INHERITANCE AND STABILITY OF SPINOSAD RESISTANCE IN A LABORATORY STRAIN OF THE TOBACCO BUDWORM

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Abstract

In 1996 and 1997 field collections of North Carolina tobacco budworm larvae, *Heliothis virescens* (F.), were established as a laboratory (parental) strain. Larvae from this colony were repeatedly selected for spinosad resistance by topical application of technical spinosad (a mixture of spinosyns A and D) over 13 generations. Topical LD50s 18 days after treatment were 0.11 µg of active ingredient per larva for the parental (susceptible) strain and 73.55 µg per larva for generation (G) 19 of the selected strain. When the resistant and the susceptible strains were crossed, and the F1(R x S) budworms then backcrossed to the resistant strain, spinosad resistance was found to be the result of a partially recessive, single gene. The F1 budworms were estimated to be 4- to 5-fold resistant compared to the susceptible strain. The stability of resistance in the absence of immigration and exposure to spinosad for 5 generations in a cohort of the resistant strain was examined. The LD50 decreased by 1.4-fold compared to the resistant budworms. Comparing the developmental biology of the susceptible (parental) and resistant strains, resistant males developed a little slower as larvae and emerged as adults later than susceptible males and had slightly smaller 1 day old pupal wet weights. When susceptible (S) and resistant (R) moths of both sexes were allowed to mate freely in a mixed population (80%R X 20%S), the majority of the offspring (84.6%) were susceptible to spinosad. This indicates reduced reproductive competitiveness for the resistant strain.

Introduction

The spinosyns were discovered in the 1980s (Thompson et al., 1997) in the Actinomycete, *Saccharopolyspora spinosa.* Spinosyns A and D demonstrated insecticidal activity against pest species with favourable 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., 1997). Although the exact mode of action of spinosyns is still under examination, they simultaneously modify the function of nicotinic acetylcholine receptors and GABA-gated chloride channels (Salgado 1997). Spinosad is a new class of insecticide chemistry with a novel mode of action and has only lately (1997) been marketed for the control of lepidopteran pests in cotton, tobacco and other crops (Thompson et al., 1996; 2000).

Our laboratory selected a laboratory strain of susceptible tobacco budworms, *Heliothis virescens*, originally established from field collections in North Carolina, by the topical application of spinosad every generation for 13 generations (Bailey et al., 1999; Roe et al., 2000; Young et al., 2000). These insects became highly resistant to the insecticide. This paper examines the inheritance of resistance, the stability of resistance in the absence of selective insecticide pressure and immigration, the biology of the resistant strain, and the relative reproductive success of resistant and susceptible budworms.

Materials and Methods

Insect Rearing

Larvae were routinely reared at 27±2°C and 60±10% relative humidity with a 14:10 (light:dark) cycle in 1 oz plastic cups containing 10-12 ml of a standard artificial diet (Burton, 1970). Adults mated freely and laid eggs in 2.3-liter plastic containers. Moths were supplied with 20% sucrose and oviposited on gauze (1mm mesh) covering the top of the container. Eggs were washed 5 min in a 2% dilution of commercial bleach and then allowed to hatch.

Determination of Spinosad Susceptibility and a Diagnostic Dose for Detecting Resistance in Individual Larvae

All larvae used to determine spinosad toxicity were from eggs collected on the 2nd day of oviposition. Spinosad applications were made to day 1 fourth stadium larvae (45±5mg). One microliter of appropriate dilutions of technical spinosad (spinosyns A and D, 88% pure, Dow AgroSciences, Indianapolis, IN) in absolute ethyl alcohol was applied to the dorsal thorax. Applications were made with a 50 µl Hamilton syringe (Hamilton Company, Reno, NV) fitted with a 1µl repeating dispenser. All treatment doses and LD50 values are given as amount of pure spinosad. In all LD50 estimations, treatments (3-6 doses) and solvent controls were replicated three times with 25 larvae each. Dosed larvae were reared singly. Mortality is defined as failure to respond to a touch from a blunt probe within 10 sec.

