# Toxicity and Sublethal Effects of Methoxyfenozide on *Spodoptera exigua* (Lepidoptera: Noctuidae)

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J. Econ. Entomol. 103(3): 662–667 (2010); DOI: 10.1603/EC09244

**ABSTRACT** The toxicity and sublethal effects of methoxyfenozide were evaluated in third instars of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), that fed on contaminated semisynthetic diet. The $LC_{50}$ value was estimated at 0.23 mg of active ingredient (AI)/kg diet (range, 95% CI: 0.17–0.37) at 264 h after treatment. The effects on development, survival, and reproduction were observed in third instars of this pest that survived exposure to an $LC_{25}$ concentration of methoxyfenozide. The larvae from the insecticide treatment exhibited lower pupal weights, an increase in both larval and pupal developmental times and a higher frequency of malformations of the wings in adults than untreated larvae. Adults from the methoxyfenozide treatment did not show reduced fecundity (mean cumulative number of eggs laid per female), but fertility as measured by the percentage of eggs hatched (fertility) was significantly reduced compared with untreated control insects. No significant effects were observed on pupal sex ratio. We concluded that the lethal and sublethal effects of methoxyfenozide are likely to have a significant impact on *S. exigua* populations on treated crops.

**KEY WORDS** *Spodoptera exigua*, methoxyfenozide, development, fecundity, fertility

Beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), originally from southeastern Asia, is now a cosmopolitan pest that is particularly abundant in North and Central America, Africa, Australia, Southern Asia, and Europe (CAB International 2000). This insect is polyphagous and is an important pest of vegetable crops such as tomato, sweet pepper, beans, cucumber, alfalfa, cotton and ornamentals, among others (Belda 1994). The intensive use of chemicals for the control of *S. exigua* has resulted in high levels of resistance to practically all conventional insecticides in many parts of the world (Moulton et al. 2002). In consequence, effective alternatives for *S. exigua* control are required for use in the development of integrated pest management programs.

Over the past three decades, efforts have been made to develop novel insecticides with selective properties that are designed to act on specific biochemical sites or physiological processes of the target pest (Ishaaya et al. 2005). The insect growth regulators (IGRs) are biorational insecticides that disturb the hormonal system and are less harmful to nontarget organism than many broad-spectrum neurotoxic insecticides (Palli and Retnakaran 2001). Methoxyfenozide is the newest and most potent synthetic ecdysone agonist so far developed, and it binds to ecdysteroid receptors, inducing a premature and lethal molt in many insects, primarily lepidopteran pests (Smagghe et al. 2003). Because of its high activity at low rates, methoxyfenozide is widely used all over the word (Palli and Retnakaran 2001). In Mexico, this insecticide was registered in 2000 for controlling several lepidopteran pests on several crops, including *S. exigua* (Gastelum 2004).

Although the toxicity and kinetics of methoxyfenozide on *S. exigua* larvae has been examined previously using different routes of exposure (Moulton et al. 2002, Smagghe et al. 2003, Osorio et al. 2008), little is known about the sublethal effects in adults emerged from larvae that have been exposed to sublethal concentrations of this compound. Thus, we have studied reproductive effects in adults emerged from larvae that had continuously fed sublethal concentrations of the compound, as a close approach to what might happen in the field. In general, sublethal effects caused by ecdysone agonists include delayed developmental rates (Adel and Sehnal 2000, Biddinger et al. 2006), reduced larval and pupal weight (Pineda et al. 2007), adult deformities (Sundaram et al. 2002), and reduced fecundity and fertility (Seth et al. 2004, Sáenz de Cabezón et al. 2005, Pineda et al. 2009). Individuals that survive sublethal dosages of an IGR are considered functionally dead because the resulting adults are often sterile or development in asynchronous manner

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with respect to their host plant or to normal breeding pest populations (Biddinger et al. 2006). Therefore, the survivors of an ecdysone agonist treatment could negatively impact the population dynamics of lepidopteran pest species (Pineda et al. 2009).

