MECHANISM OF RESISTANCE AND CROSS RESISTANCE IN A LABORATORY, SPINOSAD-SELECTED STRAIN OF THE TOBACCO BUDWORM AND RESISTANCE IN LABORATORY-SELECTED COTTON BOLLWORMS

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Abstract

A laboratory strain of the tobacco budworm, *Heliothis virescens*, was selected for high levels of resistance to spinosad, the active ingredient in Tracer®. Resistance relative to the parental strain was estimated at 1,068, 314-, and >163-fold by topical, diet, and injected routes of exposure, respectively. Experiments to determine the mechanism of resistance have shown that reduced or delayed penetration of the cuticle, metabolism, or excretion are not significantly different in the resistant strain. Neurophysiological preparations show 1.9- and 1.8-fold reductions of spinosad A-induced inward currents in the resistant strain when spinosad A is applied at *in vitro* concentrations of 10^{-8} and 10^{-7} M, respectively, suggesting that the selected strain may have decreased neural sensitivity to spinosad A. Reduced inward currents in the resistant strain may or may not be related to the mode of action of the spinosyns. In a further effort to elucidate the mechanism of resistance several pesticides were bioassayed for cross resistance. No cross resistance was found based on comparisons of the LC50s to permethrin, profenofos, indoxacarb, emamectin benzoate, or acetamiprid, suggesting that the mechanism of resistance in the laboratory selected strain is unique as is the mode of action of the spinosyns. Finally, a laboratory strain of the cotton bollworm, *Helicoverpa zea*, was selected with spinosad (Tracer®) in artificial diet for 6 generations, resulting in a strain that was 5-fold less susceptible to topically applied technical spinosad.

Introduction

Our laboratory has previously selected a laboratory strain of the tobacco budworm, *Heliothis virescens*, for resistance to spinosad, the active ingredient in Tracer®, by topical application of technical grade spinosad in solvent (Bailey et al., 1999; Roe et al., 2000b; Young et al., 2000). After 12 rounds of selection, a strain highly resistant to spinosad was established. Susceptibility to spinosad was reduced 1,068-, 314-, and >163-fold when larvae were exposed by topical application, by placement on treated diet for 48 h, and by injection with technical spinosad in the perivisceral hemocoel, respectively. In order to determine the mechanism of resistance we examined the role of reduced cuticular penetration, altered metabolism, and possible changes in the nervous system, which might reduce susceptibility to spinosad. In order to outline the basis of managing the onset of any possible resistance to this novel class of compounds in the field, we examined the response of the laboratory-selected spinosad resistant strain to alternative pesticide chemistries. We also selected a laboratory strain of the cotton bollworm, *Helicoverpa zea*, with spinosad.

Materials and Methods

Insects were raised on artificial heliothine diet (Burton, R. L., 1970) at 27ºC, 50% RH, and a 14:10 L:D photoregime.

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The original selection of the spinosad resistant strain was made by topically applying technical spinosad in 1 µl acetone to the dorsal thorax of third instars (Bailey et al., 1999; Roe et al., 2000b; Young et al., 2000). Mortality (lack of response to a blunt probe within 15 sec) was assessed from 12 d post dose to pupation or death of all larvae. After twelve rounds of selection resistance was fixed at high levels.

Penetration rates for spinosad were determined by topical application of 2'-*O*-methyl[^{14}C]spinosyn A (0.14 µg, 22,000 dpm) in 1 µl acetone to the dorsal thorax of last stadium larvae. Five fifth instars were used for each time point. Larvae were incubated after treatment in 20 ml glass scintillation vials. After 3, 6 and 12 h, the larvae were externally washed for 30 sec twice with 1 ml acetone each, the solvent from each of the two aliquots was evaporated, and the radioactivity quantified by liquid scintillation counting (lsc; Beckman 6500 liquid scintillation counter, Irvine, CA). The larvae were then homogenized in 1 ml of methanol, 2-100 µl aliquots were removed, and the radioactivity in each was quantified by lsc to provide an estimate of the internal content of spinosyn A. The radioactivity remaining in the holding vails was also quantified by lsc. Data are presented as percentage of the label present internally ± 1 standard deviation.

