Research Article

Received: 29 July 2016 Revised: 26 October 2016 Accepted article published: 19 December 2016 Published online in Wiley Online Library: 13 February 2017

(wileyonlinelibrary.com) DOI 10.1002/ps.4508

The toxicity of flonicamid to cotton leafhopper, *Amrasca biguttula* (Ishida), is by disruption of ingestion: an electropenetrography study

Kaleem Tariq,[a,b] Mah Noor,[c] Elaine A Backus,[d] Adil Hussain,[b] Asad Ali,[b] Wei Peng[a] and Hongyu Zhang[a*]

Abstract

BACKGROUND: The cotton leafhopper, *Amrasca biguttula*, is one of the most destructive pests of cotton in Asia. This species is thought to cause damage by injecting enzymatic saliva into various, presently unknown, cotton tissues and ingesting the resulting macerate. Flonicamid is a novel systemic insecticide used to control the cotton leafhopper; however, its mode of action is unknown.

RESULTS: The mechanism of action of flonicamid on cotton leafhopper was investigated using electropenetrography (EPG). EPG recordings revealed six waveforms, i.e. Np (non-probing), A1 (channel cutting), A2, A3, A4, A5 and A6. Waveforms A2 and A3 probably represent active ingestion with (A2) and without (A3) simultaneous watery salivation. The meanings of A4, A5 and A6 are presently unknown, but minor in duration. Flonicamid significantly increased the mean duration of non-probing events and strongly inhibited ingestion by treated insects, which resulted in the slow death of leafhoppers. Inhibition of ingestion was dose dependent, and near-complete suppression was observed when the flonicamid concentration was increased to 10 000 mg L^{-1}.

CONCLUSIONS: We propose that starvation caused by inhibition of active ingestion is the mechanism of toxicity for flonicamid. This knowledge could aid in applicability and use of this new insecticide for field management of leafhopper populations.
© 2016 Society of Chemical Industry

Supporting information may be found in the online version of this article.

Keywords: flonicamid; *Amrasca biguttula*; electrical penetration graph; electropenetrography; feeding behaviour

1 INTRODUCTION

Cotton (*Gossypium* spp.) is a major commercial crop, designated as the 'king of fibres', that has global importance in the production of fibre and seed, raw material for the textile industry.[1] China is one of the largest cotton-producing countries in the world and meets almost 20% of the annual worldwide cotton demand.[2] Cotton plays a pivotal role in the economic development of many countries. Insect pests and crop diseases are major yield-limiting factors in almost all cotton-growing countries.[3] The introduction of *Bacillus thuringiensis* (*Bt*) cotton reduced the problem of bollworms, but unfortunately the population of piercing-sucking insect pests gradually increased.[4] Piercing-sucking insects cause 40–50% of the damage to the cotton crop.[5]

The cotton leafhopper or cotton jassid, *Amrasca biguttula* (Ishida) (Hemiptera: Auchenorrhyncha Cicadellidae), is one of the most destructive pests of cotton in subcontinental South-east Asia, including China. The leafhopper has multiple hosts such as cotton and jute and several other plants from the family Solanaceae such as potato, eggplant, okra and brinjal (eggplant). Nymphs and adults of *A. biguttula* cause damage to the crop by sucking cell sap and injecting disruptive saliva into tissues. A severe attack of *A. biguttula* can reduce cotton yield by 24.45%.[3,6–9] Farmers adopt various strategies to control *A. biguttula*, of which insecticides are the first line of defence. Currently, several conventional and new chemical insecticides are being used to control this potential pest.

Flonicamid, a pyridine carboxamide, is a novel insecticide that is in the selective Hemiptera-feeding blocker group (mode of action group 9C; classification by IRAC, irac-online.org). The insecticidal mechanism appears to be starvation caused by irreversible

* *Correspondence to: H Zhang, State Key Laboratory of Agricultural Microbiology, Hubei Key Laboratory of Insect Resource Application and Sustainable Pest Control and Institute of Urban and Horticultural Entomology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China. E-mail: hongyu.zhang@mail.hzau.edu.cn*

a *State Key Laboratory of Agricultural Microbiology, Hubei Key Laboratory of Insect Resource Application and Sustainable Pest Control and Institute of Urban and Horticultural Entomology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China*

b *Department of Agriculture, Abdul Wail Khan University Mardan, Khyber Pakhtunkhwa, Pakistan*

c *Molecular Biotechnology Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China*

d *USDA, Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA*

inhibition of feeding activity.[10] Flonicamid inhibits the feeding activity of insects immediately after treatment and is an effective insecticide against sap-sucking insects such as whiteflies, aphids, leafhoppers (jassids), thrips, scales and mealybugs.[11] Flonicamid inhibited the feeding activity of aphids within 0.5 h of treatment, without showing symptoms of neuronal intoxication, and feeding activity was inhibited until death.[12] Ghelani *et al.*[13] reported that the application of flonicamid in cotton fields caused significant mortality of *A. biguttula* and was statistically as effective as dinotefuran and imidacloprid.

Although laboratory and field experiments provide useful information on mortality caused by the application of an insecticide, comprehensive information on how an insecticide affects the feeding behaviour of insects requires other methods.[14] Among those methods, electropenetrography (EPG) is the most rigorous method for examining the effects of an insecticide on the feeding behaviour of piercing-sucking insect pests and on the damage to plant tissues.[15–17] In EPG, an insect and feeding substrate are incorporated into an electrical circuit. Changes in voltage occur when the insect begins to feed and completes the circuit. The fluctuations in biopotentials (voltage inside the plant) and resistance produce waveforms that correspond to the feeding behaviour of the insect, such as cell puncturing, stylet insertion, salivation, ingestion and stylet location within plant tissue.[18]

Several previous studies have been conducted to evaluate the effects of insecticides on the feeding behaviour of sap-sucking insects,[15,19,20] but the effect of flonicamid on the feeding behaviour of *A. biguttula* has not yet been examined. This study characterised the effects of flonicamid on the feeding behaviour of *A. biguttula* on cotton plants treated with the systemic insecticide. The results of this research will help the scientific community interpret the toxicity mechanism of flonicamid to *A. biguttula* and may provide valuable information for field application of flonicamid.

2 MATERIALS AND METHODS

2.1 Insects

Colonies of *A. biguttula* used in this experiment were collected from a cotton field at Huazhong Agricultural University. The cultures were maintained in the laboratory without exposure to insecticides at 30 ± 4 °C with a 14:10 h light:dark photoperiod and 60–65% RH. The 25–30-day-old potted cotton plants (variety Zhong 206) were infested with insects from the field. Insects were confined on plants in cages; the leafhoppers were cultured for four generations.