Carton et al. (1998) evaluated the sublethal effects of methoxyfenozide, topically applied to last-instar larvae or injected to pupae of *S. exigua.* More recently, Osorio et al. (2008) observed effects on the reproduction of *S. exigua* adults when exposed to methoxyfenozide by ingestion. Other studies have evaluated the effects of tebufenozide, a related compound of the same family of ecdysone agonists, on larvae, pupae, and adults of *S. exigua* (Smagghe and Degheele 1994a, Gobbi et al. 2000). Here, we report the results of experiments that were performed to determine the susceptibility of *S. exigua* third instars to methoxyfenozide and the associated sublethal effects in surviving individuals when third instars were reared on methoxyfenozide-treated diet until pupation.

## Materials and Methods

**Insects and Rearing.** A laboratory colony of *S. exigua* was obtained from the Instituto de Nacional de Investigaciones Agropecuarias y Forestales (INIFAP), Ciudad Obregon, Sonora, Mexico. The larvae were reared on artificial diet (Poitout and Bues 1974) in a growth chamber at 25 ± 2°C, 75 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Adults were fed with a 15% honey solution. Brown paper was provided as an oviposition substrate and was replaced periodically, as required.

**Larval Toxicity Bioassays.** Newly moulted (0–8 h) *S. exigua* third instars were continuously fed artificial diet containing one of six concentrations (0.001, 0.0025, 0.01, 0.025, 0.05, and 0.1 mg [AI]/kg diet) of the commercial formulation Intrepid (23.26% methoxyfenozide, suspension concentrate [SC], DowAgrosciences, Zamora, Michoacan, Mexico). Serial dilutions involving the different concentrations were prepared in 20 ml of water and mixed into the diet after it had cooled to ≈50°C (Osorio et al. 2008). Treated diet was refrigerated (6°C) for no longer than 48 h before being used or discarded. Groups of 12 larvae were randomly selected and individually placed into 2.5-$cm^2$ cylindrical wells of 12-well Castor tissue culture plates containing ≈1 $cm^3$ of insecticide-treated diet. Untreated diet was provided to control individuals. The diet was replaced periodically as necessary until all larvae had completed the pupal molt. Larval mortality was scored at 24-h intervals for pupation, or if no movement was observed larvae were recorded as dead. The assays were performed four times.

**Sublethal Effects.** Sublethal effects associated with methoxyfenozide were evaluated by placing third instars on the treated diet until pupation. Larvae were allowed to feed on 0.018 mg (AI)/kg diet of methoxyfenozide. This concentration corresponded to the $LC_{25}$ value for this insecticide, which was estimated from the larval toxicity bioassay. Experimental conditions were identical to those of the toxicity bioassay described above. In this case, groups of 12 larvae were assigned randomly and exposed to the concentration and control. In total, 49 and 15 replicates were used for insecticide treatment and the control, respectively. Larvae were checked at intervals of 24 h for pupation or until mortality occurred. Larval developmental time also was determined.

Pupae were weighed and sexed 3 d after pupation and then individually placed into wells of tissue culture plates as detailed above. Pupae were examined daily for eclosion and the numbers of normal or deformed adults were registered. An adult was considered deformed if it was unable to shed the pupal exuvium or if it did not have normal wings. Pupae were considered dead if adults had not emerged after 12 d.

**Effects on Reproduction.** The influence of methoxyfenozide on fecundity and fertility was assessed by paring moths in a small mating chamber (7.5 cm in diameter by 5 cm in height) lined and covered with tissue paper. In total, 40 and 30 pairs of adults (<24 h old) resulting from larvae that had been exposed to an $LC_{25}$ of the insecticide and control were used, respectively. The mating chambers were provided with a 15% honey solution on a moist cotton trough and replaced every 2 d to prevent fungal growth. The tissue paper was replaced every 2 d and fecundity was determined by counting the total number of eggs laid by each female during the first 12 d after the onset of oviposition. Pairs that failed to reproduce were discarded. The percentage of eggs that hatched from those collected at 6 d after the first oviposition was used to evaluate fertility. The number of eggs that had hatched was assessed 4 d postcollection when egg hatch was fully complete in the control.