In order to examine possible changes in the nervous system, electrophysiological recordings were made from neurons taken from susceptible and resistant insects. Neurons from the thoracic ganglia of adult *H. virescens* were isolated using a method similar to that of Lee et al. (1999). Ganglia were desheathed and incubated in 0.5 mg/ml collagenase (Type 1A; Sigma Chemical, St. Louis, MO) at 37ºC for 7 min, then washed 3 times with saline (as below) and dissociated by trituration with pipets of descending tip diameter. Neurons were then allowed to settle to the bottom of a petri dish for at least 30 min prior to electrophysiological recordings. Whole-cell currents were recorded using the technique of Hamill et al. (1981). The internal recording solution contained (in mM): CsF (100), CsCl (40), $MgCl_2$(3), EGTA (10) and HEPES (5), pH 7.0. The external buffer solution contained (in mM): NaCl (140), KCl(4), HEPES (10), glucose (10), $CaCl_2$ (2), and $MgCl_2$ (2), pH 7.2. Spinosyn A was first dissolved in DMSO, typically at 10 mM, then diluted into saline at the noted final concentrations. Current-voltage relationships were determined by brief voltage steps from holding potential (usually -50 or -70 mV) to test potentials. Data were analyzed using Pulse/PulseFit software (ALA Instruments), and each data point represents the mean greater than or equal to 3 independent observations.

Topical toxicity of Curacron® (profenofos), Pounce® (permethrin), Denim® (emamectin benzoate), and Steward® (indoxacarb) diluted into distilled water was estimated by larvae dip. Third instars (25-35 mg) were gently grasped with soft forceps, immersed in the diluted formulation 1-2 sec, and then placed on a paper towel to dry for 2 min, after which they were placed singly on fresh diet. Mortality was assessed 72 h post dose. Seventy-five larvae were assayed per dose and water control. Mospiran® (acetamiprid) was assayed by placing neonates on dehydrated meal pads made from artificial diet (Roe et al., 2000a; Bailey et al., 2001) that had been rehydrated with Mospiran® diluted with distilled water. Twenty-eight larvae were used per dose. Doses are presented as ppm (µg/ml) for larval dips and ppm (µg/g of diet) for Mospiran®. Log dose-probit plots were made (Finney, 1970), and the fiducial limits of the median dose estimated as per Sokal and Rohlf (1995). Toxicity ratios and their fiducial limits were estimated by the methods of Steele and Torrie (1980) and Robertson and Preisler (1992), respectively.

A closely related heliothine species, the cotton bollworm, *Helicoverpa zea*, was selected for decreased susceptibility to spinosad by placing 50-100 neonates on dehydrated meal pads made from artificial diet (Roe et al., 2000a; Bailey et al., 2001) that had been rehydrated with Tracer®, starting with an initial concentration of 0.02 µg/g of diet. Those larvae that

survived to second instar were transferred individually to diet without insecticide in 1 oz. Solo cups. There were 646 neonates dosed in the first round of selection, and subsequent rounds of selection used from 450 to 1153 neonates. The selection concentration was increased to 0.05 µg/g of diet in rounds 2-4, to 0.06 µg/g in the fifth round and to 1.0 µg/g in the sixth and final round, the survivors of which produced many eggs, none of which hatched. Data presented are the percentage of surviving second instars, the concentration of active ingredient, and the number of neonates placed on diet for each generation.

Results and Discussion

Spinosad Penetration

Although there was a slight trend toward reduced penetration in the resistant as compared to the susceptible strain, these differences were small with overlapping standard deviations at each time point (Fig. 1). After 12 h, 12 and 15% of the applied label was absorbed by the resistant and susceptible strains, respectively. We previously found that the selected budworms were resistant to spinosad when the insecticide was injected directly into the hemocoel (Young et al., 2000). Therefore, the high level of spinosad resistance to the topical application of spinosad (>1,000-fold) can not be explained simply by reduced penetration. Young et al. (2000) also reported that spinosad was not metabolized in both the resistant and susceptible strain. These results suggest a possible alteration at the target site for spinsoad in the insect nervous system.

