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Development of methods to evaluate susceptibility of soybean aphid to imidacloprid and thiamethoxam at lethal and sublethal concentrations

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Abstract
The soybean aphid, *Aphis glycines* Matsumura (Homoptera: Aphididae), is a recent introduction (2000) from Asia and has become a serious soybean [*Glycine max* (L.) Merr. (Fabaceae)] pest in North America. Seed treatments using the neonicotinoid insecticides, imidacloprid and thiamethoxam, have been suggested as a method of control, and the use of these insecticides is becoming widespread. As a consequence, there is increased potential to select for resistance to these compounds. In the case of soybean aphids, baseline susceptibility to neonicotinoid insecticides and standardized methods for bioassay are lacking. A bioassay technique that uses excised soybean leaves immersed in an insecticide solution was developed to determine systemic insecticidal activity at lethal and sublethal concentrations. Mortality and population growth inhibition were evaluated after 7 days. Life table parameters were calculated by exposing 1-day-old aphids to three concentrations of thiamethoxam. Aphid mortality and nymph production were recorded daily until the entire cohort collapsed. Soybean aphid age-specific survivorship, fecundity, net reproductive rate, longevity, intrinsic rate of increase, discrete daily growth rate, and life expectancy were all significantly reduced at higher thiamethoxam concentrations. Soybean aphid response to both insecticides was similar, and both compounds were very toxic with LC_{50}s of 31.3 and 16.9 ng ml^{-1} and EC_{50}s of 6.3 and 5.4 ng ml^{-1} for imidacloprid and thiamethoxam, respectively. These results indicate that the methods developed in this study had negligible impact on the life table estimates measured and can be used to develop a baseline of susceptibility as a benchmark for subsequent resistance monitoring. Given the rapid and widespread adoption of this new insecticide class, vigilant monitoring for changes in susceptibility will be essential to its long-term sustainability.

Keywords: seed treatment, neonicotinoid, bioassay, life table, Homoptera, Aphididae, *Aphis glycines*, *Glycine max*

Introduction

After its introduction to North America, the soybean aphid, *Aphis glycines* Matsumura (Homoptera: Aphididae), has spread rapidly and poses a serious threat to soybean [*Glycine max* (L.) Merr. (Fabaceae)] production in North America (Venette & Ragsdale, 2004). Key natural enemies found in North America, such as the coccinellid *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and the minute pirate bug *Orius insidious* (Say) (Heteroptera: Anthocoridae) (Rutledge *et al.*, 2004), do not reliably reduce aphid populations in North America, and outbreaks and soybean yield losses occur regularly (Ostlie, 2001). Furthermore, soybean aphid populations can change quickly over time, doubling in less than 2 days under ideal conditions (McCornack *et al.*, 2004). Therefore, population suppression by natural enemies has been inconsistent, and various approaches that do not rely solely on natural enemy control are required.

Several different non-chemical approaches to managing soybean aphids have been examined, such as the use of parasitoids imported from Asia (Heimpel *et al.*, 2004) or introduction of resistant varieties (Li *et al.*, 2004; Mensah *et al.*, 2005). However, these control methods may

take several years to be effective, and as a consequence, soybean farmers rely mainly on chemical control.

In several areas of the midwestern USA, soybean aphid has been responsible for the first ever insecticide application to soybean fields (Rutledge & O'Neil, 2005), and there is an urgent need to develop reliable and sustainable soybean aphid management strategies. A single foliar application of λ-cyhalothrin or chlorpyrifos at R2 to R3 stages of plant development has been shown to prevent yield losses (Myers *et al.*, 2005a). However, soybean aphid populations are affected by many factors, and regional and seasonal variation is common. Therefore, a second application is sometimes required to keep aphid densities below economic threshold levels. In addition, foliar application of broad spectrum insecticides may reduce natural enemies (Galvan *et al.*, 2005) contributing to pest resurgence. In contrast, systemic insecticides applied as seed treatment may offer increased selectivity over foliar-applied insecticides (Krauter *et al.*, 2001; Albajes *et al.*, 2003) and may provide longer plant protection (Nault *et al.*, 2004) than foliar-applied insecticides.