From the estimations of the LD50, a discriminating dose of 4.4 µg of spinosad per larva was chosen. No susceptible larvae survived this dose while typically less than 7% mortality occurred for resistant larvae. This discriminating dose with mortality assessed after 18 days was used to diagnose resistance in individual larvae in the studies that follow.

Inheritance of Resistance

To determine whether resistance was a dominant or recessive trait, multiple matings were made in separate experiments of resistant males with susceptible females (R-males X S-females), R-females X S-males, S-males X S-females, and R-males X R-females. Each of the two R X S crosses consisted of two mating containers with 10 moths of each sex per container. The S X S and the R X R crosses consisted of five mating containers each with 20 moths of each sex per container. Eggs were pooled across all containers of each treatment and neonates from days 2, 3 and 4 of oviposition were reared individually. Susceptibility to spinosad at a diagnostic dose of 4.4 µg per larva was estimated for 25 offspring collected from each day of oviposition (days 2, 3 and 4) for each of the four different crosses. The LD50 was estimated from five doses in the R-female X S-male and the R-male X S-female crosses. A solvent control of 3 replicates of 25 insects per replicate was run for each cross.

To further characterize the inheritance of resistance, 5 single pair matings were made of R-males X S-females followed by single pair backcrosses of the F1 (R-males X S-females)-females X R-males (18 pairs). S-male X S-female and R-male X R-female crosses (6 and 5 pairs, respectively) were conducted as controls. Except for the backcross, all eggs were collected from the second day of oviposition through the completion of egg laying. The first approximately 300 neonates from each mating pair were placed on diet and a minimum of 50 larvae per pair were treated with the diagnostic dose of 4.4 µg of spinosad per larva. The solvent control for each cross consisted of 20 offspring per mating pair. Five female adults produced from each of the 5 R-male X S-female single pair matings were mated with R-males (single pair matings). Eighteen of the single pair matings produced eggs, and from these matings, approximately 45-150 neonates were placed on diet per mating. Budworms (17-50 larvae per backcross) were treated with the diagnostic dose of 4.4 µg of spinosad per larva. The solvent control for the backcrosses consisted of 5-10 insects per mating pair for a total of 106 control larvae.

Removal of Spinosad Selection

A cohort of spinosad resistant tobacco budworms were reared in the absence of any exposure to spinosad from generations 19 to 23. After five generations, the susceptibility to topically applied spinosad was determined. Mortality was assessed 18 days after the spinosad treatment.

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Biology

The biology of resistant tobacco budworms was compared to that of the susceptible strain by measuring in the same experiment male and female time to pupation, one-day-old pupal wet weight, and time to adult eclosion.

Relative Reproductive Success of Resistant and Susceptible Moths

Multiple-pair matings were performed with a breeding population of 80% resistant and 20% susceptible moths of both sexes. Eight breeding containers were established with 16 resistant (8 per sex) and 4 susceptible (2 per sex) adults per container. From days 2, 4, and 6 of oviposition, 100 larvae per breeding container were treated with a diagnostic dose of 4.4 µg spinosad per larva.

Statistics

Abbott's correction (Abbott 1925) was applied to all data from dose-response experiments. LD50s were determined by plotting log dose versus probit plus 5 mortality (Sokal and Rohlf, 1995; Finney, 1971; Microsoft Excel, 1997). Multiple means tests were conducted by the Tukey-Kramer test (Abacus Concepts, 1995) with $P<0.05$. Goodness of fit was determined by the one-group chi-square method using StatView 4.5 (Abacus Concepts, 1995). Confidence intervals for toxicity ratios were determined by the method of Robertson and Preisler (1992).

Results and Discussion

Diagnostic Dose for Resistance Detection

The highly resistant laboratory strain demonstrated 669-fold (95% confidence interval of 417-982) resistance to topically applied spinosad at generation (G) 19 with a LD50 value of 73.55 µg per larva. The susceptible *H. virescens* strain had a LD50 value of 0.11 µg per larva (Table 1). After 12 rounds of selection (G13), susceptibility to spinosad was reduced 314- and >163-fold when larvae were exposed by placement on treated diet for 48 h and by injection with technical spinosad in the perivisceral hemocoel, respectively (Bailey et al., 1999; Roe et al., 2000; Young et al., 2000). From the estimation of the LD50s in Table 1 of this study, a discriminating dose of 4.4 µg of spinosad per larva was chosen. No susceptible larvae survived this dose whereas resistant larvae typically had less than 7% mortality. This discriminating dose with mortality assessed after 18 days was used to diagnose resistance in individual larvae in the studies that follow.

