Synthetic feeding stimulants enhance insecticide activity against western corn rootworm larvae, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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Keywords

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Abstract

In bioassays, the addition of a synthetic feeding stimulant blend improved the efficacy of the insecticide thiamethoxam against neonate western corn rootworm, *Diabrotica virgifera virgifera* LeConte, larvae. In 4-h bioassays, the concentration of thiamethoxam required for 50% mortality (LC₅₀) was 2800 pg/ml for the insecticide alone, but was reduced to 0.0075 pg/ml when the synthetic feeding stimulant blend was added (more than a 100,000-fold difference). Dilutions of thiamethoxam and tefluthrin (ranging from 0 pg/ml to 10 µg/ml) were tested in behavioural bioassays alone and in conjunction with a feeding stimulant blend containing 30 : 4 : 4 : 1 mg/ml glucose : fructose : sucrose : linoleic acid. Larvae were placed on insecticide-treated filter paper disks in an arena that allowed them to leave the treated area at will. In 30-min bioassays, and in 4-h bioassays, significantly more larvae fed on thiamethoxam-treated disks when the feeding stimulant blend was present for every concentration of insecticide tested. Larvae fed readily on disks treated with tefluthrin when the feeding stimulants were added, but the feeding stimulant blend did not affect the activity of tefluthrin insecticide at any concentration tested.

Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, continues to be a significant pest of corn (*Zea mays* L.) in the United States. Because of recent changes in their distribution (now in Europe; Kiss et al. 2005) the impact of this pest is growing. While a variety of management tools are still available for growers, some of the current options including crop rotation (Gray et al. 2009), baits to control adults (Tollefson 1998), insecticidal seed treatments (Furlan et al. 2006), Bt corn (Lefko et al. 2008; Mehlis et al. 2008), adult sprays (Meinke et al. 1998) and granular soil insecticides (Wright et al. 2000) have issues reducing their effectiveness or do not work in certain areas. For this reason, it is worthwhile to look towards the development of new strategies that are specifically aimed at improving the efficacy of current products.

We previously demonstrated the presence of phagostimulation cues for western corn rootworm larvae in the roots of corn plants. In behavioural bioassays, neonate larvae fed vigorously on paper disks treated with liquid pressed from corn roots, or with a solvent extract of corn roots, but not on disks treated with distilled water (Bernklau and Bjostad 2005). In the same study, the efficacy of a neonicotinoid insecticide (thiamethoxam; Syngenta, Inc., Basel, Switzerland) was significantly increased when crude corn root extract was added. The concentration of
thiamethoxam required for 50% larval mortality (after 24 h) was reduced from 1 ppm without corn extract to 0.01 ppm when the corn extract was added, a 100-fold decrease.

We recently identified a specific feeding stimulant blend from corn roots as a combination of simple sugars (30 : 4 : 4 mg/ml glucose : fructose : sucrose in the corn root) plus either linoleic acid or oleic acid (0.3 mg/ml; Bernklau and Bjostad 2008). In this study, we tested the synthetic blend (glucose : fructose : sucrose : linoleic acid, 30 : 4 : 4 :1 mg/ml) in conjunction with two classes of insecticides to determine effects of the synthetic feeding stimulant blend on the efficacy of the insecticides against western corn rootworm larvae.

Materials and Methods

Insects

Western corn rootworm eggs (non-diapausing strain) were shipped in soil-filled Petri dishes from the USDA-ARS Plant Genetics Research Unit in Columbia, Missouri and from the USDA-ARS Laboratory in Brookings, SD. In field studies, survivorship and infestation levels of these colonies were not significantly different from wild type insects (Hibbard et al. 1999). To our knowledge, there are no reports of resistance in these colonies to either tefluthrin or thiamethoxam. The source insects were reared on corn plants grown in soil using methods described by Jackson (1985) and modified by Hibbard and Bjostad (1988). The eggs were incubated in soil at 27°C and larvae were used for bioassays within 16 h of hatching.

