STUDIES ON THE MECHANISM(S) OF TOBACCO BUDWORM RESISTANCE TO SPINOSAD (TRACER®)

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Abstract

A laboratory strain of the tobacco budworm, *Heliothis virescens*, originally collected from North Carolina was selected topically with technical spinosad (spinosyns A and D) for successive generations, producing high levels of resistance. The toxicity ratio for spinosad based on differences in the LC_{50} between the parental and selected strains in a larval dip bioassay (48 h after treatment) was 245-fold. Insects were also resistant when fed on spinosad-treated artificial diet for 48 h. The resistance ratio increased drastically depending on when mortality was assessed. At 1 day after the treatment the resistance ratio was 150-fold while at 4 days the value increased to 2670-fold. When the selected budworms were placed on spinosad treated cotton for 48 h, the resistance ratio was only 91.1-fold. The selected strain was also resistant by injection. The parental strain six day LD_{50} was 0.059 µg/larva while 10 µg/larva produced only 30% mortality in the selected strain. Preliminary studies indicated no differences in the rate of penetration across the cuticle or metabolism of 2′-*O*-[^{14}C]-methyl spinosyn A between susceptible and selected larvae suggesting that these mechanisms are not contributing to the observed differences in spinosad susceptibility.

Introduction

The spinosyns, derived from the Actinomycete, *Saccharopolyspora spinosa*, were discovered in the 1980s. Spinosyns A and D, have strong insecticidal activity against pest species, with very favorable mammalian and off target toxicity profiles (Borth et al. 1996; Hendrix et al., 1997; Salgado et al., 1997; Sparks et al., 1996; Thompson et al., 1996). Although the exact site of action of spinosyns is still under investigation, they simultaneously alter the function of nicotinic acetylcholine receptors and GABA-gated chloride channels. Spinosad is becoming widely used for the control of lepidopteran pests after being introduced in 1997. Because the spinosyns represent a new insecticide chemistry with a novel mode of action, there is no reason to immediately expect control problems with this insecticide due to insect resistance, and there have been no reports of resistance in the field (Leonard et al., 1996; Moulton et al., 1999).

Bailey et al. (1999) developed the first laboratory strain of the tobacco budworm, *Heliothis virescens*, resistant to spinosad. This strain was obtained by selecting budworm larvae each generation topically with technical spinosad. As reported elsewhere in this proceedings (Roe et al., 2000), the resistance ratio in generation 14 of this strain was >763-fold based on differences in the day fifteen LD_{50}s between the selected and parental strains. Resistance was measured using topical doses of technical spinosad and occurred in both the larval and adult stage. Since this is the first documentation of reduced sensitivity in an economically important target for spinosad, studies were initiated to further characterize the reduction in toxicity and to identify the resistance mechanism(s).

Materials and Methods

Insects

Tobacco budworm larvae are routinely reared on artificial diet (Burton, 1970). All insects used in these experiments were maintained at 27˚C and 40–70% RH under a 14:10 L:D photoperiod. A laboratory (parental) colony of the budworm used in our studies was established from field collections from North Carolina in 1996 and 1997. Tobacco budworms from the parental strain were selected with technical spinosad starting in November of 1997. This colony is designated as the resistant (laboratory selected) strain. Spinosad was applied topically on the dorsal thorax of 14-45 mg larvae, and from 1300 to 2300 larvae were selected in each generation (G). For more details on the history of selection, see Bailey et al. (1999) and Roe et al. (2000). The LD_{50} (15 days after treatment of 30±5 mg larvae) for technical spinosad in the parental strain is 0.131 (95% confidence interval 0.028-0.778) µg of spinosad per larva. In the 14th generation of selection, 100 µg of technical spinosad per larva produced 47.1% mortality (15 days after treatment). This is a resistance ratio >763-fold based on the differences in the LD_{50}s between the two strains.

The studies that follow were conducted with selected budworms in G14 and later. When a cohort of the selected budworms were reared in the laboratory in the absence of selection and the immigration of new genes into the population from G9 through G14, the relaxed strain did not revert back to the susceptibility of the parental strain. Based on these experimental results, we expect resistance in our selected strain was stable at high levels throughout the experiments described in this paper (G14 through G20).

Chemicals

Technical spinosad (spinosyns A and D, 88% pure) was a gift from Dow AgroSciences, Indianapolis, IN. Formulated

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Tracer®, 44.2% active ingredient, was a gift from Dr. Clyde Sorenson, Department of Entomology, North Carolina State University or was provided by Dow AgroSciences. Radiotracer experiments were performed with 2′-*O*-[^{14}C]-methyl spinosyn A (51.6 mCi/mmole) provided by Dow AgroSciences. All other chemicals were from Fisher Scientific Co., Pittsburgh, PA, with the highest purity possible.