Neonicotinoid insecticides are commonly used as systemic insecticides and are highly effective in controlling piercing–sucking insects (Tomizawa & Casida, 2005). Similar to nicotine, neonicotinoids act as agonists at the postsynaptic acetylcholine receptor (Tomizawa & Casida, 2003). Imidacloprid and thiamethoxam are two neonicotinoids that have been utilized as seed treatments to reduce soybean aphid densities. Because the use of these insecticides is becoming more widespread, particularly as seed treatments, there is increased potential for selection of target pests for resistance. For this reason, baseline susceptibility of target pest species should be established to provide a mechanism for early detection of resistance development. Additionally, because these insecticides are used as seed treatments, less insecticide is available as the plant grows, and the insect pest may then become exposed to sublethal concentrations. Most toxicological studies focus on dose/response estimates to establish lethal doses or concentrations, but a toxicant may have broader, more subtle effects (Stark & Banks, 2003). Moreover, a reduction in population growth as a result of exposure to sublethal concentrations may provide more time for natural enemies to affect population dynamics (Satoh *et al.*, 1995).

In the case of soybean aphids, methods for exposing aphids to neonicotinoid insecticides are lacking such that measurement of baseline susceptibility or determining effects of sublethal exposure have not been possible. This study was designed to provide information on soybean aphid susceptibility to two neonicotinoid insecticides, imidacloprid and thiamethoxam. Additionally, the impact of thiamethoxam on soybean aphid life-history characteristics at different concentrations was determined. Importantly, the methodology developed provides a basis to establish baseline susceptibility from geographically distinct populations that can be used to detect future changes in susceptibility that may occur in response to increased selective pressures.

Materials and methods

Soybean aphid and plant material

A soybean aphid laboratory colony was initiated in July 2005 from individuals collected from infested fields near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory (Dixon Co., NE, USA; 42°23′N, 96°57′W). The colony was maintained on a continuous supply of soybean seedlings (V4–V6 stages) (Syngenta S23-Z3) (Research Triangle Park, NC, USA). New plants were provided weekly to the colony and aphids were transferred by placing infested leaves on uninfested plants. The colony was maintained at 25 ± 2 °C, 75 ± 5% r.h., and a photoperiod of L16:D8 h in a plant growth chamber.

Four seeds were planted in 15 cm diameter × 17 cm deep pots in a soil mix containing sand soil–peat–perlite in a 2:1:3:3 ratio. After germination, the seedlings were thinned to three to reduce competition. Plants were watered daily and fertilized weekly with a soluble fertilizer (20N:10P:20K). Plants were grown in greenhouses at 25 ± 7 °C under 400-W high-intensity discharge lamps with an L16:D8 h photoperiod.

Insecticides

Technical grade imidacloprid and thiamethoxam were purchased from Chem Service (West Chester, PA, USA) and maintained at –20 °C. Insecticidal stock solutions were prepared in acetone and further diluted to each concentration in double distilled water for use in systemic bioassays. Acetone concentrations at the highest insecticidal concentrations were less than 0.01% and did not significantly affect mortality.

Systemic bioassay

A systemic bioassay was developed that allowed excised soybean leaves immersed in insecticide solution to take up the insecticide through their petioles. Similar bioassays have been used to measure the susceptibility of sucking insect pests to systemic insecticides in cotton (Nauen *et al.*, 2002; Prabhaker *et al.*, 2005), cabbage (Nauen & Elbert, 1997), and citrus (Prabhaker *et al.*, 2006).

The cut petioles of excised soybean leaves were immersed in insecticide solution with different concentrations of imidacloprid or thiamethoxam. The control leaves had their petioles immersed in water alone and there were at least four replicates and five different con-

centrations per insecticide bioassay. To reduce soybean leaf variation, only the first and second trifoliate from healthy V4 stage soybean seedlings were used. The petioles were kept aphid-free overnight (approximately 12 h) to allow insecticide uptake and recovery of leaf turgidity.

An opaque, plastic tray (CD International, Pitman, NJ, USA) containing eight square cells (10 cm per side × 8 cm in depth) was used to keep the aphids separated in each treatment. To avoid aphid escapes, each cell was sealed with a porous transparent plastic lid (CD International). Five milliliters of each concentration was dispensed into a glass tube (1 cm in diameter × 5 cm depth) and the tube was attached to the tray with adhesive tape.