Preparation of insecticide/feeding stimulant blends

The following sugars and free fatty acids were purchased from Sigma-Aldrich, Inc., St. Paul, MN: D-(+)-glucose (reagent grade), sucrose (standard), β-D-fructose (reagent grade), linoleic acid 99%, oleic acid 99%. Technical grade tefluthrin (organophosphate insecticide) and thiamethoxam (neonicotinoid insecticide) were obtained from Syngenta Crop Protection, Inc., Basel, Switzerland.

In preliminary tests, feeding stimulant blends containing 1 mg/ml linoleic acid elicited slightly stronger feeding than blends containing 0.3 mg/ml linoleic acid or any blends containing oleic acid and, therefore, 1 mg/ml linoleic acid was used in all subsequent tests, in combination with 30 : 4 : 4 mg/ml glucose : fructose : sucrose. All test solutions (insecticide alone and insecticide plus feeding stimulants) were prepared in 50 : 50 acetone : water.

Filter paper (Whatman No. 4; Springfield Mill, Maidstone, Kent, UK) was cut with a gasket punch (Blue Point 11 piece gasket punch set; Snap-On Tools Corp., Kenosha, WI) to make small (1.5 cm in diameter) or large (4 cm in diameter) disks. The disks were washed by agitating them in distilled water for 8 min, and then air-dried. An aliquot (30 µl for the small disks and 90 µl for the large disks) of a test blend was applied to a disk and the disks were air-dried for 24 h to ensure removal of any solvent.

Larval bioassays

Bioassays were conducted in a chamber designed to maintain a humid and closed environment conducive to larval health and optimal feeding (Bernklau and Bjostad 2005). A ring of blotter paper (Steel Blue; Anchor Paper Company, St. Paul, MN, cut 1 cm wide and 5 cm in diameter) was moistened with distilled water, placed inside the inverted lid of a Petri dish (5 cm diameter), and the Petri dish bottom was placed lightly (with the flat surface down) on top of the paper. The ring of blotter paper provided a 1 mm gap between the stacked halves of the Petri dish. This gap maintained the humidity and provided minimal space for larvae to feed and move freely. For bioassays, a treated filter paper disk was placed, treated side down, in the bioassay arena and re-moistened with distilled water (30 µl for small disks and 90 µl for large disks). Watersoluble fluorescent powder (D-282, UV-blue optical brightener; DayGlo Color Corp., Cleveland, OH) was added to the distilled water to make a concentration of 0.1%.

Using a camel’s hair brush, neonate western corn rootworm larvae (less than 16 h old, unfed) were placed in the centre of the moistened disk and the arena cover was replaced. One larva at a time was placed on the small disk and five larvae were placed on the large disk. Larvae that left the treated disk during the bioassay were not replaced onto the disk. After the allotted time, larvae were removed from the chamber and each larva was evaluated individually. All observations were collected as binomial data (0 = no feeding, no staying, dead; 1 = feeding, staying, alive). For staying behaviour, it was noted whether or not the larva had stayed on the treated disk. For feeding evaluation, each larva was observed under ultra-violet light (GE Model 23301-A with F15T8 Blacklamp bulb; General Electric Co., St.
Louis, MO) in a darkened room. The digestive tracts of larvae that had fed on the filter paper substrate fluoresced (Bernklau and Bjostad 2008) and larvae that had not fed did not fluoresce.

For mortality evaluation, each larva (regardless of condition) was transferred onto the root of an individual 3-day-old germinating corn seed. Each seed was placed in a small (5 cm in diameter), covered Petri dish lined with moist blotter paper. After 24 h, larvae that survived the feeding bioassay had burrowed into the corn root and were recovered using a modified Berlese technique (Bernklau and Bjostad 2005). Larvae that crawled completely out of the arena during the bioassay were immediately transferred to a corn root.