2.2 Insecticide

The insecticide flonicamid 96% TC (Hangzhou Tialong Biotechnology Co., Ltd, Hangzhou, China) was diluted with distilled water to make a series of concentrations: 0, 20, 200, 1000, 5000 and 10 000 mg AI L^{-1}. To minimise any chemical decomposition, the solutions were used immediately after preparation.

2.3 Bioassay methods with *A. biguttula*

As developed and recommended by the Insecticide Resistance Action Committee (IRAC method No. 8), and previously described by Preetha *et al.*,[21] a leaf dip bioassay was conducted with *A. biguttula*. In the bioassay, two plastic cups were used, one as the inner test chamber and the other as the outer water reservoir. Fresh, uncontaminated cotton leaves were collected from the cotton field and were cleaned with a wet cotton swab. The leaf petiole was cut to a length of approximately 5 cm. The leaves were dipped into freshly prepared insecticide solutions for 5 s and hung vertically for 5–10 min in the open shade to dry. The leaf petiole was passed through a small hole on the side of the inner cup bottom. The outer cup containing water was placed inside to prevent overdrying and to keep the leaf in an upright position.

Third-instar nymphs of *A. biguttula* were removed from the culture with a fine camel hair brush, and ten nymphs were released into the test cup. The cup was covered with a perforated lid. A total of 50 nymphs (five cups) were tested for each concentration. The control treatment contained a cotton leaf dipped in distilled water. Each treatment was replicated 3 times, including the control. The cups were maintained in laboratory conditions as described above, and mortality was assessed daily.

Insect starvation treatment was given as described by Yueping *et al.*[19] Briefly, ten third-instar nymphs of *A. biguttula* were confined inside a plastic cup containing a water-saturated cotton swab (no leaf). A total of 50 nymphs (five cups) were tested for starvation treatment. The experiment was repeated 3 times, and the rest of the data on mortality were recorded as described above. The bioassay experiment was conducted for 7 days.

2.4 Electropenetrography of *A. biguttula* feeding behaviour

The objective of this study was to determine the effects of flonicamid on the probing behaviour of *A. biguttula* compared with water-treated control plants. EPG of *A. biguttula* on cotton was recorded using one Giga-4 DC EPG amplifier with a 10^9 Ω input resistance and less than 1 pA input bias current (EPG Systems, Wageningen, The Netherlands). Treatments were five concentrations of flonicamid (20, 200, 1000, 5000 and 10 000 mg AI L^{-1}) and distilled water as the control. The leaves of 40–50-day-old cotton seedlings were treated by dipping in the different concentrations of flonicamid. After being starved for 2 h, third-instar nymphs of *A. biguttula* were chilled on ice for 2–3 min. The immobilised insects were individually connected, via the thoracic notum, to a gold wire (2 cm length, 18 µm diameter) with silver conductive glue (Pelco colloidal silver paint; Ted Pella, Inc., Redding, CA). The ground electrode was placed into the soil near the stems of treated cotton plants to provide the voltage, and the insect was connected to the electrical circuit by gluing the gold wire to a copper wire (0.5 mm diameter) with conducting silver paint, which was then soldered to a brass nail inserted into the input lead of the head stage amplifier. The insect attached to the gold wire was then carefully placed on the leaf of the treated cotton plant.

Voltage fluctuations representing feeding behaviours of nymphs of *A. biguttula* on insecticide-treated and control leaves were recorded onto a computer hard disk through an analog-to-digital conversion for further analysis. To reduce interference from external electrical noise, the entire experimental set-up was performed inside a large Faraday cage (2 × 2 × 4 ft, aluminium frame with a steel base) at 30 ± 2 °C and 60–65% RH under continuous light for 8 h using PROBE 3.4 software (Wageningen Agricultural University, Wageningen, The Netherlands) operating in Windows-XP. New, freshly treated cotton plants and insects were used for each replication. A total of ten 8 h EPG readings were recorded for each concentration of flonicamid, which were used for final data analysis.

2.5 Statistical analyses

Six primary types of waveform produced by cotton leafhoppers probing cotton leaves were determined (Figs 1A to D), based on their similarities to those described by Jin *et al.*[22] for the related