Thirty aphids (including all developmental stages) were then transferred to the leaves using a fine camel hair paintbrush. After 7 days, the dead and living aphids were counted. Aphids were considered dead when they did not respond to prodding. The trays were held in a growth chamber at 25 °C, L16:D8 h photoperiod, and 75 ± 5% r.h. LC_{50} values were calculated based on aphid mortality and EC_{50} values were calculated based on the percentage reduction in living aphids relative to controls.

Sublethal effects

Only thiamethoxam was tested for sublethal effects, and concentrations were chosen based on preliminary response curves and corresponded to the LC_{25}, LC_{50}, and LC_{75}. In order to synchronize nymph production, several apterous adults were transferred to excised leaves immersed in water. Twenty-four hours later, a cohort of 25 1-day-old aphids were selected and then carefully transferred to the upper side of the leaves using a fine camel hair paintbrush. Leaves and insecticide solutions were replaced at 7-day intervals to maintain an adequate food source for the aphids. The number of dead aphids, living aphids, and nymphs produced were recorded daily until the initial cohort of 25 aphids had died. Aphids were considered dead when they did not respond to prodding. Neonate nymphs produced were removed daily to avoid miscalculations. The experiment was repeated four times. The trays were maintained in a walk-in growth chamber at 26 ± 2 °C, L16:D8 h photoperiod, and 75 ± 5% r.h.

Calculation of life table parameters

Age-specific survivorship (l_x) and age-specific fecundity (m_x) (Gotelli, 2001) were calculated for each concentration. Age-specific survivorship was calculated based on the formula $l_x = S_x/S_0$, where x is the age interval (days), S_x is the number of survivors at age x and S_0 is the size of the original cohort. Age-specific fecundity (m_x) is the average number of nymphs produced at a specific age x by all aphids living at that age. However, m_x was transformed to cumulative fecundity to facilitate graphical visualization and interpretation of the difference among treatments. Cumulative fecundity is the average number of nymphs born per adult at a specific age x, plus the nymphs born in previous days. Other life table parameters, such as the intrinsic rate of increase (r), adapted from the Euler equation $\Sigma\, e^{-rx} l_x m_x = 1$, the net reproductive rate, $R_0 = \Sigma\, l_x m_x$, generation time or number of days to reproductive maturity, $G = \Sigma\, l_x m_x x / R_0$, doubling time, $t_d = \ln(2)/r$, finite rate of increase (λ), $r = \ln(\lambda)$, and life expectancy, $e_0 = \Sigma\, l_{x+1}\, l_x^{-1}$ were also estimated.

Statistical analysis

The mortality data were analyzed by probit analysis (Finney, 1971) using POLO-PC (LeOra Software, 1987) and corrected using Abbott's formula (Abbott, 1925). Nymphs produced were transformed to percentage population growth inhibition relative to controls. These data were then analyzed by non-linear regression (PROC NLIN; SAS Institute, 2002). LC_{50}, LC_{90}, and EC_{50} values were considered significantly different when confidence limits and intervals did not overlap (Prabhaker *et al.*, 2005).

To generate standard errors for each parameter, the population statistics r, R_0, G, t_d, λ, and e_0 were estimated for each cohort (4). Cohorts that did not produce nymphs were excluded from the analysis of reproductive parameters r, G, t_d, and λ. The parameters were analyzed by one-way analysis of variance (ANOVA) using the mixed procedure (PROC MIXED; SAS Institute, 2002). Once the treatment effect was significantly different ($P < 0.05$) by ANOVA, then Fisher's protected least significant difference was performed to identify differences among treatment means (PROC MIXED; SAS Institute, 2002). PROC LIFETEST (SAS Institute, 2002) using Kaplin-Meier estimator for survival was used to calculate survivorship curves as a function of days. Significant differences among survivorship curves were determined by Wilcoxon's χ^2-test of equality. Pearson's χ^2 analysis was used to compare the different cumulative fecundity dose curves with control.