30-Minute bioassays with thiamethoxam

Six concentrations of thiamethoxam were tested with and without feeding stimulants: 0 pg/ml, 0.1 pg/ml, 10 pg/ml, 1 ng/ml, 100 ng/ml and 10 μg/ml. The small filter paper disks (see Preparation of Insecticide/Feeding Stimulant Blends) were used for this experiment and larvae were evaluated after 30 min. Twenty replications were conducted (20 larvae total) for each insecticide concentration with and without feeding stimulants.

30-Minute bioassays with tefluthrin

Eight concentrations of tefluthrin were tested, with and without feeding stimulants: 0 pg/ml, 0.00001 pg/ml, 0.001 pg/ml, 0.1 pg/ml, 10 pg/ml, 1 ng/ml, 100 ng/ml and 10 μg/ml. The small filter paper disks (see Preparation of Insecticide/Feeding Stimulant Blends) were used for this experiment and larvae were evaluated after 30 min. Twenty five replications were conducted (25 larvae total) for each insecticide concentration with and without feeding stimulants.

4-Hour bioassays with thiamethoxam

Five concentrations of thiamethoxam were tested with and without feeding stimulants: 0 pg/ml, 10 pg/ml, 1 ng/ml, 100 ng/ml and 10 μg/ml. The large filter paper disks (see Preparation of Insecticide/Feeding Stimulant Blends) were used for this experiment to allow larvae to wander and still maintain contact with the insecticide. Larvae were evaluated after 4 h. Four replications were conducted (20 larvae total) for each insecticide concentration with and without feeding stimulants.

Statistical analysis

For each experiment, mortality data were log transformed (log(x + 0.001)) and the LC50 and 95% confidence intervals were estimated (for the insecticide alone and the insecticide with feeding stimulants) using the PROBIT procedure of SAS. The untransformed LC50 values are reported. Mortality, feeding and staying data (binomial) from all experiments were analyzed with PROC GENMOD of the SAS statistical package (SAS Institute, 2000) using a logit link function and a binomial distribution. Individual comparisons between data with and without feeding stimulants at a particular insecticide dose were determined with the Least Square Means by using the LSMEANS statement within GENMOD (SAS Institute 2000, Cary, NC). For the tefluthrin experiment, where the higher doses resulted in 100% mortality and staying, data for one of the 25 replications was changed (from a ‘1’ to a ‘0’) for the GENMOD analysis because the model requires variation to run.

Results

30-Minute bioassays with thiamethoxam

In 30-min bioassays, the LC50 of thiamethoxam with feeding stimulants was 206 pg/ml (95% CI: 9.06–3.06 × 103) and the LC50 without feeding stimulants was 84 500 pg/ml (95% CI: 1.83 × 103–3.57 × 108; table 1). Significantly more (P < 0.05) western corn
rootworm larvae fed on the treatment disks with feeding stimulants than on disks without feeding stimulants for every concentration of thiamethoxam tested (0 pg/ml: $\chi^2 = 15.15$; d.f. = 1, 39; P < 0.0001; 0.1 pg/ml: $\chi^2 = 6.39$; d.f. = 1, 39; P = 0.0115; 10 pg/ml: $\chi^2 = 13.54$; d.f. = 1, 39; P = 0.0002; 1 ng/ml: $\chi^2 = 16.31$; d.f. = 1, 39; P < 0.0001; 100 ng/ml: $\chi^2 = 11.14$; d.f. = 1, 39; P = 0.0008; 10 μg/ml: $\chi^2 = 7.59$; d.f. = 1, 39; P < 0.0059) (fig. 1a). Significantly more larvae fed on the treatment disks with feeding stimulants than on disks without feeding stimulants for every concentration of thiamethoxam tested, except the 0 pg/ml, for which there was no feeding either with or without feeding stimulants (0 pg/ml: $\chi^2 = 3.52$; d.f. = 1, 39; P = 0.0608; 0.1 pg/ml: $\chi^2 = 2.50$; d.f. = 1, 39; P = 0.1142; 10 pg/ml: $\chi^2 = 12.18$; d.f. = 1, 39; P = 0.0005; 1 ng/ml: $\chi^2 = 15.35$; d.f. = 1, 39; P < 0.0001; 100 ng/ml: $\chi^2 = 15.35$; d.f. = 1, 39; P < 0.0001; 10 μg/ml: $\chi^2 = 10.23$; d.f. = 1, 39; P = 0.0014) (fig. 1b). For the 100 ng concentration of thiamethoxam, larval mortality was significantly higher when feeding stimulants were present (80% mortality) than when feeding stimulants were not present (40% mortality) ($\chi^2 = 6.16$; d.f. = 1, 39; P = 0.0130; fig. 1c).