 © 2016 Society of Chemical Industry

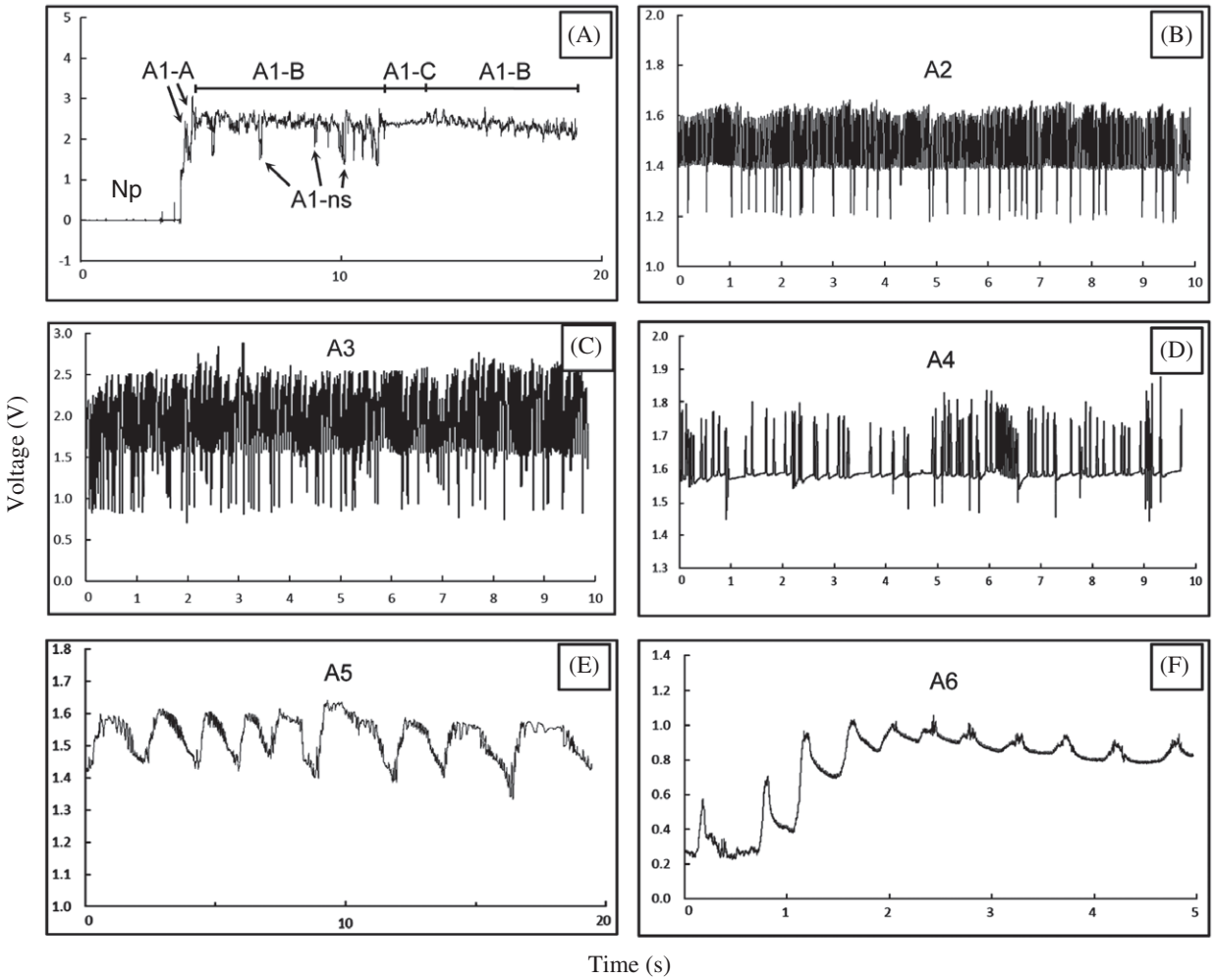

Figure 1. Characteristic EPG waveforms recorded from *A. biguttula* on cotton leaves. (A) non-probing (Np) and waveform A1 (including subtypes) early in a probe (only a portion of A1 is shown; A1-C continued after this excerpt); (B) A2, performed after A1; (C) A3 after A2; (D) A4, interspersed with A2 and A3; (E) A5; (F) A6. The *y*-axis indicates the amplitude (voltage) at standardised gain for all examples. The *x*-axis indicates time in seconds. Note that the *y*-axis indicates that waveforms have very different amplitudes despite being portrayed as similar heights.

leafhopper species *Empoasca vitis*. Biological meanings of these waveforms were hypothesised, again on the basis of Jin *et al.*[22] Each EPG waveform was summarised using four non-sequential variables: the number of events and the percentage, total duration and average duration of each waveform during the entire recording time. These variables were calculated similarly to those in Backus *et al.*[23] The number of events for each waveform was the number of times that a waveform appeared during the recording time. The percentage of each waveform was calculated by dividing the total duration of each waveform over the whole recording by the total recording time. The total waveform duration for each EPG waveform was calculated by summing the durations of all waveform events of all insects that occurred during the observation time. The average duration of each EPG waveform represented the mean duration of each waveform per insect. Sequential variables of EPG waveforms were also analysed similarly to Sarria *et al.*,[24] including the following: the duration of non-probing (A) from the start of the EPG recording to the first probe, the duration between the first and second probes of A1, the number of probes before the first probe containing A3, the duration of the first probe containing A3 and the interval from the start of the first probe to the first A3 event. Furthermore, the percentages of the insects that reached A3, after being treated with different concentrations of flonicamid and water (CK), were also calculated.

EPG recording data were analysed with SAS v.9.2 for Windows (SAS Institute, Cary, NC) statistical software. The least significant difference (LSD) test was used to evaluate the differences in the EPG variables between control (CK) and treatments at various concentrations of flonicamid. Regression analyses were conducted to determine the relationship between EPG variables and concentrations of flonicamid (log transformed). The mortality data from dose–response bioassays and the calculations of LC_{50} values were determined using PoloPlus software (PoloPlus: probit and logit).

3 RESULTS

3.1 Characterisation of waveforms from *A. biguttula* nymphs feeding on cotton leaves

When *A. biguttula* probed on cotton leaves, six different types of waveform (Fig. 1 and Table 1) were identified in the EPG out. The characterisation of identified waveforms is as follows.

3.1.1 Np

When insects were not probing, the recording always showed a flat baseline (0 V reference level) (Fig. 1A). The output voltage of

 © 2016 Society of Chemical Industry

Table 1. Electrical characteristics of waveforms from *A. biguttula* nymphs feeding on cotton leaves during ten recordings of 8 h each

EPG waveform	Subtypes	Relative amplitude[a]	Repetition rate	Voltage level[b]	Electrical origin
Np[c]		Low	0	0	
A1	A1-A	20–40	Mixed	e	R
	A1-C	100	Mixed	e	R
	A1-B	30–60	6–12	e	R
	A1-ns			Unknown	emf
A2		20–60	5–6	e	emf
A3		80–100	8–10	e	emf
A4		3–20	4–5	e	emf
A5		5–18	0.4–1.4	e	emf
A6		4–30	1–6	e	emf

[a] Voltage deflection (minimum to maximum) is a percentage of the maximum waveform amplitude.
[b] e = extracellular voltage level.
[c] Np = non-probing.

all the detected waveforms was higher than the baseline, and thus the baseline was used to differentiate probing from non-probing activities of insects. The baseline with *A. biguttula* recordings was relatively flat; however, small irregularities were also detected that were due to the movement of leafhoppers on the leaf surface (Fig. 1A).

3.1.2 A1

The voltage level changed suddenly to a positive value (depending upon the applied voltage to the electrode) as the stylets were inserted. Stylet penetration always started with the A1 waveform (Fig. 1B). The A1 waveform produced by *A. biguttula* was composed of waveform subtypes A1-A, A1-B, A1-C and A1-ns (brief negative spikes) (Fig. 1A). Usually, the median voltage of A1 was equal to or higher than the median voltage of the A2 waveform and was lower than the A3 waveform (Table 1). The repetition rate of the A1 waveform (including subtypes) was variable. The fast Fourier transform (FFT) spectrum of the A1 waveform showed a spectral band of low frequency between 0 and 20 Hz; however, the frequencies between 0.4 and 0.8 Hz predominated.

All the subtypes of the A1 waveform were extracellular (above 0 V) and originated as R component. The waveform A1-A was always generated just after initial stylet penetration, which indicates that this subtype appeared when stylets contacted the leaf epidermis. Among the A1 waveform subtypes, A1-C was (to some extent) regular in frequency, with a wide frequency band of 6–12 Hz (Table 1). There was alternating occurrence of A1-B and A1-C, and the A1 event always ended with A1-C. Brief events of A1-ns always appeared within the A1-B waveform, as very brief potential drops. Although A1-ns seemed to have an appearance similar to that of the drop caused by a calibration pulse (which can be used to distinguish probing activities from non-probing activities), there were differences in the details between them. It is noteworthy that A1 always occurred in every probe and also appeared in alternating moments of two different waveforms.