Results

Bioassays

Soybean aphids were highly susceptible to both neonicotinoid insecticides based on responses to treated soybean foliage with LC_{50}s of 31.3 and 16.9 ng ml^{-1} for imidacloprid and thiamethoxam, respectively (Table 1). Both compounds also caused significant sublethal effects based on calculated EC_{50}s of 6.3 and 5.4 ng ml^{-1} imidacloprid and thiamethoxam, respectively (Table 1). These results suggest that soybean aphids respond at concentrations well below those that cause mortality.

Table 1. *Aphis glycines* susceptibility exposed to imidacloprid and thiamethoxam systemic bioassays as measured by growth inhibition and mortality

Insecticide	No.	Slope ± SE	LC_{50} ng ml^{-1} (95% CL)	LC_{90} ng ml^{-1} (95% CL)	χ^2 (d.f.)	EC_{50} ng ml^{-1} (95% CI)[1]
Imidacloprid	3891	2.21 ± 0.214	31.29 (21.75–41.95)	118.87 (84.04–203.1)	7.15 (5)	6.29 (4.85–8.12)
Thiamethoxam	4349	3.12 ± 0.373	16.91 (7.38–26.74)	43.54 (27.63–76.06)	4.14 (3)	5.38 (1.53–12.61)

[1] Concentration of imidacloprid and thiamethoxam that produces 50% population growth inhibition relative to untreated controls. Calculated by non-linear fitted probit model. CL, confidence limit; CI, confidence interval.

Aphid net reproductive rate and longevity

Consistent with the sublethal effects observed previously, thiamethoxam had a significant impact on soybean aphid net reproductive rate (R_0) and longevity especially at the highest concentration (Figures 1 and 2, respectively). At the lower concentrations, the number of nymphs produced per adult (R_0) was not significantly different from control ($P = 0.5868$ at the LC_{25} and $P = 0.1410$ at the LC_{50}). However, at LC_{75}, significantly fewer nymphs were produced relative to the control treatment ($P = 0.0096$). In general, as the thiamethoxam concentration increased, the net reproductive rate decreased (Figure 1). A similar trend was observed for longevity with increasing thiamethoxam concentrations (Figure 2). Aphids not exposed to insecticide (control) lived significantly longer ($P < 0.0001$) than aphids exposed to concentrations corresponding to the LC_{50} and LC_{75}, although there was no significant difference between the control and LC_{25} treatment ($P = 0.8566$).

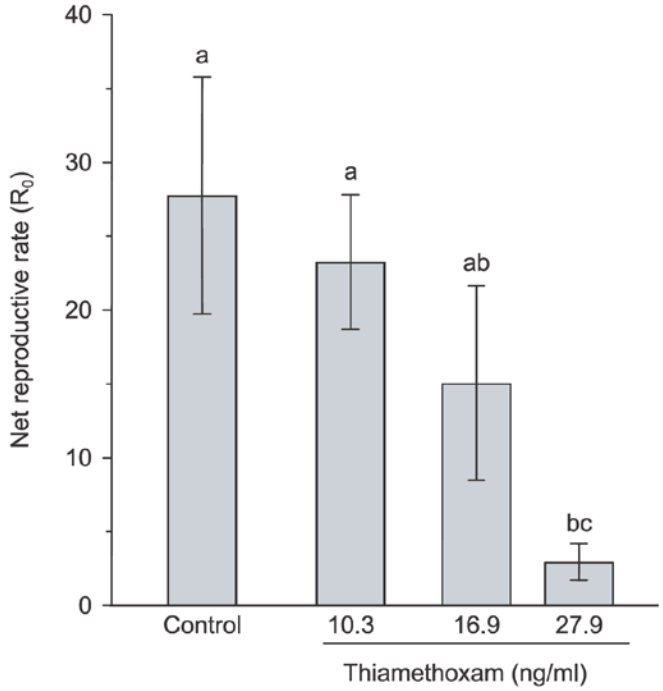

Figure 1. Fecundity (mean ± SE) of *Aphis glycines* (net reproductive rate or number of nymphs produced by each adult until the cohort had died) reared on thiamethoxam concentrations corresponding to the LC_{25} (10.3 ng ml^{-1}), LC_{50} (16.9 ng ml^{-1}, and LC_{75} (27.9 ng ml^{-1}) compared to control. Bars capped with a different letter are significantly different (Fisher's protected least significant difference: $P < 0.05$; $n = 4$).