Electrophysiological Response to Spinosyn A

At negative holding potentials, spinosyn A produced a slowly developing inward chloride current in neurons from susceptible (adult) tobacco budworms. Current-voltage plots revealed that this current reversed polarity at approximately -25 mV on average, which is very near the reversal potential for Cl^- (-30 mV) as derived from the Nernst equation. Currents were observed at concentrations as low as 0.1 nM spinosyn A, and were consistently observed at concentrations above 1 nM. The amplitude of currents increased with increasing concentrations of spinosyn A (540 pA at 10^{-8} M, 1400 at 10^{-7}, and 1800 at 10^{-6}).

Dose-dependent spinosyn A-induced currents were also observed from neurons from spinosad resistant (adult) tobacco budworms. At both 10 nM and 100 nM, however, the amplitude of these currents were significantly smaller (160 and 760 pA, respectively) than the amplitude of currents observed from neurons from susceptible budworms. This suggests that neurons from resistant insects have decreased sensitivity to spinosyn A. Roe et al. (2000b) previously showed that the selected strain was resistant to spinosad at both the larval and adult stage. Reduced inward currents in the resistant strain may or may not be related to the mode of action of the spinosyns.

Permethrin (Pounce®) Toxicity

The results of the larval dip assay of Pounce® (permethrin) are shown in Fig. 2. There was no significant difference in the LC50s between the resistant and susceptible strains based on 95% confidence intervals. The toxicity ratio for the median lethal doses (the LC50 for the spinosad resistant strain divided that for the susceptible strain) was 0.66-fold which was not significantly different from 1. The slopes of the fitted lines were also not significantly different.

Profenofos (Curacron®) Toxicity

The results of the larval dip assay of Curacron® (profenofos) are shown in Fig. 3. There was no significant difference in the LC50s between the resistant and susceptible strains based on 95% confidence intervals. However, the toxicity ratio for the median lethal doses of 1.68 was statistically significant from 1, the lower fiducial limit being 1.14-fold. The slopes of the fitted lines were not significantly different. Given the small (less than two-fold) difference in LC50s as compared to the >1,000-fold difference in spinosad susceptibility between these two strain, cross-resistance to profenofos is of no practical significance.

Emamectin Benzoate (Denim®) Toxicity

The results of the larval dip assay of Denim® (emamectin benzoate) are shown in Fig. 4. Again, there was no difference between the two strains in the LC50s, but the resistance ratio of 1.90-fold was significant, the lower fiducial limit being 1.07-fold. The slopes of the fitted lines were not significantly different and cross-resistance to emamectin benzoate has no practical significance.

Indoxacarb (Steward®) Toxicity

The results of the larval dip assay of Steward® (indoxacarb) are shown in Fig. 5. No difference in the LC50s were found between the resistant and susceptible strains, the resistance ratio of 1.68-fold was not statistically significant, and no differences were found in the slopes of the fitted lines.

Acetamiprid (Mospiran®) Toxicity

The results of the diet exposure of neonates to Mospiran® (acetamiprid) are shown in Fig. 6. Although there was no difference in the LC50s, the resistance ratio of 0.41-fold was marginally significant, the upper fiducial limit being 0.84-fold. Inverting the toxicity ratio, the spinosad-susceptible strain is 2.44-fold less susceptible to acetamiprid, with fiducial limits of 1.19- to 5.00-fold. The slopes of the fitted lines are not significantly different.

Selection for Spinosad Resistance in the Cotton Bollworm

Fig. 7 shows the outcome of six generations of selection for first instars of the cotton bollworm on diet containing Tracer® (spinosad). By the fifth generation the selection concentration had to be increased 5-fold from the initial dose of 0.02 µg/g of diet. The sixth round of selection, at 1.0 µg/g diet, resulted in 78 viable pupae. The adults that emerged, however, produced no viable eggs, resulting in the loss of the strain.

Summary

- There was no significant difference in cuticular penetration of radiolabeled spinosyn A between the spinosad susceptible and resistant strains of the tobacco budworm.
- There was a decreased electrophysiological response to spinosyn A by neurons of the spinosad resistant adult tobacco budworm relative to the susceptible strain, although this can not be interpreted as being directly related to the mode of action.
- There were no practical differences in the toxicities of permethrin (Pounce®), profenofos (Curacron®), emamectin benzoate (Denim®), or indoxacarb (Steward®) between the spinosad susceptible and resistant strains of the tobacco budworm when estimated topically (by larval dip).
- There was a small (2.4-fold) but statistically signiicant increase in susceptibility of the spinosad-resistant strain of tobacco budworm to acetamiprid (Mospiran®), relative to the spinosad-susceptible parental strain, when placed on treated diet as first instars.
- A laboratory strain of the cotton bollworm, *Helicoverpa zea*, selected for six generation as neonates on artificial diet containing Tracer®, demonstrated a 5-fold reduction in susceptibility to the insecticide. The strain was lost after the sixth generation when the eggs failed to hatch.