3.1.3 A2

The A2 waveform was highly regular, resembling a loose square wave with variable amplitude (Fig. 1B, compressed view), and was characterised by a repetitive and regular overlying pattern of square peaks of high amplitude. Moreover, the A2 waveform showed a sequence of underlying, short spikes appearing at regular intervals, producing a 'ruffle'. Consequently, A2 showed a narrow spectral band of frequency ranging between 5 and 6 Hz. The A2 waveform of *A. biguttula* was always preceded by A1 and mostly followed by A3. However, A2 appearing alone, without subsequent A3, was also observed. The mean voltage level of A2 was always positive (extracellular), similarly to waveform A1. Additionally, when the applied substrate voltage was changed from positive to negative, no change in characteristics of the A2 waveform was seen. This result demonstrates that electromotive force (emf) was the main component of the A2 waveform (Table 1).

3.1.4 A3

Waveform A3 produced by *A. biguttula* was also a highly regular waveform (Fig. 1C) and always appeared after an A2 waveform event; the amplitude of A3 was much higher with less variation in depth compared with A2 waveform. The frequency range of A3 was 8–10 Hz. A3 was consistently of the same shape, except at times varying in amplitude. Usually, A3 would slowly evolve over time into higher amplitude and lower frequency (Fig. 1C). The mean voltage level of A3 was always positive (extracellular) and higher than all the other detected waveforms. The A3 waveform appeared frequently and lasted more briefly than A2. The electrical origin of A3 was emf.

3.1.5 A4

The A4 waveform was regular and complex with tall spikes (Fig. 1D). A4 appeared occasionally and usually was preceded by the A1 waveform. A rapid rate of repetition (4–5 Hz) with a higher amplitude (0.5–0.7 V) was recorded at the beginning of an A4 event, although not at the end. This waveform evolved gradually over time with a lower amplitude (0.2–0.3 V) and a slower rate of spike repetition (0.5–1 Hz). The A1 and/or A5 waveform usually followed A4. Like the A3 waveform, the electrical origin of A4 was emf (Table 1).

3.1.6 A5

The A5 waveform produced by *A. biguttula* was characterised by a high-amplitude sequence of peaks with negative deflections (Fig. 1E). A higher frequency (0.8–1.4 Hz) occurred at the beginning of this waveform, with a tendency to decline (0.4–0.6 Hz) over time. During the recording of *A. biguttula* feeding behaviour,

 © 2016 Society of Chemical Industry

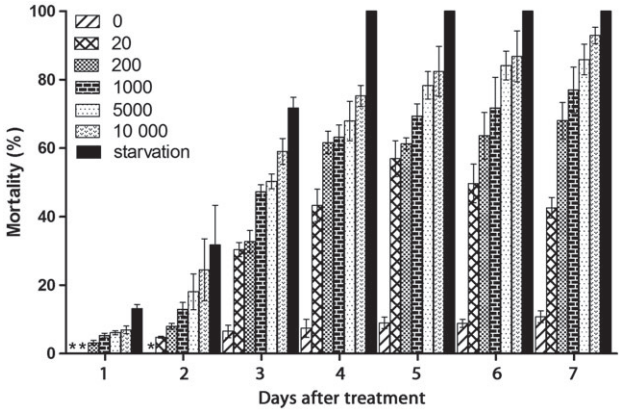

Figure 2. Mortality of third-instar nymphs of *A. biguttula* treated with flonicamid or starvation. * indicates 0%.

A5 generally followed an A4 event, but occurred only occasionally. Events of the A5 waveform often had shorter duration (up to 2–3 min) than events of A1, A2 and A3. The positive level of voltage indicated an extracellular position of stylets during A5.

3.1.7 A6

The A6 waveform was characterised by upward spikes with a highly variable amplitude and frequency (1–6 Hz) (Fig. 1F). This waveform was very stereotypical, appearing with the same shape which was easily distinguished from other waveforms. A6 was not detected in all tested leafhoppers, but when it happened, it was of relatively short duration (1.5–2 min). Also, A6 was always preceded by A1, and usually followed by A2.

3.2 Activity of flonicamid against *A. biguttula*

The toxicity of flonicamid 96% TC to third-instar nymphs of *A. biguttula* was compared with the control (CK: 0 mg L^{-1} of flonicamid) and starvation (water only, no cotton leaf for feeding) treatments (Fig. 2 and Table 2). After 24 h, the mortality of third-instar nymphs to flonicamid at different concentrations was less than 10%, whereas mortality of 0 and 13.16% was observed in the CK and starvation treatment respectively. After 48 h, mortality for all tested concentrations of flonicamid was less than 30%, whereas mortality was zero and 31.7% in the CK and starvation treatment respectively (Fig. 2). After 72 h, mortality was 5.6 and 71.7% in the CK and starvation treatment respectively. The LC_{50} values of flonicamid to *A. biguttula* at 24, 48 and 72 h were 7510.94, 974.10 and 595.71 mg L^{-1} respectively (Table 2). Mortality in the starvation treatment reached 100%, whereas in the CK the mortality was 7.3% after 96 h of flonicamid treatment. After 96 h of treatment, mortality for the highest concentration (10 000 mg L^{-1}) of flonicamid was 75.2%, and the LC_{50} value was 358.11 mg L^{-1} (Fig. 2 and Table 2). After 120 h, less than 10% mortality was observed in the CK, and the LC_{50} value (239.22 mg L^{-1}) of flonicamid was approximately half the value at 72 h. At 168 h after treatment, 8.8% mortality occurred in the CK, whereas mortality of 86.8% was recorded at the highest concentration (10 000 mg L^{-1}) of flonicamid. The LC_{50} value at this time point was 155.27 mg L^{-1}, which was 3.8-fold lower than the value at 72 h. After 216 h of treatment, mortality in the CK was 10.7%, whereas mortality at 10 000 mg L^{-1} of flonicamid reached 92.9%, with an LC_{50} value of 153.96 mg L^{-1} (Fig. 2 and Table 2). Bioassays demonstrated that flonicamid was slow acting and was significantly slower than starvation in causing mortality of *A. biguttula*.