Aphid survivorship and fecundity

Age-specific survivorship was significantly reduced as the concentration of thiamethoxam increased (Figure 3). Thiamethoxam at the LC_{25} and LC_{50} did not significantly reduce soybean aphid survivorship ($\chi^2 = 5.159$, d.f. = 2, $P = 0.0758$), but the LC_{75} treatment survivorship was significantly reduced compared with control treatment ($\chi^2 = 48.2$, d.f. = 1, $P < 0.0001$) (Figure 3). Sublethal concentrations of thiamethoxam caused a reduction in aphid fecundity with increasing concentrations (Figure 4). Aphids in the thiamethoxam LC_{25} treatment had a cumulative fecundity similar to control ($\chi^2 = 27.09$, d.f. = 29, $P = 0.5671$). However, both the LC_{50} and LC_{75} of thiamethoxam significantly reduced aphid fecundity ($\chi^2 = 17.09$, d.f. = 8, $P = 0.0291$ and $\chi^2 = 20.32$, d.f. = 7, $P = 0.0049$) after 9 and 8 days, respectively.

Other population growth parameters

In general, higher thiamethoxam concentrations caused greater impact on soybean aphid growth parameters (Table 2). With the exception of generation time ($F = 0.45$, d.f. = 3, $P = 0.7201$) and doubling time ($F = 0.54$, d.f. = 3, $P = 0.6655$), all parameters were significantly affected by thiamethoxam. Intrinsic rate of increase, discrete daily growth rate, and life expectancy were all significantly reduced.

Discussion

Although LC_{50} and EC_{50} values for thiamethoxam were not significantly different, thiamethoxam appeared to be slightly more toxic than imidacloprid. However, imidacloprid may have had a greater effect on *Aphis glycines* populations than thiamethoxam at lower concentrations based on the similar EC_{50} values. Additionally, thiamethoxam significantly affected soybean aphid life-history traits at lower concentrations. These results suggest that both compounds have lethal and sublethal effects that impact reproductive capacity and survivorship.

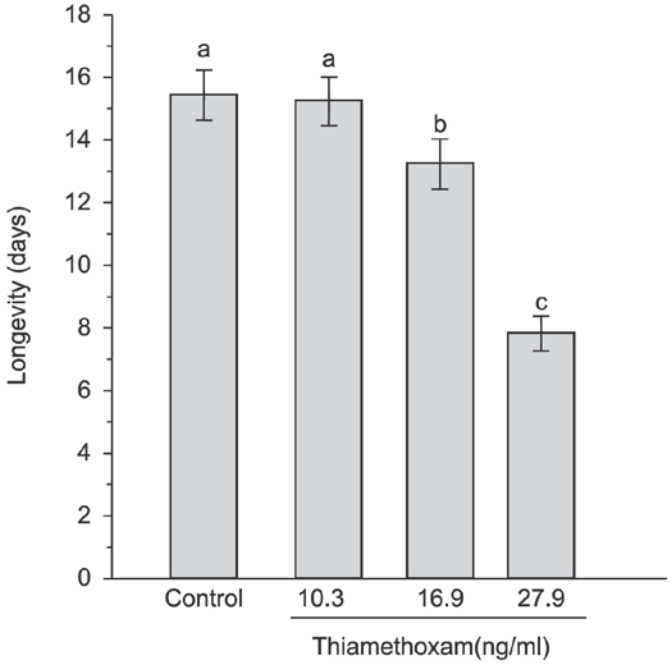

Figure 2. Longevity (mean ± SE) of *Aphis glycines* reared on thiamethoxam concentrations corresponding to the LC_{25} (10.3 ng ml^{-1}), LC_{50} (16.9 ng ml^{-1}), and LC_{75} (27.9 ng ml^{-1}) compared to control. Bars capped with a different letter are significantly different (Fisher's protected least significant difference: $P < 0.05$: $n = 4$).