Inheritance of Spinosad Resistance

The offspring of multiple-pair matings of resistant (R) females with susceptible (S) males (n=75) and R-males with S-females (n=75), were all susceptible to spinosad. The diagnostic dose of 4.4 µg of spinosad per larva killed 100% of the larvae (data not shown). The resistance ratios for these crosses were 4.9- (95% confidence interval of 2.5-9.2) and 4.0-fold (2.2-6.6), respectively (Table 1), indicating that the hybrids were only slightly more resistant than the susceptible (parental) strain. There were also no apparent sex-linked differences in the inheritance of resistance since the LD50s of the hybrids were statistically the same for the two crosses (Table 1). It appears from these studies that spinosad resistance is the result of a non-sex linked, incompletely recessive pattern of inheritance.

To determine the number of genes involved in resistance, single pair matings between R-females and S-males were conducted. The 250 offspring tested were all susceptible to the diagnostic dose. These results confirmed our earlier study with multiple pair matings. We next conducted a backcross of F_1 females with resistant males and treated 476 of the offspring with the diagnostic dose of spinosad (data not shown). If resistance is controlled by one locus with two alleles (our null hypothesis), the backcross should produce 50% resistant and 50% susceptible offspring. Using Tabashnik's (1991) formula to determine the expected mortality, the goodness-of-fit X^2 between the backcross observed response and the expected response is calculated as described by Sokal & Rohlf (1981). We estimated a $X^2 = 0.09$ and $P = 0.76$, which leads us to accept the hypothesis of a one-gene, two-allele, recessive mode of inheritance for the resistant phenotype.

Relaxation of Selection

When a cohort of the resistant strain was not exposed to spinosad for five generations (G19-G23) and in the absence of immigration, only minor reversion to susceptibility was found. The difference was 1.4-fold (95% confidence interval of 1.12-1.76) with LD50 values of 73.55 µg per larva at generation 19 and 52.39 µg per larva at generation 23 (Table 1). The estimated resistance ratio after 5 generations without any selection with spinosad is still 476-fold (286.2-728.2) as compared to G19 (669-fold). This was not surprising. Apparently, the result of more than 12 generations of selection with spinosad was a population mostly homozygous for resistance. In the absence of any immigration of susceptible budworms into the population, resistant is stable. These results also suggest that there are no significant biological disadvantages for being resistant when reared in the laboratory.

Biology of the Resistant Strain

Our experience has been that rearing the spinosad resistant budworms in the laboratory is no more difficult than rearing the susceptible strain. This and the fact that resistance was stable in the absence of spinosad selection for 5 generations, suggested that the biology of resistant and susceptible budworms must be similar. To examine this question further, both strains were reared under identical conditions. The time to pupation was estimated using a regression of the probit of cumulative percent pupation on the time (days) after egg hatch. The TP50, the time (days) required for 50% of the larvae to pupate, was estimated from this regression. The TP50 was 14.6 d for resistant males as compared to 13.8 d for susceptible males and 13.5 d for the resistant females as compared to 13.8 days for susceptible females (Table 2). Although no statistically significant differences could be found in the TP50s between resistant and susceptible budworms for both sexes, the regression line showed that pupation was slightly delayed for resistant males as compared to the other treatments.

Pupal wet weights (less than 24 h after pupation) were compared between resistant and susceptible budworms (Table 3). No difference was found between resistant and susceptible females, but resistant males weighed slightly less than susceptible males.

The first adults emerged from the pupa 27 days after egg hatch. The period of adult emergence for the susceptible budworms of both sexes and the resistant females overlapped broadly with a peak of eclosion on days 28 through 30 (data not shown). Adult emergence for resistant males was delayed with the first adults emerging by day 29 and with the peak in adult emergence occurring on days 30 through 32, about two days later than the others (data not shown). This delay in male emergence from the pupa in the resistant strain is consistent with the delay in larval development for resistant males noted above. The effect this might have on possible resistance evolution under field conditions is not clear but could be of some concern (Liu et al., 1999).