3.3 EPG and feeding behaviour in flonicamid-treated *A. biguttula*

During the 8 h recording period, the average and total duration of the Np waveform of the CK were 8.2 ± 2.4 min and 46.2 ± 17.07 min respectively. The average and total durations of the Np waveform of the CK were significantly shorter than those on plants that were treated with flonicamid ($P < 0.05$) (Fig. 3A and supporting information Fig. S1A). Regression analysis showed a significant linear relationship between flonicamid concentrations and the average and total duration of the Np waveform ($P < 0.0001$, $R^2 = 0.85$; $P < 0.002$, $R^2 = 0.88$ respectively). The duration of Np waveforms increased as the concentration of flonicamid increased (Fig. 3A and supporting information Fig. S1A). The number of events of Np waveforms were not significantly different between the CK and the flonicamid treatments ($P = 0.35$) (supporting information Fig. S2A). Analysis of the average duration revealed no significant correlation between the CK and flonicamid treatment for A1 and A4 waveforms, and the fit to either a linear or an exponential model was poor (linear: $P = 0.15$, $R^2 = 0.046$ and $P = 0.035$, $R^2 = 0.77$; exponential decay: $P = 0.21$, $R^2 = 0.069$ and $P = 0.035$, $R^2 = 0.76$ respectively) (Figs 3B and E). However, a significant difference was observed in the average duration of the A2 and A3 waveforms among the treatments ($P = 0.0001$, $R^2 = 0.91$ and $P = 0.0001$, $R^2 = 0.96$ respectively) (Figs 3C and D). No significant difference was observed in the total duration of the A1 and A4 waveforms among the treatments ($P = 0.35$) (supporting information Figs S1B and E). A significant difference in the total duration of the A2 and A3 waveforms was found among the treatments ($P = 0.0001$, $R^2 = 0.99$ and $P = 0.0001$, $R^2 = 0.87$ respectively) (supporting information Figs S1C and D).

Table 2. Toxicity of flonicamid to *A. biguttula* after different time periods

Time after treatment (h)	Slope (± SE)	LC_{50} (95% CL)(mg L^{-1})	N^a	$\chi^{2\,b}$	df
24	0.39(±0.055)	7510.94 (3764.78–20125.65)	150	2.12	2
48	0.31(±0.049)	974.10 (478.50–2057.10)	150	0.86	2
72	0.33(±0.050)	595.71 (283.71–1181.8)	150	2.08	2
96	0.36(±0.050)	358.11 (166.54–677.48)	150	3.71	2
120	0.37(±0.051)	239.22 (108.08–446.78)	150	5.22	2
168	0.35(±0.051)	155.27 (60.16–309.85)	150	5.02	2
216	0.38(±0.052)	153.96 (63.47–279.20)	150	7.30	2

[a] *N*: number of *A. biguttula* tested.
[b] Chi-square testing linearity of dose–mortality response: $P < 0.05$.

 © 2016 Society of Chemical Industry

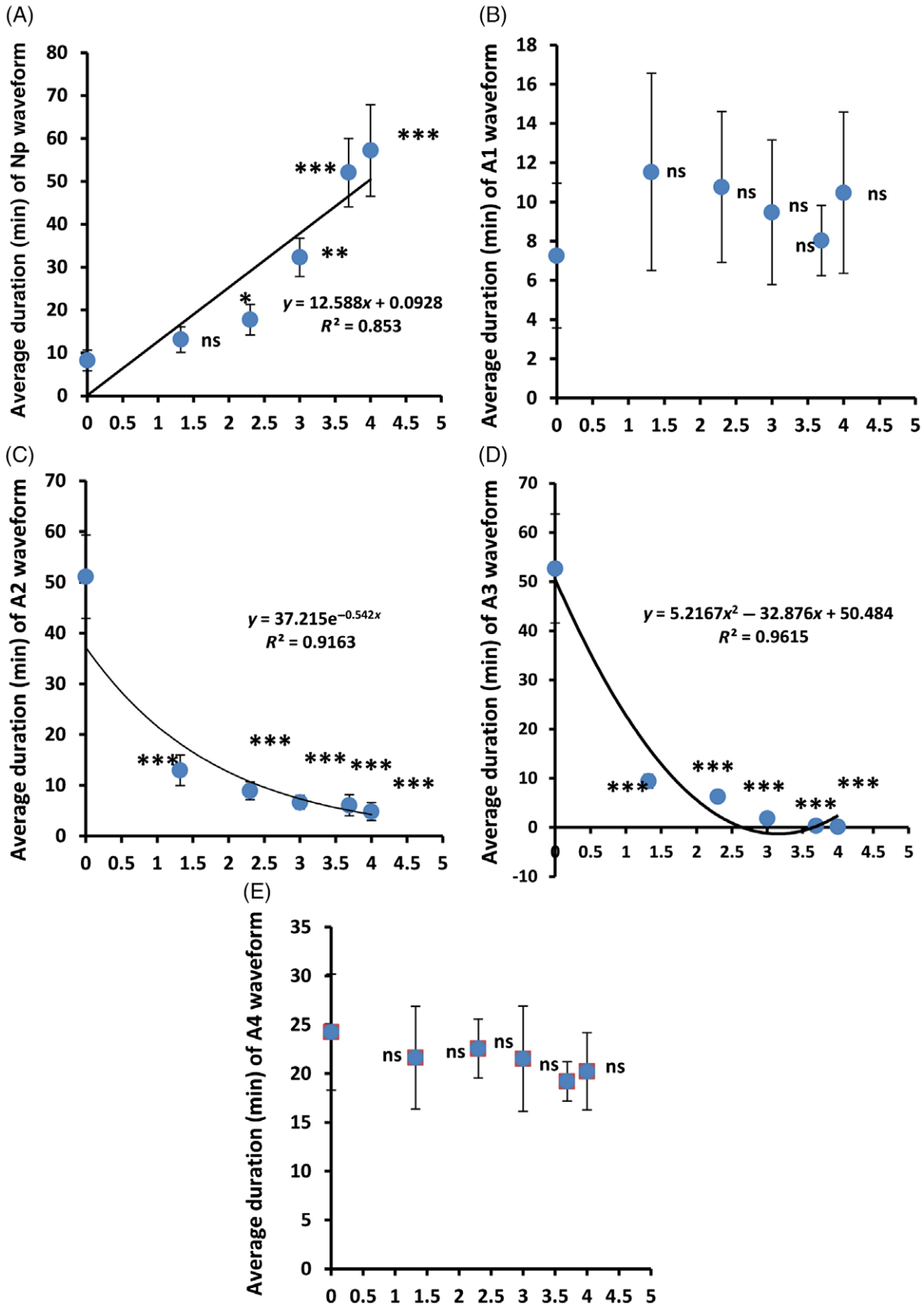

Figure 3. Relationship between flonicamid concentrations (log) and the average duration of each EPG waveform, including the average duration of the Np waveform (A), A1 waveform (B), A2 waveform (C), A3 waveform (D) and A4 waveform (E). Each data point is presented as the mean ± SE. Asterisks *, ** and *** by a data point show a significant difference between the CK and these values ($P < 0.05$, $P < 0.01$ and $P < 0.001$) respectively, and 'ns' denotes non-significance ($P > 0.05$).