30-Minute bioassays with tefluthrin

In 30-min bioassays, the LC50 of tefluthrin with feeding stimulants was 3.38 pg/ml (95% CI: 3.50 × 10⁻⁸–3.36 × 10⁻¹) and the LC50 without feeding stimulants was 9.98 pg/ml (95% CI: 1.23–8.71 × 10⁻¹; table 1). Significantly more larvae fed on the treatment disks with feeding stimulants than on disks without feeding stimulants for every concentration of tefluthrin tested, except the 10 μg/ml, for which there was no feeding either with or without feeding stimulants (0 pg/ml: $\chi^2 = 17.46$; d.f. = 1, 49; P < 0.0001; 0.0001 pg/ml: $\chi^2 = 16.13$; d.f. = 1, 49; P < 0.0001; 0.001 pg/ml: $\chi^2 = 19.93$; d.f. = 1, 49; P < 0.0001; 0.1 pg/ml: $\chi^2 = 10.63$; d.f. = 1, 49; P = 0.0011; 10 pg/ml: $\chi^2 = 4.80$; d.f. = 1, 49; P = 0.0285; 1 ng/ml: $\chi^2 = 9.02$; d.f. = 1, 49; P = 0.0027; 100 ng/ml: $\chi^2 = 4.80$; d.f. = 1, 49; P = 0.0285; 10 μg/ml: $\chi^2 = 0.00$; d.f. = 1, 49; P = 1.00; fig. 2a). Significantly more larvae stayed on the treatment disks with feeding stimulants than on disks without feeding stimulants for the 0 and 0.001 pg/ml concentrations (0 pg/ml: $\chi^2 = 7.84$; d.f. = 1, 49; P = 0.0051; 0.001 pg/ml: $\chi^2 = 3.90$; d.f. = 1, 49; P = 0.0484; fig. 2b). There were no significant differences in larval mortality for any dose when feeding stimulants were not present compared to when feeding stimulants were present (fig. 2c).

4-Hour bioassays with thiamethoxam

In 4-h bioassays, the LC50 of thiamethoxam with feeding stimulants was 0.0075 pg/ml (95% CI: 0.00–7.82) and the LC50 without feeding stimulants was 2,800 pg/ml (95% CI: 3.95 × 10⁻⁸–1.68 × 10⁻⁴; table 1). Significantly more larvae fed on the treatment disks with feeding stimulants than on disks without feeding stimulants for every concentration of thiamethoxam tested (0 pg/ml: $\chi^2 = 13.74$; d.f. = 1, 39; P = 0.0002; 10 pg/ml: $\chi^2 = 16.44$; d.f. = 1, 39; P < 0.0001; 1 ng/ml: $\chi^2 = 11.68$; d.f. = 1, 39; P = 0.0006; 100 ng/ml: $\chi^2 = 11.14$; d.f. = 1, 39; P = 0.0008; 10 μg/ml: $\chi^2 = 4.76$; d.f. = 1, 39; P = 0.0291; fig. 3a). Significantly more larvae stayed on the treatment disks with feeding stimulants than