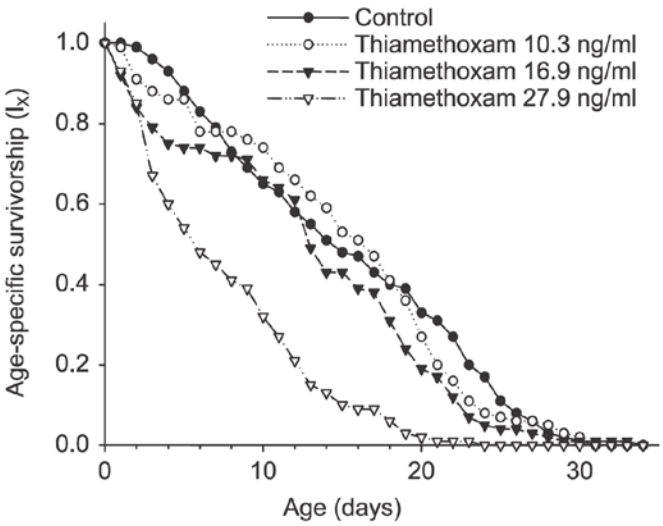

Figure 3. Age-specific survivorship of *Aphis glycines* reared on thiamethoxam concentrations corresponding to the LC_{25} (10.3 ng ml^{-1}), LC_{50} (16.9 ng ml^{-1}), and LC_{75} (27.9 ng ml^{-1}) compared to control.

Such effects could be explained not only by direct insecticide toxicity but also by possible neonicotinoid antifeedant properties (Nauen, 1995; Devine *et al.*, 1996; Nauen & Elbert, 1997). Neonicotinoids have been shown to cause feeding inhibition in *Myzus persicae* (Sulzer) and *Myzus nicotianae* (Blackman) at concentrations in the parts per billion range (Devine *et al.*, 1996).

The LC_{50}s obtained for soybean aphids for both compounds are in the range of reported for neonicotinoids using systemic bioassays for other plant-sucking insects. The LC_{50} for imidacloprid against the glassy-wing sharpshooter, *Homalodisca coagulate* (Say), ranged from 0.087 to 53.09 ng ml^{-1} (Prabhaker *et al.*, 2006). However, LC_{50} values for *Bemisia tabaci* (Gennadius) were 264 and 108 μg ml^{-1} for imidacloprid and thiamethoxam, respectively (Prabhaker *et al.*, 2005). *Myzus nicotianae* also exhibited generally higher LC_{50} values for imidacloprid [16 μg ml^{-1} (Nauen & Elbert, 1997)], which are much higher than values reported here for soybean aphids. In these other studies, mortality was evaluated after only 2 days, as opposed to the 7-day exposure in the present study, which may explain the generally higher soybean aphid susceptibility compared to *Bemisia tabaci* and *Myzus nicotianae*.

The methodology in the present study did not seem to affect soybean aphid performance because population growth estimates in the control treatment were similar to those previously reported for soybean aphids (Li *et al.*, 2004; McCornack *et al.*, 2004; Myers *et al.*, 2005b; Rutledge & O'Neil, 2006) under both laboratory and field conditions. Our calculated net reproductive rate (Figure 1) was very close to that reported by Rutledge & O'Neil (2006) and McCornack *et al.* (2004) but lower than the rate reported by Myers *et al.* (2005b). The longevity measured by Li *et al.* (2004) in the susceptible genotype (Pana) is almost identical to our estimates (Figure 2). The intrinsic rate of increase (r) and discrete daily growth rate (λ) obtained in this study (Table 2) presented intermediate values compared to those observed in these other studies (Myers *et al.*, 2005b; Rutledge & O'Neil, 2006) and were surprisingly similar to those calculated by McCornack *et al.* (2004) for aphids reared at 30 °C. Other estimates for soybean aphid life table parameters were also within the range reported in previous studies.