Reproductive Competition Between Resistant and Susceptible Moths

When multiple pair matings were conducted with 80% resistant and 20% susceptible moths of both sexes, the majority of the offspring produced were susceptible (Fig. 1). Of the 780 larvae treated with a diagnostic dose, only 120 survived (15.4%). Theoretically, if random mating occurs, with neither strain having any advantages or disadvantages over the other, the offspring should be 64% resistant and 36% susceptible (a 16:9 ratio) with the expected genotypes rr:rS:SS = 64:32:4 (4(0.8rr + 0.2rS) + 1x(0.8rS + 0.2SS)). Considering that spinosad resistance is the result of one-locus with two alleles and is recessive, there must be a reproductive disadvantage for resistant adults relative to the susceptible strain. Further research is needed to determine the reason for this competitive disadvantage.

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Table 1. Comparison of LD50s 18 days after topical application of technical spinosad to day 1 fourth stadium susceptible, resistant, relaxed and hybrid tobacco budworms. The relaxed strain were larvae that were not selected with spinosad for 5 generations (G19-G23). The hybrids were obtained in separate experiments by crossing resistant males with susceptible females (10 each) and by crossing resistant females with susceptible males (10 each). LD50s are expressed as µg of 100% spinosad per larva[a].

Cross	LD50	Regression curve	R^2	95% CI	N	RR
HvS X HvS	0.11	y=2.84x+7.67	0.95	0.03-0.49	300	
HvR-G19 X HvR-G19	73.55	y=1.85x+1.55	0.99	56.26-101.91	225	669
HvR-G23 X HvR-G23	52.39	y=1.76x+2.0	0.98	29.26-103.29	375	476
HvSfem X HvRmale	0.44	y=1.26x+5.45	0.99	0.14-1.35	300	4.0
HvSmale X HvRfem	0.54	y=1.35x+5.35	0.98	0.1-2.87	300	4.9

[a]Resistance ratio (RR) is the LD50 for the cross indicated divided by the LD50 for the susceptible strain (HvS X HvS). If the sex is not indicated, then both males and female were used. CI, confidence interval; fem, female; G, generation; Hv, *Heliothis virescens*; n, number of individuals tested; R, Hv from the resistant strain; S, Hv from the susceptible (parental) strain.

Table 2. Time needed for 50% of the larvae to pupate.

Strain	Time for 50% Pupation (days)	Confidence interval (95%)
Susceptible males	13.8	13.5-14.0
Resistant males	14.6	13.7-15.6
Susceptible females	13.8	13.5-14.2
Resistant females	13.5	11.8-14.7

Table 3. Pupal weight within 24 h of pupation.

Strain	Pupal weight (mg)[a]	+/- one standard error of the mean
Susceptible males	253.1 (a)	248.7-257.5
Resistant males	224.4 (b,c)	217.7-231.0
Susceptible females	234.4 (a,c)	228.1-240.7
Resistant females	228.0 (b,c)	222.7-233.2

[a]Pupal weights followed by the same letter were not significantly different as determined by a Tukey-Kramer test (P<0.05).

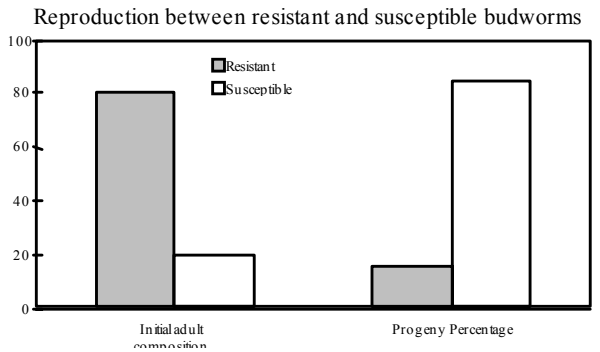

Figure 1. Resistant (R) and susceptible (S) tobacco budworms of both sexes were allowed to mate freely (80%R X 20%S). The offspring from this mating were exposed to a diagnostic topical dose of spinosad (4.4 µg/larva) and mortality assessed after 18 days.