Acknowledgments

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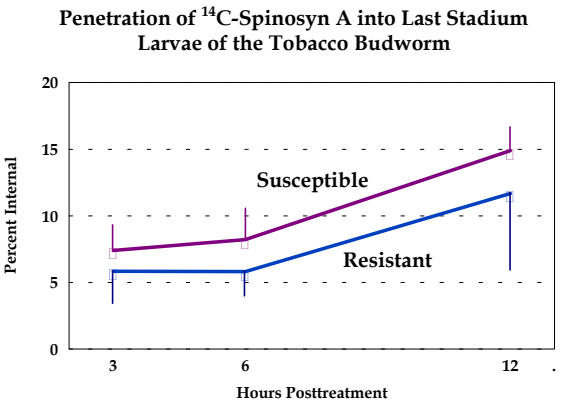

Figure 1. Penetration of [14C]spinosyn A through the dorsal cuticle of last stadium tobacco budworms. Data are the means of 5 larvae; dispersion bars are 1 standard deviation.

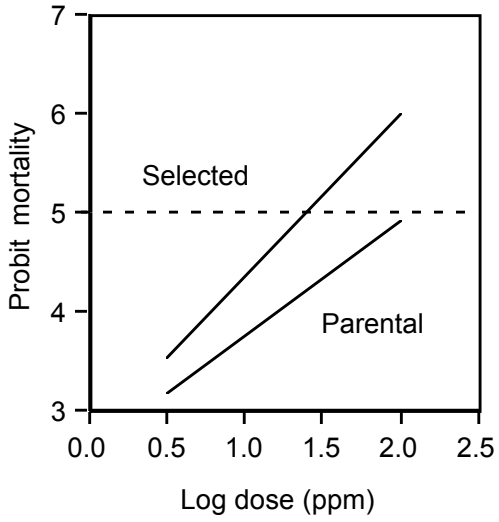

Figure 2. Log dose-probit plot of toxicity of permethrin (Pounce®) applied by larval dip to tobacco budworm 3rd instars. Dose is in ppm (µg/ml) of active ingredient.

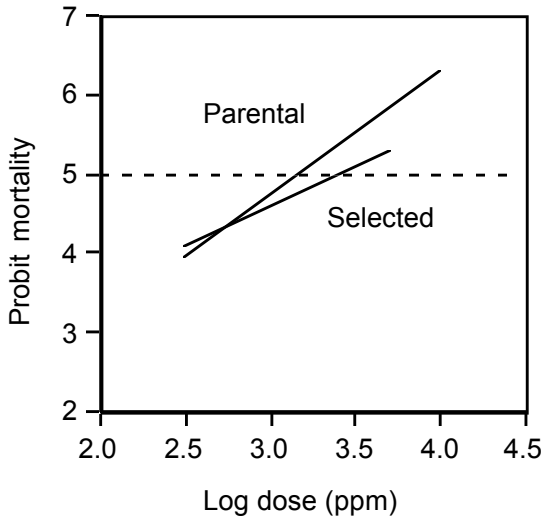

Figure 3. Log dose-probit plot of toxicity of profenofos (Curacron®) applied by larval dip to tobacco budworm 3rd instars. Dose is in ppm (µg/ml) of active ingredient.

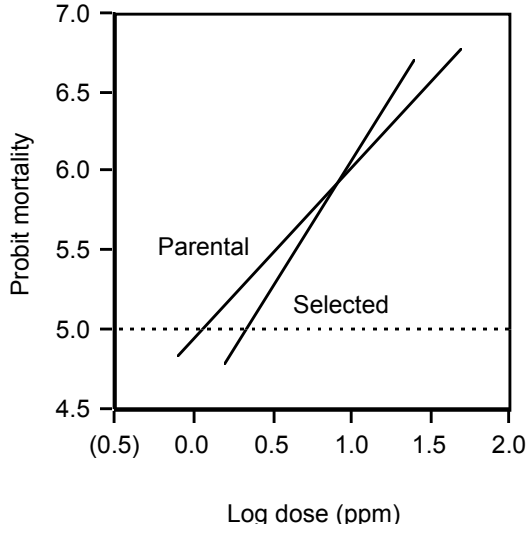

Figure 4. Log dose-probit plot of toxicity of emamectin benzoate (Denim®) applied by larval dip to tobacco budworm 3rd instars. Dose is in ppm (µg/ml) of active ingredient.