 © 2016 Society of Chemical Industry

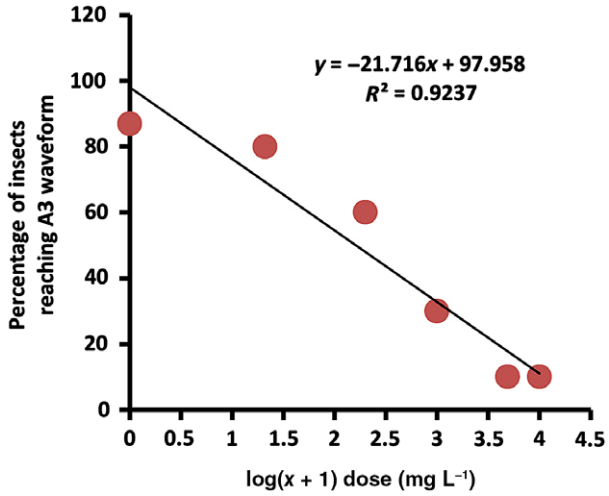

Figure 4. Percentage of insects reaching the A3 waveform after being treated with water (CK) and different concentrations of flonicamid.

The number of events of Np, A1 and A4 waveforms were statistically similar in the CK and flonicamid treatments ($P = 0.34$) (supporting information Figs S2A, B and E). However, significant differences were found for the A2 and A3 waveforms between the CK and flonicamid treatments ($P = 0.0001$, $R^2 = 0.84$ and $P = 0.001$, $R^2 = 0.83$ respectively) (supporting information Figs S2C and D).

A significantly negative correlation was observed between the concentration of flonicamid and the percentage of tested insects that reached the A3 event ($P = 0.0001$, $R^2 = 0.92$) (Fig. 4). Additionally, five sequential variables were analysed (Figs 5A to E). No significant difference was found ($P > 0.05$) among the treatments for the transition time from the starting point to the first A1 event (Fig. 5A), for the interval from the first to the second A1 probe (Fig. 5B) or for the number of probes before the first A3 event (Fig. 5C). However, a significant exponential regression was observed between the log dose of flonicamid and the first A3 period ($P = 0.0001$, $R^2 = 0.99$) (Fig. 5D). The analysis from A1 to the first A3 event revealed a significant linear relationship between the log dose and the first A3 event ($P = 0.022$, $R^2 = 0.82$) (Fig. 5E).

Thus, the EPG results showed that, as the concentration of flonicamid increased, the percentage of recording time represented by Np increased, while the percentage of A2 and A3 decreased. Accordingly, the effect of flonicamid was significantly negative on A2 and A3. However, no significant effects were detected on A4 waveforms (Fig. 6).

4 DISCUSSION

4.1 Hypothesised biological meanings of *A. biguttula* EPG waveforms

Different EPG waveforms produced by *A. biguttula* were biologically interpreted on the basis of the previously defined correlation[22] of waveforms from *E. vitis*, a species in the same subfamily (Typhlocybinae) as *A. biguttula*. All previously studied typhlocybine leafhoppers use the cell rupture feeding strategy (formerly known as the lacerate-and-flush[25]), rather than the salivary sheath feeding strategy typical of other leafhoppers. In cell rupture feeding, only a partial salivary sheath is produced and the insect actively moves its stylets through many cells (termed 'channel cutting'), actively ingesting from multiple cell types in vascular and/or non-vascular tissues.[22,25] The *E. vitis* waveforms E1-A, E1-B, E1-C and E1-ns have been shown to be strongly correlated with stylet movements and secretion of watery saliva during channel cutting.[22] We hypothesise that similar waveforms detected in this study, A1-A, A1-B, A1-C and A1-ns (respectively), also represent channel cutting by stylets.

The *E. vitis* E2 waveform represents watery salivation combined with active ingestion, while the E3 waveform represents active ingestion alone.[22] We propose that waveforms A2 and A3 identified in this study (Figs 1B and C) also represent active ingestion, with and without watery salivation respectively. At present, the leaf tissue(s) in which such channel cutting, salivation and ingestion occur are unknown for both *E. vitis*[22] and *A. biguttula*. Previous EPG studies of *E. fabae*[25] suggest channel cutting in either mesophyll/parenchyma or general phloem tissues. *E. fabae* has never been observed to ingest from xylem cells. Here, the waveforms A4, A5 and A6 were only occasionally observed, similarly to E4, E5 and E6 in Jin *et al.*[22] However, both of these were minor waveforms detected for both the species, and they were not biologically characterised either by Jin *et al.*[22] or in our study.

4.2 Effects of flonicamid on *A. biguttula* feeding behaviour

Flonicamid is a novel insecticide that is a selective Hemiptera-feeding blocker, with the stated insecticidal mechanism being starvation caused by irreversible inhibition of feeding activity.[12,26] The present study revealed that the feeding behaviour of *A. biguttula* on flonicamid-treated cotton plants was significantly inhibited. A dose-dependent correlation was observed between Np percentage and flonicamid concentration, which indicated that insects on flonicamid-treated plants spent more time in Np or resting and were not as active in feeding as those on the CK. A dose-dependent negative correlation was detected between active ingestion (A2 and A3 waveforms) and flonicamid. Furthermore, a significant decrease was observed in the number of test insects able to reach the active ingestion phase (A3 waveform) as the flonicamid concentration increased, which indicated clearly that flonicamid toxicity to *A. biguttula* was due to inhibition of ingestion.

A significant negative correlation was observed between total and average durations of A2 and A3 waveforms and concentration of flonicamid. However, the number of Np and A1 events, the waiting times to first probe, the time intervals between the first and second probes and the number of A1 probes before the first A3 were not significantly altered by flonicamid.

Flonicamid is a systemic insecticide that circulates within the plant[27] and is translocated in the xylem and phloem. Our bioassay revealed that flonicamid-treated *A. biguttula* leafhoppers survived longer when compared with starved leafhoppers. We hypothesise that *A. biguttula* could not survive long on water alone (thus these insects may be unlikely to ingest from xylem on plants). We hypothesise that the concentration of flonicamid may be lower in mesophyll/parenchyma cells than in vascular cells. If so, then *A. biguttula* may have preferred to lacerate and ingest from phloem, but may have switched their ingestion to mesophyll/parenchyma on flonicamid-treated leaves to survive slightly longer than starved insects. Cho *et al.*[28] found that flonicamid induced starvation in aphids with the inhibition of phloem sap ingestion, and that lower LC_{50} values of flonicamid were associated with a decrease in the duration of phloem ingestion and increase in the Np period; these findings support the results obtained in the present study.