**Data Analysis.** Mortality data from larval toxicity bioassays were subjected to probit regression analysis against log [insecticide concentration] using the POLO PC program (LeOra Software 1987). Differences in larval, pupal, and total mortality; pupal formation and pupal weight; adult emergence; duration of the larval, pupal, and adult stages; and the effects on fecundity and fertility were analyzed by Student's *t*-test using SPSS 12.0 (SAS Institute, Cary, NC). In cases where the assumptions of normality were violated even after transformation, a nonparametric Mann–Whitney *U* test was applied. Sex ratio was analyzed by contingency tables (chi-square test).

## Results

**Larval Toxicity Bioassays.** Third instars of *S. exigua* were susceptible to methoxyfenozide incorporated into the diet. The range of concentrations used in our study was such that 50% mortality was observed at 240 h posttreatment, but an improved fit to the probit model was observed at 264 h. At this time, the estimated $LC_{50}$ value was 0.23 mg (AI)/kg diet (range, 95% CI: 0.17–0.37) ($n = 5$, b = 1.83 ± 0.35; a = 1.18 ± 0.33; $\chi^2 = 1.47$).

To determine the sublethal effects on the development, sex ratio, and reproduction in surviving individuals to methoxyfenozide treatment, we calculated

**Table 1. Effects of methoxyfenozide on larval and pupal mortality, pupal weight, and sex ratio of *S. exigua* exposed from the third instar until pupation to an $LC_{25}$ concentration through diet incorporation**

| Concn ([AI]/kg diet) | Mortality (%) ± SE | | | | Pupal wt (mg) ± SE (*N*) | | Sex ratio[f] (% male) |
|---|---|---|---|---|---|---|---|
| | *N* | Larvae[a] | Pupae[b] | Total[c] | Males[d] | Females[e] | |
| Control | 177 | 10.88 ± 1.34a | 6.05 ± 2.07a | 16.95 ± 2.13a | 82.72 ± 1.91a (82) | 84.1 ± 1.74a (68) | 45.33a |
| 0.018 | 585 | 24.35 ± 2.07b | 17.79 ± 2.05b | 37.66 ± 2.18b | 78.31 ± 0.80b (256) | 77.3 ± 1.06b (173) | 59.67a |

Within the same column, values followed by the same letter are not significantly different from each other ([a] Mann–Whitney $U$, $P > 0.05$; [b–e] Student $t$-test, $P > 0.05$; [c] $\chi^2$ test). [a] $U = 118.0$, $P < 0.001$; [b] $t$-test $= -2.91$, df $= 61$, $P < 0.05$; [c] $t$-test $= -4.85$, df $= 61$, $P < 0.001$; [d] $t$-test $= 2.39$, df $= 322$, $P < 0.05$; [e] $t$-test $= 3.44$, df $= 253$, $P < 0.01$; [f] $\chi^2 = 1.15$, $P = 0.28$.

*N* is number of larvae initially tested.

the $LC_{25}$ value in the period between treatment and pupation. In this case, the $LC_{25}$ value was estimated at 0.018 mg (AI)/kg diet (range, 95% CI: 0.0042–0.029) ($n = 6$, b $= 1.41 \pm 0.37$; a $= 1.79 \pm 0.49$; $\chi^2 = 1.72$).

**Larval and Pupal Mortality.** Third instars treated with the $LC_{25}$ of methoxyfenozide had significantly higher mortality (24%) compared with those in the control (11%) when examined until pupation (Table 1). Similarly, pupae from larvae treated with the insecticide had significantly higher mortality (18%) than untreated larvae (6%) (Table 1). Larvae treated with methoxyfenozide often showed morphological changes such as double head capsule formation and they were unable to shed the old cuticle.