![Fig. 1 Response of neonate western corn rootworm larvae in 30-min bioassays with thiamethoxam or thiamethoxam plus feeding stimulants. (a) Larvae feeding on treated disks. (b) Larvae staying on treated disks. (c) Larval mortality (evaluated after 24 h). Significant differences (Least Square Means Test, P < 0.05) between an insecticide concentration with feeding stimulants and the same concentration of insecticide alone are indicated with different lower case letters.](image-url)
on disks without feeding stimulants for every concentration of thiamethoxam except 1 ng/ml (0 pg/ml: $\chi^2 = 9.98$; d.f. = 1, 39; P = 0.0016; 10 pg/ml: $\chi^2 = 6.92$; d.f. = 1, 39; P = 0.0085; 1 ng/ml: $\chi^2 = 0.00$; d.f. = 1, 39; P = 1.0; 100 ng/ml: $\chi^2 = 7.55$; d.f. = 1, 39; P = 0.006; 10 µg/ml: $\chi^2 = 7.59$; d.f. = 1, 39; P = 0.0059; fig. 3b).

For the 10 pg/ml concentration of thiamethoxam, larval mortality was significantly higher when feeding stimulants were present than when feeding stimulants were not present (10 pg/ml: $\chi^2 = 13.82$, d.f. = 1, 39, P = 0.0002). There were no significant differences in mortality for any other concentrations of thiamethoxam (0 pg/ml: $\chi^2 = 0.47$, d.f. = 1, 39, P = 0.4917; 1 ng/ml: $\chi^2 = 3.60$, d.f. = 1, 39, P = 0.0577; 100 ng/ml: $\chi^2 = 0.35$, d.f. = 1, 39, P = 0.5557; 10 µg/ml: $\chi^2 = 0.35$, d.f. = 1, 39, P = 0.5557; fig. 3c).

**Discussion**

In this study, the addition of a synthetic feeding stimulant blend (containing glucose, fructose, sucrose and linoleic acid) improved the efficacy of the insecticide
thiamethoxam against western corn rootworm larvae. In 4-h bioassays, the concentration of thiamethoxam required for 50% mortality (LC50) was 2800 pg/ml for the insecticide alone, but was reduced to 0.0075 pg/ml when the feeding stimulant blend was added (more than a 100 000-fold difference). This reduction in the LC50 with the addition of feeding stimulants is consistent with the results of previous experiments in which larvae fed on a solvent extract of germinating corn (Bernklau and Bjostad 2005). The decrease in the LC50 is reflected in the concentration response whereby significantly higher mortality was observed when feeding stimulants were present for the lowest concentration of thiamethoxam tested. For the 30-min bioassay with thiamethoxam, we did not observe a significant difference in the LC50 with or without the feeding stimulant blend present (although there was a single significant difference in mortality at the 100 ng dose).

Thiamethoxam is a neonicotinoid insecticide that acts by binding to nicotinic acetylcholine receptors. It has been shown to have contact, stomach and systemic activity (Maienfisch et al. 2001) and is equally effective whether ingested or applied topically (Harrewign et al. 1998). Mason et al. (2000) demonstrated that thiamethoxam does not have an anti-feedant effect on the whitefly, Bemisia tabaci (Gennadius), and the results of our study are consistent with this previous report. In the 30-min and the 4-h bioassays, 50–90% of larvae fed upon the treatment disks when the feeding stimulants were present, indicating that thiamethoxam did not have an inhibitory effect on rootworm feeding.