 © 2016 Society of Chemical Industry

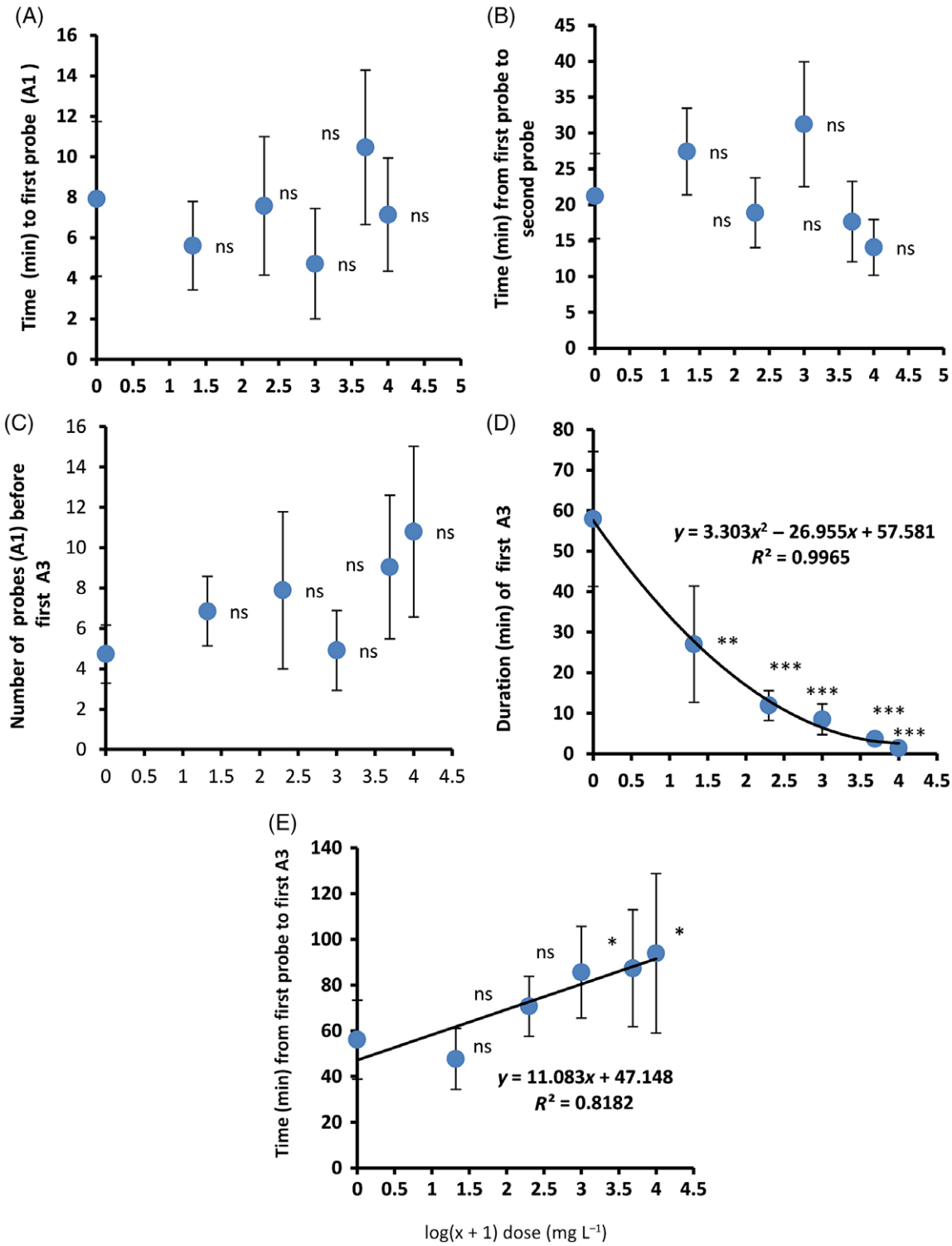

Figure 5. Relationship between flonicamid concentrations (log) and sequential variables of each EPG waveform, including the time from the start of the EPG recording to the first probe (A), the time interval from the first probe to the second probe (B), the number of probes before the first A3 (C), the duration of the first A3 (D) and the time from the first probe to the first A3 (E). Error bars indicate ± SE. Asterisks *, ** and *** by a data point show a significant difference between the CK and these values ($P < 0.05$; $P < 0.01$ and $P < 0.001$) respectively, and 'ns' denotes non-significance ($P > 0.05$).

In the present study, after leaf dipping application with flonicamid, a difference between the effects of flonicamid on active ingestion was observed, and thus effects should not be related only to the translocation of flonicamid within plant. Therefore, in addition to the leaf dipping, other methods such as injection and topical application could be used for further verification. In addition, further studies are necessary to determine any effect on the plant from flonicamid, and the speculation is that flonicamid-treated plants are distasteful to insects.

 © 2016 Society of Chemical Industry

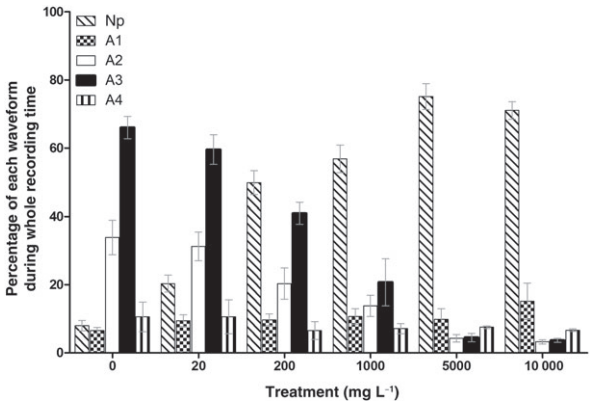

Figure 6. Percentage of each waveform phase in the whole recording time after being treated with water (CK) and different concentrations of flonicamid.

In conclusion, our EPG data showed that flonicamid significantly increased the duration of Np and decreased the active ingestion period (A2 and A3 waveforms). It may also have stimulated insects to change from phloem ingestion to mesophyll/parenchyma ingestion before dying.