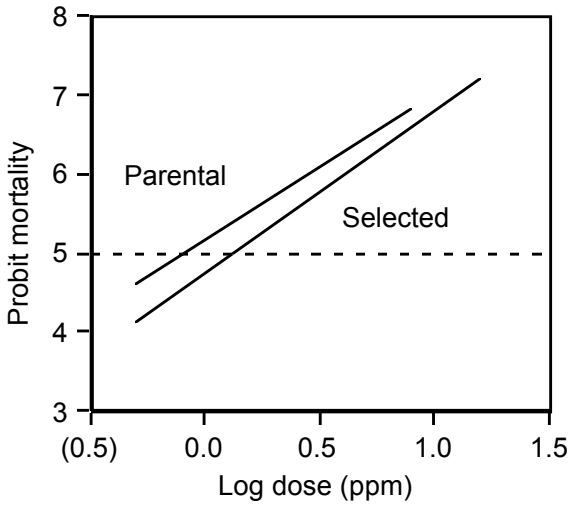

Figure 5. Log dose-probit plot of toxicity of indoxacarb (Steward®) applied by larval dip to tobacco budworm 3rd instars. Dose is in ppm (µg/ml) of active ingredient.

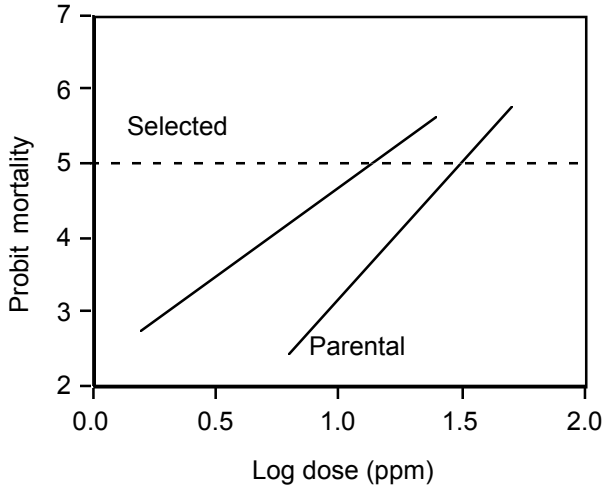

Figure 6. Log dose-probit plot of toxicity of acetamiprid (Mospiran®) incorporated into diet of tobacco budworm 1st instars. Dose is in ppm (µg/g of diet) of active ingredient.

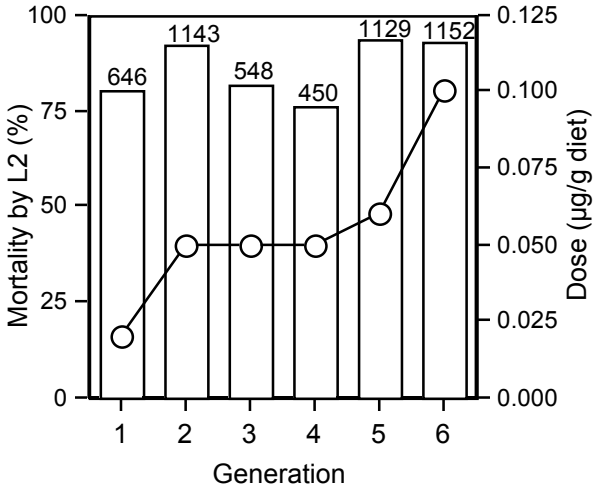

Figure 7. History of the laboratory selection of cotton bollworm exposed to Tracer® incorporated into heliothine diet. Larvae were exposed as neonates. Open bars are the mortality of first instars; open circles are the dose in ppm (µg/g of diet) of active ingredient. Numbers over columns are number of neonates dosed per generation.