probe frequency and duration of the primarily salivation phase, compared with the susceptible rice, which was also similar to the barnyard grass. The primarily ingestion phase was observed on the resistant rice as frequently as on the susceptible rice, unlike on the barnyard grass, but in most cases, ingestion was interrupted within 10 min. A similar response was recorded on other resistant varieties of rice with several differ-
ent resistance genes, i.e. IR56 (Bph3) (Velusamy and Heinrichs, 1986), ASD7 (bph2) (Khan and Saxena, 1988) and IR46 (Bph1) (Kimmins, 1989). Sogawa and Pathak (1970), using plant histology and honeydew analysis, confirmed that N. lugens can successfully insert its stylets into the vascular bundles of Mudgo (Bph1) as frequently as those of TN1 (susceptible rice), but excretes less honeydew and probably ingests less. Thus, our results strongly suggest that the resistance of Babawee (bph4) to N. lugens is basically due to the same mechanism as other resistant varieties, i.e. interruption of ingestion after insertion of the stylets into the phloem.

On the barnyard grass, the planthoppers performed many probes, but the mean duration of a probe and probing duration per insect were shorter than on the resistant and susceptible rice, resulting in increased durations per insect of non-probing (complementary to probing). Additionally, on the barnyard grass more than 50% of all probes consisted of S waveform only. These results suggest that continuous probing was deterred during the primarily salivation phase in the parenchyma and, ingestion activity may be diminished on the barnyard grass. As a result, N. lugens rarely showed the primarily ingestion phase on the barnyard grass. In aphids probing on non-host plants, an absence of phloem ingestion, but occurrence of non-phloem ingestion has frequently been observed (e.g. McLean and Kinsey, 1968; Campbell et al., 1982). However, the presence or location of chemicals that could interrupt access to the vascular bundle or ingestion event were not fully investigated in these cases.

Aconitic acid is contained in leaf blades and leaf sheaths of barnyard grass at a concentration of 0.44% and 0.26% on a fresh weight basis, respectively, and is virtually not detected in its phloem sap (Katsuhara et al., 1994). Its concentration in the phloem sap is less than 0.007% (Nagata and Hayakawa, 1998). Nagata and Hayakawa (1998) also reported that aconitic acid inhibited feeding of N. lugens at a concentration above 1% in 2.5% sucrose. In the present study, insects ingested 1% aconitic acid dissolved in distilled water for very short durations and showed relatively long durations of salivation. This result suggests that when N. lugens comes in contact with aconitic acid in parenchyma cells during the primarily salivation phase, it should withdraw its stylets before reaching the vascular tissues. Eventually, insects may be less likely to locate the phloem sieve elements.

The primarily ingestion phase in the phloem was rarely observed on barnyard grass, and was interrupted within 5 min. On the other hand, insects performed ingestion on 1% aconitic acid solution with sucrose for 18 min on average, although this concentration is higher than that in the leaf blade or sheath described above. These findings suggest that ingestion from the sieve element cannot be inhibited rapidly even if aconitic acid should flow into the phloem sap containing sucrose, from parenchyma or other tissues ruptured during probing. Interruption of ingestion caused by resistant plants, therefore, seems to be due to factors other than aconitic acid. This situation may be similar to that observed with aphids, which spend longer times searching phloem in wheat with higher concentrations of hydroxamic acid. But, once they find a sieve element in which this substance is absent, they ingest from it for long durations (Givovich and Niemeyer, 1995). Therefore, the barnyard grass might have a defense mechanism similar to that of resistant rice which interrupts the feeding activity of N. lugens after the primarily ingestion phase, in addition to interruption in the primarily salivation phase by aconitic acid.

ACKNOWLEDGEMENTS

I thank Drs. K. Sogawa, E. A. Backus, and J. C. Reese for critical reading of the manuscript. I also thank Dr. S. Kawabe for invaluable suggestions about EMS. Thanks are due to Mr. A. Koarai for gifts of barnyard grass seeds and Mrs. M. Sakairi for rearing insects.

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