CHARACTERIZATION OF SPINOSAD (TRACER®) RESISTANCE IN A LABORATORY STRAIN OF THE TOBACCO BUDWORM AND DEVELOPMENT OF NOVEL DIAGNOSTICS FOR RESISTANCE MONITORING IN THE FIELD

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Abstract

A highly spinosad-resistant strain of the tobacco budworm was developed in the laboratory by selecting each generation topically with technical spinosad. The LD_{50} at 15 days after treatment for the parental (susceptible) strain originally collected from North Carolina and reared in the laboratory on artificial diet was 0.131 µg/larva. Resistance was detected as early as the 6th generation. In the 14th generation, a dose of 100 µg/larva produced 47.1% mortality, a resistance ratio >763 fold based on a comparison of the LD_{50}s. The parental strain demonstrated a similar susceptibility to spinosad as additional field strains from MS, LA, GA and NC. The susceptibility of the resistant strain did not revert back to that of the susceptible strain when reared for six generations in the laboratory in the absence of spinosad selection, immigration of new genes, and fitness challenges particular to the field environment. Spinosad resistance in the selected strain is present in both the larval and adult stage, validating the use of an adult bioassay for resistance monitoring in this strain. A larval feeding disruption assay was developed for spinosad resistance detection. One advantage of this assay is the rapid detection of resistance in neonates collected as eggs from a specific location or field prior to insecticide application. Other advantages of the feeding disruption assay technology are discussed.

Introduction

Spinosad was originally discovered in the actinomycete, *Saccharopolyspora spinosa*, and has been developed into an important new class of insecticides for control of lepidopterous pests in cotton and other crops (Thompson et al., 1997). The principle active components of the commercial product, spinosad (Tracer®), are spinosyns A and D, which contain a tetracyclic core consisting of a 12-membered macrocyclic lactone fused to a 5,6,5-*cis-anti-trans*-tricyclic ring system. Also attached to the core is the amino sugar forosamine and a neutral sugar, 2,3,4-tri-*O*-methylrhamnose. Spinosyns A and D differ only by the presence of a methyl group at C_6 (Sparks et al., 1998). Spinosad has only recently (1996) come into widespread use as an insecticide.

We have been interested in the toxicology of the spinosyns because they represent a new class of insecticide chemistry with a novel mode of action. The insecticide simultaneously alters the function of nicotinic acetylcholine receptors and GABA-gated chloride channels, but the exact site of action is unknown and appears to be unique to the spinosyns. Tracer is especially important to agriculture, because of its high activity toward pest species and low toxicity to non-targets (Thompson et al., 1997). We have also been interested in spinosyns because, previous to our research, there were no laboratory or field studies demonstrating insect resistance to this control agent. Leonard et al. (1996) did find a 21.4-fold range in susceptibility of the tobacco budworm to spinosad among field-collected strains from the Southeastern U.S., and Moulton et al. (1999) found as high as a 70-fold range in susceptibility of the beet armyworm from the Southern U.S. and Southeast Asia. We reported in the 1999 Beltwide Cotton Conference (Bailey et al., 1999) that selection of a laboratory colony of the tobacco budworm collected from the field in North Carolina with technical spinosad applied topically every generation, resulted in high levels of resistance to spinosad. The current paper reports on the status of selection on this strain, its comparison to other field strains, the effect of rearing of the selected strain in the absence of spinosad treatments, and the development of larval and adult bioassay techniques for monitoring resistance in the field.

Materials and Methods

Insects and Spinosad Selection

Tobacco budworm larvae were routinely reared at 27±1°C with a 14:10 (light:dark) cycle on artificial diet (Burton, 1970). A laboratory (parental) colony of the tobacco budworm used in our selection studies was established from field collections from North Carolina in 1996 and 1997. Four additional field populations were collected in 1998 and maintained as separate colonies in the laboratory on artificial diet. Hv Johnston was collected as larvae on tobacco in Johnston Co., NC; Hv Franklin was collected as larvae on velvetleaf in Franklin Parish, LA; Hv Washington was collected as eggs on geranium in Washington Co., MS; and Hv Quitman was collected as eggs from cotton refugia for Bt cotton in Quitman Co., GA.

Tobacco budworms from the parental strain described above were selected with technical spinosad (88.0% active ingredient, provided by Dow AgroSciences, Indianapolis, IN) starting in November of 1997. This colony is designated as the resistant (laboratory selected) strain. Spinosad was applied in 1 µl of acetone with a repeating Hamilton syringe (Hamilton Co., Reno, NV) to the dorsal thorax of ice-chilled

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larvae in the weight range of 15-45 mg/larva. Treated insects were reared singly in 30-ml plastic cups (Solo Cup Co., Urbana, IL) on standard artificial diet. From 1300 to 2300 larvae were selected in each generation. The starting selection dose was 0.05 µg of technical spinosad/larva that was increased to 60 µg/larva by the 11th generation (G). For more details on the history of selection, see Bailey et al. (1999).