During the 30-min test with thiamethoxam, 85% of larvae stayed on the 100 ng/ml concentration of insecticide when feeding stimulants were present. This staying behaviour was likely responsible for the high mortality (80%) that resulted from this treatment because, for the same concentration of insecticide alone, only 15% of larvae stayed, resulting in only 40% mortality. Most larvae (at least 60%) stayed on the disks treated with thiamethoxam and feeding stimulants for every concentration of insecticide tested. When feeding stimulants were not present, 40–45% of larvae stayed on disks with low concentrations of insecticide (0–0.1 pg/ml), but with concentrations of 10 pg/ml or higher, most of the larvae (65–95%) quickly crawled off the disks when feeding stimulants were not present. Rapid movement away from thiamethoxam alone was observed in a previous study with neonate western corn rootworm larvae (Bernklau and Bjostad 2005). This activity could be a manifestation of excitatory symptoms commonly seen in insects as a result of nicotinoid poisoning (Nauen et al. 2003). However, the fact that most larvae stayed on the insecticide-treated disks when the feeding stimulants were present suggests, instead, that thiamethoxam has a mild repellent effect that is overcome by the feeding stimulants. Other studies have shown that thiamethoxam is not repellent to whiteflies, B. tabaci (Gennadius) (Mason et al. 2000) or wireworms, Agriotes obscurus (L.) (Van Herk et al. 2008).

Although the synthetic blend elicited robust feeding by neonate larvae on tefluthrin, the feeding stimulants had no effect on the efficacy of this insecticide. Although tefluthrin has been reported to cause short-lived feeding inhibition in larvae of the southern corn rootworm (Diabrotica undecimpunctata howardi Barber) at both lethal and sublethal concentrations (Michaelides 1995; Michaelides and Wright 1997), we observed no evidence of antifeedant effects of tefluthrin on neonate western corn rootworm larvae. Larvae fed readily (as high as 90% feeding) on disks treated with tefluthrin and feeding stimulants, and even larvae that exhibited obvious toxicity (writhing and curling) were observed chewing on the feeding stimulant-treated disks.

Tefluthrin is a pyrethroid insecticide and its primary mode of action is on the nervous system where it interferes with the sodium channel and disturbs the function of neurons. It has a ‘knock down’ effect and provides good toxicity upon contact, but has been shown to be less toxic when ingested (Michaelides et al. 1997). In this study, rootworm larvae exhibited ‘knock down’ at a concentration of 10 pg/ml, but mortality for this dose (after 24 h) was no greater than for the control, because the affected larvae recovered after being removed from the treated disk and were subsequently extracted alive from inside the corn roots. A few larvae exhibited mild toxic effects (lethargy) even at the lowest concentration tested (0.00001 pg/ml) and most larvae were rendered immobile when placed on disks with 0.1 pg tefluthrin. Sublethal doses of tefluthrin have been reported to cause curling, writhing and regurgitation in insect larvae, but these symptoms commonly diminish when exposure to tefluthrin ends (Michaelides et al. 1997). In preliminary experiments, moribund larvae that had been feeding on tefluthrin-treated disks recovered completely when removed from the treated disks and placed on corn roots. In this study, we transferred all larvae (regardless of condition) to the roots of corn and reported larvae as dead only if they were not recovered alive from the corn roots after 24 h.
In this study, 50% or more larval mortality was achieved with low (10 pg/ml) concentrations of thiamethoxam when larvae ingested the insecticide. This suggests the possibility of combining feeding stimulants and thiamethoxam to form a toxic bait for rootworm larvae similar to adult baits with cucurbitacinics incorporated as feeding stimulants that are used in areawide management programs (Tollefson 1998; Pingel et al. 2001). In field trials, hand-made granules coated with thiamethoxam (2% ai) and feeding stimulants (crude extract of germinating corn) were effective in preventing damage to corn roots, but the feeding damage was not significantly different (P < 0.05) from damage to roots treated with insecticide granules without feeding stimulants (Bernklau 2003). The concentration of thiamethoxam in the granules was relatively high (2% active ingredient) compared with the effective concentrations in this study (10–100 ng/ml), suggesting that further soil or field tests should be conducted using much lower concentrations of thiamethoxam. The earlier field experiment was conducted using a crude (acetone) extract of germinating corn as a source of the feeding stimulants (Bernklau 2003). With the recent identification of the active components (Bernklau and Bjostad 2008), it is now possible to prepare treatments that contain the proportions of feeding stimulant components that provide the strongest feeding and then add materials to optimize longevity in the soil.

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