ACKNOWLEDGEMENTS

This research was supported by the earmarked fund for the China Agriculture Research System No. CARS-27 and the Fundamental Research Funds for the Central Universities (No. 2014PY005). Efforts by EA Backus were supported by in-house funding from the Crop Diseases, Pests and Genetics Research Unit of the USDA Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 *Directorate of Economics and Statistics*, Department of Agriculture and Cooperation, New Delhi, India (2013).
2 Wu KM and Guo YY, The evolution of cotton pest management practices in China. *Annu Rev Entomol* **50**:31–52 (2005).
3 Luttrell RG, Fitt GP, Ramalho FS and Sugonyaev ES, Cotton pest management: Part 1. A worldwide perspective. *Annu Rev Entomol* **39**:517–526 (1994).
4 Mohan S and Nandini S, A promising entry for cotton leafhopper. *Pestology* **35**:11–13 (2011).
5 Karar H, Babar TK, Shahazad MF, Saleem M, Amjad Ali and Akram M, Performance of novel vs. traditional insecticides for the control of *Amrasca biguttula biguttula* (Homoptera, Cicadellidae) on cotton. *Pak J Agric Sci* **50**:223–228 (2013).
6 Borah RK, Insect pest complex in brinjal (*Solanum melongena* L.). *Ann Agric Res* **16**:93–94 (1995).
7 Patel Z and Patel JR, Re-surveyed of Jassid (*Amrasca biguttula biguttula*) in brinjal. *Indian J Entomol* **60**:152–164 (1998).
8 Rafique MA and Shah HA, Cotton pest scouting of farmers' fields at Multan during 1996. *Pak Entomol* **20**:40–42 (1998).
9 Sudahkar K, Punnaiah, KC and Krishnawa PC, Efficacy of certain selected insecticides on the sucking pest complex on Brinjal. *Ind Entomol* **60**:214–244 (1998).
10 Roditakis E, Fytrou N, Staurakaki M, Vontas J and Tsagkarakou A, Activity of flonicamid on the sweet potato whitely *Bemisia tabaci* (Homoptera: Aleyrodidae) and its natural enemies. *Pest Manag Sci* **70**:1460–1467 (2014).
11 Kodandaram MH, Rai AB and Haldar J, Novel insecticides for management of insect pest in vegetable crops: a review. *Veg Sci* **37**:109–123 (2010).
12 Morita M, Ueda T, Yoneda T, Koyanagi T and Haga T, Flonicamid, a novel insecticide with a rapid inhibitory effect on aphid feeding. *Pest Manag Sci* **63**:969–973 (2007).
13 Ghelani M, Kabaria B and Chhodavadia S, Field efficacy of various insecticides against major sucking pests of *Bt* cotton. *J Biopestic* **7**:7–32 (2014).
14 Cameron R, Lang EB, Annan IB, Portillo HE and Alvarez JM, Use of fluorescence, a novel technique to determine reduction in *Bemisia tabaci* (Hemiptera: Aleyrodidae) nymph feeding when exposed to *Benevia* and other insecticides. *J Econ Entomol* **106**:597–603 (2013).
15 Civolani S, Cassanelli S, Chicca M, Rison JL, Bassi A, Alvarez JM *et al.*, An EPG study of the probing behavior of adult *Bemisia tabaci* biotype Q (Hemiptera: Aleyrodidae) following exposure to cyantraniliprole. *J Econ Entomol* **107**:910–919 (2014).
16 He Y, Zhang J, Chen J, Wu Q, Chen L, Xiao P *et al.*, Influence of pymetrozine on feeding behaviors of three rice planthoppers and a rice leafhopper using electrical penetration graphs. *J Econ Entomol* **104**:1877–1884 (2011).
17 Jacobson AL and Kennedy GG, Effect of cyantraniliprole on feeding behavior and virus transmission of *Frankliniella fusca* and *Frankliniella occidentalis* (Thysanoptera: Thripidae) on *Capsicum annuum*. *Crop Prot* **54**:251–258 (2013).
18 Jacobson AL and Kennedy GG, Electrical penetration graph studies to investigate the effects of cyantraniliprole on feeding behavior of *Myzus persicae* (Hemiptera: Aphididae) on *Capsicum annuum*. *Pest Manag Sci* **70**:836–840 (2014).
19 Yueping H, Li C, Jianming C, Juefeng Z, Liezhong C, Jinliang S *et al.*, Electrical penetration graph evidence that pymetrozine toxicity to the rice brown planthopper is by inhibition of phloem feeding. *Pest Manag Sci* **67**:483–491 (2011).
20 Cui L, Sun L, Yang D, Yan X and Yuan H, Effects of cycloxaprid, a novel *cis*-nitromethylene neonicotinoid insecticide, on the feeding behaviour of *Sitobionavenae*. *Pest Manag Sci* **68**:1484–1491 (2012).
21 Preetha G, Stanley J, Manoharan T and Kuttalam S, Baseline toxicity of chloronicotinyls against cotton leafhopper, *Amrasca biguttula biguttula*, and detection of insecticide resistance. *Arch Phytopathol Plant Prot* **47**:2095–2105 (2014).
22 Jin S, Chen ZM, Backus EA, Sun XL and Xiao B, Characterization of EPG waveforms for the tea green leafhopper, *Empoascavitis* Göthe (Hemiptera: Cicadellidae), on tea plants and their correlation with stylet activities. *J Insect Physiol* **58**:1235–1244 (2012).
23 Backus EA, Cline AR, Ellerseick MR and Serrano MS, *Lygus hesperus* (Hemiptera: Miridae) feeding on cotton: new methods and parameters for analysis of nonsequential electrical penetration graph data. *Ann Entomol Soc Am* **100**:296–310 (2007).
24 Sarria E, Cid M, Garzo E and Fereres A, Excel Workbook for automatic parameter calculation of EPG data. *Comput Electron Agric* **67**:35–42 (2009).
25 Backus EA, Serrano MS and Ranger CM, Mechanisms of hopperburn: an overview of insect taxonomy, behavior, and physiology. *Annu Rev Entomol* **50**:125–151 (2005).
26 Casida JE and Durkin KA, Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annu Rev Entomol* **58**:99–117 (2013).
27 Roditakis E, Fytrou N, Staurakaki M, Vontas J and Tsagkarakou A, Activity of flonicamid on the sweet potato whitely *Bemisia tabaci* (Homoptera: Aleyrodidae) and its natural enemies. *Pest Manag Sci* **70**:1460–1467 (2014).
28 Cho SR, Koo HN, Yoon C and Kim GH, Sublethal effects of flonicamid and thiamethoxam on green peach aphid, *Myzus persicae*, and feeding behavior analysis. *J Korean Soc Appl Biol Chem* **54**:889–898 (2011).

 © 2016 Society of Chemical Industry