**Sublethal Effects.** Larval exposure to a sublethal concentration of methoxyfenozide resulted in a significant reduction in pupal weight compared with untreated larvae in both male (5%) and female (8%) insects (Table 1). The sex ratio was not significantly different between treated and control insects (Table 1). No significant differences were observed in the percentage of deformed pupae ($2.7 \pm 0.86\%$, $N = 443$) from survivors of the methoxyfenozide treatment compared with the control ($0.7 \pm 0.7\%$, $N = 157$) (Mann–Whitney $U = 316$, $P = 0.21$). However, the percentage of deformed adults from the insecticide treatment ($21.5 \pm 2.8\%$, $N = 363$) was significantly higher than that of the control ($0.0 \pm 0.0\%$, $N = 142$) (Mann–Whitney $U = 105$, $P < 0.001$).

The durations of larval and pupal development in insects that survived methoxyfenozide treatment were significantly increased compared with the control (Table 2). The duration of the larvae stage was 1.8 d and 2 d greater in insecticide-treated male and female larvae, respectively, whereas the duration of pupal stage was 0.7 d greater in both sexes. Both male and female adults had similar life spans (11–12 d) in methoxyfenozide treatment that were not significantly different from those of the controls (11–12 d) (Table 2).

**Effects on Reproduction.** The number of eggs laid by methoxyfenozide-treated insects ($608.91 \pm 25.52$) was not significantly different compared with the control ($532.57 \pm 31.67$) ($t = 1.71$, df $= 59$, $P = 0.09$). However, the number of eggs masses laid per female in the insecticide treatment ($3.4 \pm 0.22$) was significantly lower compared with the control ($4.2 \pm 0.22$) (Mann–Whitney $U = 181.25$, $P < 0.01$). Similarly, the percentage of eggs that hatched in the insecticide treatment ($92.25 \pm 1.36$) was significantly reduced compared with the control ($98.12 \pm 0.71$) ($t = 4.81$, df $= 47$, $P < 0.001$).

## Discussion

The toxicity of methoxyfenozide via ingestion has been reported in several important lepidopteran pest species (Saénz de Cabezón et al. 2005, Pineda et al. 2007). Previous studies on *S. exigua* have reported $LC_{50}$ values for third instars fed on incorporation-contaminated diet as between 0.05 and 0.09–0.30 mg (AI)/kg diet at 120 h posttreatment for laboratory and field strains, respectively (Osorio et al. 2008). Similarly, Moulton et al. (2002) reported $LC_{50}$ values of 0.28 (96 h after treatment) and 0.39 mg (AI)/liter (120 h after treatment) for *S. exigua* third instars continuously fed on diet and cotton, *Gossypium hirsutum* L., leaves contaminated with methoxyfenozide, which are similar to the value (0.23 mg [AI]/kg diet) that we estimated at 264 h posttreatment.

**Table 2. Effects of methoxyfenozide on larval, pupal, and adult developmental times of *S. exigua* exposed as third instar until pupation to an $LC_{25}$ concentration through diet incorporation**

| Concn ([AI]/kg diet) | Duration of larval stage, (d ± SE) (*N*) | | Duration of pupal stage, (d ± SE) (*N*) | | Adult longevity, (d ± SE) (*N*) | |
|---|---|---|---|---|---|---|
| | Male[a] | Female[b] | Male[c] | Female[d] | Male[e] | Female[f] |
| Control | 15.9 ± 0.4a (68) | 15.5 ± 0.2a (82) | 11.2 ± 0.1a (64) | 10.1 ± 0.1a (81) | 11.7 ± 0.4a (23) | 11.4 ± 0.3a (24) |
| 0.018 | 17.7 ± 0.2b (252) | 17.5 ± 0.3b (168) | 12.0 ± 0.1b (224) | 10.8 ± 0.1b (150) | 11.6 ± 0.5a (36) | 10.7 ± 0.5a (42) |

Within the same column, values followed by the same letter are not significantly different from each other ([a–d] Mann–Whitney $U$, $P > 0.05$; [e, f] Student $t$-test, $P > 0.05$). [a] $U = 5832.0$, $P < 0.001$; [b] $U = 4172.5$, $P < 0.001$; [c] $U = 4823.5$, $P < 0.001$; [d] $U = 3437.0$, $P < 0.001$; [e] $t$-test $= 0.49$; df $= 57$; $P = 0.62$; [f] $t$-test $= 1.03$, df $= 64$, $P = 0.30$.