Although we used excised leaves and a controlled environment in the bioassays, the life table estimates obtained were comparable with other studies. Most of the soybean aphid studies where life table parameters are reported are based on clip cages and/or whole plants (Li *et al.*, 2004; McCornack *et al.*, 2004; Rutledge & O'Neil, 2006). However, it would be very difficult to obtain leaves with similar insecticide concentrations from plants originating from treated seeds. The methods used in the present study seem to have had negligible impact on the life table estimates measured. Leaves were re-

Table 2. Comparison of life table estimates (means ± SE) for *Aphis glycines* reared on thiamethoxam concentrations corresponding to the LC_{25} (10.3 ng ml^{-1}), LC_{50} (16.9 ng ml^{-1}), and LC_{75} (27.9 ng ml^{-1})

Parameter	Dimension	Control	Thiamethoxam		
			LC_{25}	LC_{50}	LC_{75}
Intrinsic rate of increase (r)[1]	Aphids per day	0.374 ± 0.02a	0.327 ± 0.02a	0.242 ± 0.06ab	0.045 ± 0.09bc
Finite rate of increase (λ)[1]	Per day	1.455 ± 0.03a	1.387 ± 0.03a	1.280 ± 0.07a	1.059 ± 0.09b
Doubling time (t_d)[1]	Days	1.874 ± 0.12	2.148 ± 0.14	4.330 ± 2.01	4.386 ± 3.11
Generation time (G)[1]	Days	10.071 ± 0.92	11.400 ± 0.46	10.820 ± 0.86	10.081 ± 1.36
Life expectancy (e_0)	Days	14.935 ± 3.07a	14.715 ± 2.22a	12.790 ± 2.92ab	7.320 ± 1.51bc

Means within a row followed by a different letter are significantly different (Fisher's protected least significant difference test: $P < 0.05$).
Nymphs were removed daily after being counted.
[1] One cohort in the imidacloprid 10.3 ng ml^{-1} and two cohorts in the imidacloprid 16.9 ng ml^{-1} were excluded from the analysis because no nymphs were produced.

placed weekly and did not show any visible evidence of degradation, such as chlorosis, wilting, or disease. Such leaf degradation would reduce food quality and consequently impact the soybean aphid life table parameters obtained.

Although imidacloprid and thiamethoxam are very toxic to soybean aphid, the utility of seed treatments for soybean aphid management is still uncertain. Until recently, neonicotinoids have been used only as systemic insecticides in commercial soybean production. However, this could change due to inconsistent plant protection and limited economic return (McCornack & Ragsdale, 2006).

The methodology reported in this investigation could be used to develop a baseline of susceptibility that can be used as a benchmark for subsequent resistance monitoring. Generating baseline susceptibility data is especially important for systemic insecticides. Seed treatments may provide an important management option that does not significantly impact natural enemies. However, the use of treated seeds should be carefully considered, because selection pressure may be higher, resistance may develop faster, and consistent benefit to soybean farmers is uncertain. Seed treatment may also increase selection pressure, because all developmental stages are exposed and as the insecticide degrades over time, the pest is exposed to sublethal concentrations (Taylor & Georghiou, 1982). The sublethal effects estimated here may be useful in determining subtle changes in susceptibility that a LC_{50} may not detect. Furthermore, it may detect specific changes, such as reproductive parameter(s), survival, and longevity.

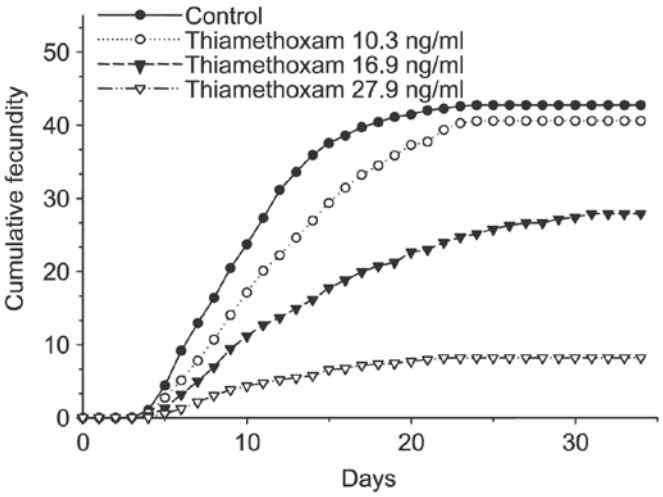

Figure 4. Cumulative fecundity of *Aphis glycines* reared on thiamethoxam concentrations corresponding to the LC_{25} (10.3 ng ml^{-1}), LC_{50} (16.9 ng ml^{-1}), and LC_{75} (27.9 ng ml^{-1}) compared to control.

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