Larval Toxicity

The LD_{50} from the topical application of spinosad was obtained for the parental and additional field strains described earlier. Mortality was assessed at 5 doses of technical spinosad applied in acetone. Treatments and controls were replicated 3 times with 25 larvae per replicate (2 replicates for Hv Washington and Hv Quitman). The larvae used in these studies were 30±5 mg as determined by weighing representative insects. Mortality was assessed at 6 d post-application unless otherwise stated. For highly resistant budworms, it was not possible to obtain a topical dose that would kill 50% of the population in 6 days. In order to obtain data as close to the LD_{50} as possible, mortality in some cases was assessed at 15 days after spinosad application and compared to the parental strain at this same time point. Larval mortality in all experiments is defined by a lack of movement after 10 sec when touched with a blunt probe. Control mortality was subtracted using Abbott's formula (Abbott, 1925), and the treatment mortality data were analyzed using log-transformation of dose and probit-transformation of mortality (SAS, 1998; Sokal and Rohlf, 1995; Microsoft, 1995), by linear regression.

Relaxation from Spinosad Selection

Studies were conducted to examine the effect of removal of selection pressure from the resistant strain on spinosad susceptibility. A separate colony of resistant tobacco budworms was maintained in generations 9 through 14 in the absence of any treatments with technical spinosad, and larval susceptibility to the topical application of spinosad determined in the 14th generation by the methods described earlier.

Adult Toxicity

Different concentrations of technical spinosad in 1 µl of acetone or acetone alone (controls) were applied to the frons region of the head of 1-2 day old female adults of parental and selected (resistant, G19) tobacco budworms. Mortality was determined 24 h after the application. Treatments and controls were replicated at least 4 times with 5 moths per replicate at each dose. Dead moths were rigid while survivors demonstrated normal behavior.

Larval Feeding Disruption Assay

Our feeding disruption assay technology has been described in detail elsewhere (Bailey et al., 1998; Roe et al., 1999; Roe et al., 2000) and was originally developed for monitoring tobacco budworm and cotton bollworm resistance to the insecticide, *Bacillus thuringiensis*. The bioassay is conducted with a blue indicator artificial diet with a diagnostic dose of insecticide, and the end point measured is the production of blue feces. Trypan Blue (Direct Blue 14, Matheson Coleman and Bell, Norwood, OH) was blended into artificial diet (described earlier) at the rate of 20 mg of dye per 100 ml diet. This diet also was formulated with 1.6 µg of spinosad active ingredient per ml diet. The spinosad was added to the diet as part of a 44.2% aqueous formulation provided to us by Dr. Clyde Sorenson (NC State University, Raleigh, NC). Several different concentrations of spinosad were investigated, but 1.6 µg/ml proved to be an optimum diagnostic dose. The blue indicator diet with spinosad was dispensed at the rate of 100 µl per well into 8-well microtitre plate strips (Nalge Nunc Int., Napersville, IL). The wells were sealed with strip caps (Nunc). In order to reduce condensation within the wells, caps were punctured twice with a #3 insect pin. Hv Franklin (spinosad susceptible) and spinosad resistant (selected) neonates of the tobacco budworm were used in all assays and were added to the assay diet within 24 h of hatch. Bioassays were conducted at 27±1°C with a 14:10 L:D cycle, and the number of blue fecal pellets produced were counted after a 24 h exposure of the neonates to the diet. Results were taken from two replicates consisting of 24 insects per replicate.

Results and Discussion

Laboratory Selection for Resistance to Spinosad

Selection of our laboratory colony of tobacco budworms which were originally collected from various locations in North Carolina, with technical spinosad applied topically each generation, produced insects that were highly resistant to spinosad. The first indication of resistance was noted in the 6th generation (Bailey et al., 1999); a dose of 0.05 µg of technical spinosad per 15-45 mg larva from the parental strain produced approximately 75% mortality while a dose of 0.075 µg produced <40% mortality in generation 6. By the 10th and 11th generations, 10 and 60 µg of technical spinosad per larva produced only 21 and 39% mortality, respectively (Bailey et al., 1999). The LD_{50} (15 days after treatment) for technical spinosad in the parental strain is 0.131 (95% C.I. 0.028-0.778) µg of spinosad per larva (Table 1). In the 14th generation of selection, 100 µg/larva produced 47.1% mortality (15 days after treatment)(Table 1). This is a resistance ratio >763-fold based on the differences in the LD_{50}s between the two strains. Fifteen day post-treatment data were used in this comparison since this produced mortality in the resistant strain that most closely approached the LD_{50}. There was only 4.2% mortality in the resistant strain six days after treatment with 100 µg of technical spinosad/larva.