*N* is number of specimens for with development was followed.

In our study, the incorporation of the $LC_{25}$ of methoxyfenozide into the diet resulted in reductions on the pupal weight in both sexes of between 5 and 8%. Similarly, larvae of *Spodoptera littoralis* (Boisduval), *Spodoptera litura* (F.) (Lepidoptera: Noctuidae), and *Platynota idaeusalis* (Walker) (Lepidoptera: Tortricidae) that fed on contaminated synthetic diet with tebufenozide or methoxyfenozide exhibited reductions in the pupae weight (Adel and Sehnal 2000, Seth et al. 2004, Biddinger et al. 2006). Recently, we also determined that both male and female pupae of *S. frugiperda* that had been treated with sublethal concentrations of methoxyfenozide in the fifth instar, weighed less that untreated larvae with more pronounced reductions in females than in males (unpublished data). Such effects also have been observed in studies performed with laboratory-age leaf residues of methoxyfenozide on *S. littoralis.* In this case, a reduction in the weight of fourth instars that fed on treated pepper, *Capsicum annum* L., leaves was observed (Pineda et al. 2007). It is well known that the cessation of feeding occurs before the ecdysis as a consequence of ecdysteroid secretion (Smagghe et al. 2004). Therefore, the decrease in weight of *S. exigua* larvae treated with methoxyfenozide could be due to an ecysonergic activity of the compound. In addition, Smagghe et al. (1997) reported that *Chrysodeixis chalcites* (Esper) (Lepidoptera: Noctuidae) larvae treated with the earlier ecdysteoroid tebufenozide suffered gut alterations, and consequently larvae stopped feeding and lose weight.

Here, we found that the larvae of *S. exigua* treated with a sublethal concentration of methoxyfenozide did not produce deformed pupae. In contrast, a drastic increase in the prevalence of deformed pupae (53%) were observed when third instars of *S. exigua* were treated with tebufenozide (Gobbi et al. 2000), but in this case the effect was observed at a single concentration of 0.1 mg (AI)/kg diet. Such differences in this effect may be due to differences between compounds and/or concentrations used for the bioassays. In another laboratory study, we observed an increase of deformed pupae (32.5%) and adults (50%) when fourth instars of *S. littoralis* were fed with 6-d-old residues of pepper leaves treated with 1 mg (AI)/liter methoxyfenozide (S.P., unpublished data). In lepidopteran species treated with lethal concentrations of ecdysone agonists, the larvae died in a double cuticle without successful ecdysis (Saénz de Cabezón et al. 2005), whereas at lower or sublethal concentrations many apparently healthy larvae underwent abnormal and lethal pupation.

Larvae or pupae treated with ecdysone agonists often exhibit deformations in the adult stage. In these cases, the wings may be conspicuously malformed, miniature and curled, although other external body parts such as legs and antennae were not affected (Sundaram et al. 2002, Seth et al. 2004), which also was observed in the current study. In this respect, Sundaram et al. (2002) reported that tebufenozide caused premature adult cuticle formation, degeneration wing epithelial cells and inhibition of scale development. It seems that the imaginal wing disc of Lepidoptera are more sensitive to this group of insecticides than other body parts and that the concentration needed to induce wing development is related to the potency with which the compound binds to ecdysteroid receptors (Carton et al. 1998, Smagghe and Degheele 1994a).