Assessment of Mortality

The endpoint for mortality was larval failure to respond within 10 sec to a touch from a blunt probe (Zhao et al., 1996).

Whole Larval- and Cotton Leaf-Dip Bioassays

Parental (susceptible) and resistant third instars held with soft forcepts were immersed for 2 sec in 4 to 5 different concentrations of spinosad (active ingredient) added to water as formulated material. Treated larvae were placed on a paper towel for 2 min to dry and transferred individually to the surface of a 1 inch diameter, cotton leaf disc which exactly fit into the bottom of a plastic rearing container. For the leaf dips, the 1 inch cotton leaf disc was treated by the same procedure as that for larvae, the treated leaf disc transferred to the bottom of a plastic rearing container as already described, and an untreated (parental or resistant) third instar placed on the surface of the treated leaf (one larva per cup). Ten insects were used per replicate, and each dose replicated twice. Observations were made after 48 h. The cotton plant variety was Acala SJ2.

Feeding Bioassay on Artificial Diet

Parental and resistant fourth instars were placed on heliothine diet containing different concentrations of spinosad (active ingredient) added to the artificial diet as formulated material. Larvae remained on the treated diet for 48 h, all living larvae were then transferred to untreated diet, and mortality assessed daily from the time of the last transfer until all insects had died or pupated. Six doses were used for the selected strain and eight for the susceptible strain. Twenty-five larvae were used per dose (replicated twice).

Injection of Larvae with Spinosad

Parental and resistant fourth and fifth instars (250–325 mg) were injected with different amounts of technical spinosad in 1 µl acetone. Injections were made with a 10 µl Hamilton syringe fitted with a 2 cm, 22-guage needle which was inserted into the hemocoel, lateral to the first two pairs of prolegs, below the spiracles. Any larvae that bled were discarded. Insects were maintained on artificial diet and observed daily until all had either died or pupated. Five doses were used for the resistant strain and six for the susceptible strain. Ten insects were used per dose (replicated six times).

Spinosyn A Penetration Through the Cuticle

An initial examination of possible differences in cuticular penetration between susceptible and resistant strains was conducted by topically applying approximately 0.1µg (ca. 0.07 µCi) of 2′-*O*-methyl[^{14}C]spinosyn A in 1 µl acetone to the dorsal thorax of third instars. Larvae in groups of two were treated topically with the radiolabel and held in 20 ml glass scintillation vials until assayed. Each treatment of two larvae was replicated 5-10 times. At 3 and 6 h after the topical application of spinosyn, the two larvae were externally rinsed together for 30 sec with 1 ml acetone and the rinse repeated. The larvae were then homogenized in 500 µl of methanol. Two 100 µl aliquots of the methanolic homogenate were then added to scintillation cocktail, vortexed and counted. The radioactivity in the external wash (solvent evaporated) and the vial used to hold the insects for 3 and 6 h were also quantified by liquid scintillation counting. From these results, the percentage penetration of spinosyn A was calculated. The percentage recovery of the total applied radioactivity in these experiments was >92%.

Spinosyn A Metabolism

2′-*O*-[^{14}C]-methyl spinosyn A (0.034 µCi, 0.5 µg) in 0.5 µl acetone was injected directly into the hemocoel (ventrally between the last two pairs of prolegs) of susceptible and resistant fifth instars with a 30 ga Hamilton syringe needle. Larvae were incubated in 24-well microtiter plates at 2, 4, 8, 16, 24, 48 and 96 h (five larvae per treatment). The microtiter plate wells were rinsed with 3 ml of isopropanol and each larva homogenized in the rinsate. The homogenate was centrifuged 15 min at 3000 rpm at room temperature, and the supernatant was removed and concentrated with a Savant microconcentrator. The yellow, oily extract was dissolved in 100 µl of ethanol by sonication, and the ethanol solution clarified for high performance liquid chromatography (HPLC) by centrifugation (high speed setting in a microfuge). Radio-HPLC was conducted on a C-18 reverse phase column (YMC-Pack-ODS-AQ column (250 x 4.6 mm id, 5 µm)) using an isocratic solvent system consisting of a 4:4:1 mixture of acetonitrile:ethanol:2% ammonium acetate at 1.5 ml/min.

Data Analysis and Statistics

Abbott's correction (Abbott, 1925) was applied to all data from dose-response experiments. LD_{50}s and LC_{50}s were determined by plotting log dose versus probit mortality (Sokal & Rohlf, 1995; SAS, 1998) or by probit analysis (Finney, 1971) for the larval and leaf dip assays.

Results and Discussion

Spinosad Resistance: Topical and Feeding Bioassays

Selected (G17) tobacco budworm larvae were resistant to spinosad both topically and by feeding. In larval dip assays, the LC_{50} (48h after treatment) for the susceptible strain was 7.2 ppm (w/v; 95% confidence interval (C.I.)=3.5-11.9) while

the LC_{50} for the resistant strain was 1766 ppm (C.I.=606-)(Table 1). This represents a resistance ratio of 245-fold based on the differences in the LC_{50}s between the two strains. In these experiments, larvae treated by dipping were allowed to feed on cotton plants to better approximate field conditions. In comparison, the resistance ratio (G14) for the topical application of technical spinosad was >763-fold (Roe et al., 2000). In this latter study, the insects were reared on artificial diet and mortality was assessed 15 days after treatment. Mortality at 15 days for a 100 µg per larvae treatment was 47.1%. However, only 4.2% mortality occurred in the resistant strain at six days, making it difficult to estimate a resistance ratio.

The tobacco budworm larvae were also highly resistant when allowed to feed on treated diet or cotton leaves. The oral toxicity for larvae on treated artificial diet is shown in Fig. 1. The LC_{50} of the parental strain 4 d after a 48 h exposure to spinosad-treated diet was 0.055 µg of active ingredient per ml (95% C.I.=0-2.36), and the LC_{50} for the resistant strain (G14) was 146.8 µg/ml (95% C.I.=0.32-), a 2670-fold resistance ratio. It is interesting to note, however, that at 1 d after treatment (24 h after being removed from a 48 h exposure on treated diet), the LC_{50} values were 0.197 and 29.6 µg/ml for parental and selected strains, respectively, a resistance ratio of 150-fold. When larvae were allowed to feed on treated cotton leaves to simulate conditions in the field, the LC_{50} (after 48 h on treated cotton leaves) for the parental strain was 12.1 ppm (95% C.I.=4.0-20.4) while the LC_{50} of the resistant strain (G17) was 1102 ppm (95% C.I.=589-), a resistance ratio of 91.1-fold (Table 1). It is clear from these studies that the selected tobacco budworm larvae are resistant to spinosad when the insecticide is applied by different methods.

Role of Penetration in Spinosad Resistance

There are several potential insecticide resistance mechanisms including altered behavior, reduced penetration, enhanced metabolism, increased excretion, altered target site and sequestration. To exclude penetration from our analysis of resistance, different amounts of technical spinosad were injected into susceptible and resistant fifth instars and the mortality determined at 6 d after treatment (Fig. 2). The parental strain demonstrated an LD_{50} of 0.058 µg/larva (95% C.I.=0.02-0.12) while the LD_{50} of the selected budworms (G19) was 29.6 µg (by extrapolation), a resistance ratio of 510-fold. At the highest dose injected of 10 µg per larva, the mortality was 30.3%. This study showed that spinosad resistance was at least partly the result of changes in the internal physiology of the selected budworms.

To examine specifically the role for penetration in resistance, ^{14}C-labeled spinosyn A was topically applied to third instars, and 3 and 6 h after treatment, the percentage of applied spinosyn A was measured. Preliminary data suggest that at both time points measured, no differences were noted in the rate of penetration of ^{14}C-spinosyn between the resistant and susceptible insects (Fig. 3). Apparently, the mechanism of spinosad resistance does not involve differences in the rate of penetration of spinosad but changes in metabolism, sequestration, excretion and/or target site.

Role of Metabolism in Spinosad Resistance

A common mechanism of insecticide resistance in insects to a number of insecticide classes is increased metabolism. However, when ^{14}C-spinosyn A was injected into both susceptible and resistant (G17) fifth instars, no metabolism was found 96 h after injection in both strains. Fig. 4 is a representative HPLC radiochromatogram for metabolism at 96 h. The radioactivity corresponded exactly with the retention time on HPLC of ^{14}C-spinosyn A. It is clear that increased metabolism is not important in spinosad resistance and in fact, spinosad is not metabolized in both resistant and susceptible budworms. These data are consistent with previous studies demonstrating that spinosyn A is not metabolized in tobacco budworm larvae (Sparks et al., 1997).

Mechanism(s) of Spinosad Resistance in the Tobacco Budworm

A laboratory strain of the tobacco budworm selected for over 11 generations with topically applied spinosad was highly resistant as compared to the parental strain when the insecticide was administered by topical application and dipping, feeding on treated artificial diet and cotton leaves, and injection. In light of the significant injection toxicity and apparent lack of differences in penetration, the resistance mechanism(s) do not appear to include reduced penetration. Metabolism apparently is also not a factor in resistance to spinosad. Behavioral resistance can also be ruled out as an important contributing factor since high levels of resistance was measured when spinosad was applied topically and the insects reared on both artificial diet and cotton leaves. The importance of behavior in feeding studies or under field conditions was not assessed. Available data at this time suggest that other mechanisms such as excretion, sequestration and altered target site may explain the lack of susceptibility to spinosad.

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Table 1. Hv susceptible and resistant strains exposed to spinosad (active ingredient) by dipping either leaves or larvae as above.