We observed that a concentration of 0.018 (AI)/kg diet of methoxyfenozide caused an increase in both larval and pupal development times in *S. exigua.* Adel and Sehnal (2000) observed a delayed in *S. littoralis* larvae when they were exposed as second and fourth instars to methoxyfenozide, respectively. When sixth instars of *S. litura* were exposed to different concentrations of tebufenozide, ranging from 0.5 to 2 ppm, pupal developmental times increased by 14–18 h (Seth et al. 2004). Delayed development may be due to the induction of an additional larval molt after the application of ecdysone agonists, as has been observed in *S. littoralis* (Adel and Sehnal 2000).

For adult longevity, we observed that methoxyfenozide did not have any effect on *S. exigua.* In contrast, when fifth and sixth instars of *S. litura* were treated with RH-5849, a reduction of ≈50% in adult longevity was observed (Seth et al. 2004). The reason for this is unclear. It seems that more information on the adult endocrine system is required to understand the mechanism by which ecdysone agonists can affect adult longevity (Pineda et al. 2009).

Little has been published about the effects on adults derived from larvae treated with these compounds. Studies with *P. idaeusalis* have demonstrated that when exposed to tebufenozide as third instars, this compound did not affect fecundity or fertility (Biddinger et al. 2006). In contrast, marked reductions in fecundity and fertility were observed in *S. litura* and *S. littoralis* that had been treated with RH-5849, tebufenozide, or methoxyfenozide during the larval stages (Adel and Sehnal 2000, Seth et al. 2004). Smagghe and Degheele (1994b) reported that when *S. exigua* females were fed with tebufenozide it interfered with ovulation, perhaps thought a readsorption of the ovarioles. In our study, fertility was affected, but not fecundity. We also have observed that the fecundity of *S. littoralis* adults derived from fourth instars that fed on laboratory leaf residues of pepper treated with methoxyfenozide was not affected (S.P., unpublished data).

Other studies have observed that the ecdysone agonists have important antireproductive effects that are mediated thought effects on male insects by the failure of sperm to be transferred, malformed spermatophores, nonmotile sperm, and/or poor development of the testes (Carpenter and Chandler 1994, Seth et al. 2004). To validate the sublethal effects observed in the reproduction of *S. exigua*, further research under semifield conditions is in progress and will help to increase the practical relevance of the laboratory-based results.

Finally, we observed that methoxyfenozide treatment did no affect the sex ratio in pupae of *S. exigua.* In contrast, tebufenozide produced sex ratios biased toward males in following treatment of neonates and

third instars of *P. idaeusalis* (Biddinger et al. 2006). At present, little is known about how ecdysone agonists influence the sex ratio of treated insects. One possibility is that *P. idaeusalis* females are more susceptible to tebufenozide treatment than males, although in such experiments the sex of pupae from treated larvae was not determined due to their morphological abnormalities, which often interfered with the determination of insect sex ratios.

The high effectiveness of methoxyfenozide against lepidopteran larvae has been widely recognized when applied at the maximum field recommended concentration (MFRC) (144 mg [AI]/liter). However, even though in toxicology the ability to predict the lethal effects of toxicants on crop infestation by the target pest is normally the principal objective (Moe et al. 2001), estimation of the sublethal effects provides valuable information on insecticide toxic action in that portion of the pest population that does not receive a lethal dose. Under field conditions, is known that methoxyfenozide is degraded over time (Knight 2000), so that after several days of application larvae of different ages are likely to be present feeding on concentrations of the compound below the MFRC, which can impair their performance. In conclusion, we found that *S. exigua* larvae fed methoxyfenozide experienced lethal effects, whereas surviving insects suffered a wide range of sublethal effects, including reproductive effects, that have the potential to play a complimentary role in the overall pest control activity of this ecdysone agonist.

## Acknowledgments

This manuscript was improved by the comments from Trevor Williams (Instituto de Ecología A.C., Xalapa, Veracruz, Mexico) and Elisa Viñuela (Universidad Politécnica de Madrid, Spain). This research was financially supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT-proyect 53089-Z); Programa de Mejoramiento del Profesorado of the Public Education Ministry of Mexico; and an grant from the Coordinacion de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo (to A.M.M.).

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*Received 25 July 2009; accepted 23 December 2009.*