Strain and Route of Exposure	LC50 (ppm)	95% C.I.	Toxicity Ratio @ LC_{50}
Larval dip[a]			
Susceptible	7.2	3.5 - 11.9	
Resistant (G17)	1766	606 -	245.3
Leaf dip[b]			
Susceptible	12.1	4.0 - 20.4	
Resistant (G17)	1102	589 -	91.1

[a]Mortality was measured 48 h after dip.
[b]Mortality was measured after 48 h of feeding on the treated leaf.

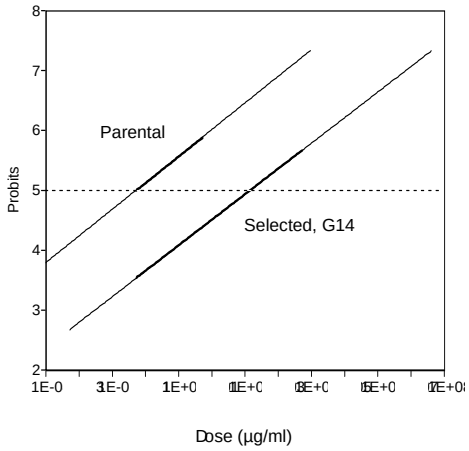

Figure 1. Oral toxicity of the active ingredient of formulated Tracer, incorporated into artificial diet. Third instars were allowed to feed freely for 48 h on the treated diet and then transferred to diet without spinosad. Mortality was measured 4 days later. Dose in µg of active ingredient per ml of diet. Extrapolations of the data are indicated by thin lines.

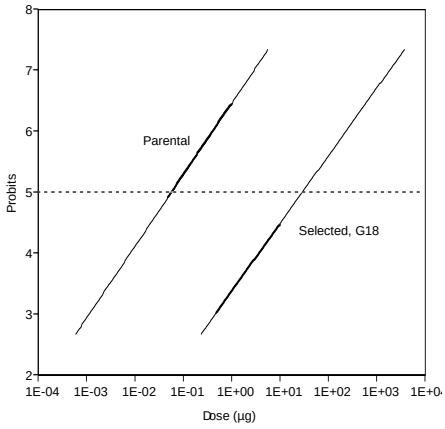

Figure 2. Toxicity of technical spinosad injected into the abdominal hemocoel of fourth-fifth instars. Mortality was measured 6 d after injection. Dose in µg of technical spinosad per larva. Extrapolations of the data are indicated by thin lines.

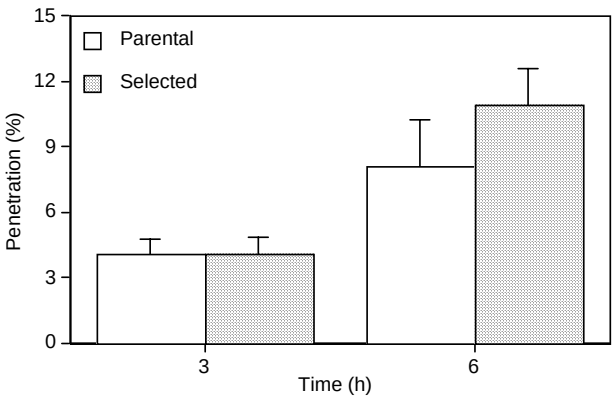

Figure 3. Penetration of 2′-*O*-[^{14}C]-methyl spinosyn A through the cuticle of third instars. The error bars represent 1 standard error of the mean.

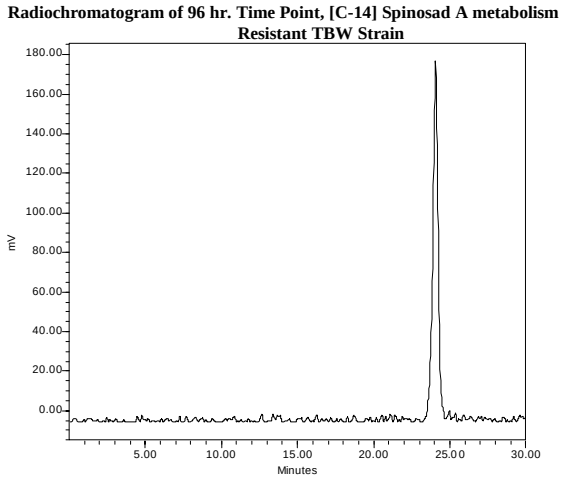

Figure 4. HPLC radiochromatogram of 2′-*O*-[^{14}C]-methyl spinosyn A, 96 h after injection into fifth stadium, resistant budworms. The peak at approximately 24 min exactly corresponds to the retention time of 2′-*O*-[^{14}C]-methyl spinosyn A standard (non shown). No metabolism of spinosyn A was found. NCSU, North Carolina State University; TBW, tobacco budworm.