There appear to be no dramatic differences in the biology of the parental versus the resistant strain that make it difficult to maintain in the laboratory. Currently, we are rearing G21 of the resistant strain. However, the symptomology of poisoning appears to differ between strains. The susceptible (parental) insects when intoxicated with spinosad become inactive, stop feeding and eventually die. The resistant insects become intoxicated and unable to walk but still feed, molt and complete their development.

Comparison to Field Strains

The susceptibility to spinosad of our parental tobacco budworm strain originally collected from the field from North Carolina appears to be similar to additional field strains collected in 1998 from NC, MS, LA and GA (Table 1). The range in LD_{50}s for mortality six days after treatment was 0.098 µg/larva for the Washington strain to 0.517 µg/larva for the Quitman, GA strain (a 5.3-fold range) with an intermediate LD_{50} for the parental strain of 0.182 µg/larva. The Quitman, GA strain was interesting for two reasons, i.e., it was a newly collected field strain, and its difference in susceptibility to spinosad was similar to selected G6 budworms that later (at G10 of selection, see Fig. 1 and related text) became highly resistant to technical spinosad. It may be informative in the future to compare the potential for resistance development in this field strain as compared to the more susceptible strains like our NC parental budworms.

Resistance in the Absence of Spinosad Selection

Preliminary observations (discussed earlier) indicated that the biology of the resistant tobacco budworms was not greatly different from that of the susceptible strain under laboratory rearing conditions; the resistant budworms have been maintained in the laboratory for 21 generations with no difficulty. However, more detailed research on the biology of the resistant versus the susceptible strain is needed to determine if there are any negative tradeoffs for carrying the resistance gene(s). Based on the ease by which we were able to rear the resistant strain in the laboratory, our hypothesis was that resistance would be stable in the absence of selection with spinosad and in the absence of the immigration of new genes. A cohort of the selected tobacco budworm strain was reared without spinosad selection for G9 through G14 and the susceptibility of this relaxed population compared to the selected and parental strains (Fig. 1). During the course of this experiment, resistance in the selected strain increased drastically after G6 (Fig. 1). In contrast, the relaxed strain reared for six generations from G9 through G14 in the absence of spinosad selection did not revert back to the susceptibility of the parental strain and was intermediate in its susceptibility to that of G6 and G10. Except for the parental, G6 and G11 strains, we were unable to find a topical dose of spinosad that would cause 50% mortality. Apparently, resistance is stable in the laboratory in the absence of selective pressure, the immigration of new genes into the resistant population, and challenges to fitness particular to the field environment.

Adult Bioassay for Spinosad Resistance

A common method for monitoring resistance in Lepidoptera collected from the field is the adult vial assay (Plapp et al., 1987) or a modification of this technique. The principle behind this bioassay is to examine the effect of a diagnostic dose of an insecticide applied topically by various methods to the adult and to examine the effect of the application on the ability of the insect to fly and/or mortality. This approach is currently being used for monitoring field resistance to spinosad. Since the spinosad-resistant tobacco budworm strain described above is the first insect strain found to be resistant to this novel insecticide class, it is important to validate whether the topical application of a diagnostic dose of spinosad can be used for resistance detection, as is commonly practiced in an adult vial assay. When applied topically to the frons of susceptible versus resistant tobacco budworms, 1.0, 5.0 and 10.0 µg of technical spinosad/insect successfully distinguished a homogenous population of susceptible budworms from the selected strain which demonstrated 0% mortality at these same doses (Fig. 2). In these studies, the 5 and 10 µg/insect doses produced the greatest differences in percentage mortality between the resistant and susceptible strains. A dose of 0.5 µg per adult produced no statistically significant difference in mortality between the two strains (t-test, α=0.05) while approximately 50% of the susceptible budworms survived the 1.0 µg dose. These studies demonstrated that the mechanism for larval resistance to spinosad is present in both the larval and adult stages.

Larval Feeding Disruption Assay for Resistance

An alternative method for resistance detection is the larval feeding disruption assay. The advantages of this approach to resistance detection are that it targets the developmental stage which is being controlled by spinosad, it targets neonates collected as eggs from a specific locality or field, and it provides timely information about resistance and other pest population parameters prior to insecticide application. For more detailed information on the uses of the feeding disruption assay see Bailey et al. (1998) and Roe et al. (1999, 2000). In contrast, adult bioassays assume that resistance is expressed at the same level in multiple life stages. In addition adult insects are able to migrate long distances before laying eggs. Also, adults are genetically different from their offspring with potential differences in insecticide susceptibility between generations. Other advantages of the feeding disruption bioassay include a rapid assay time as compared to a mortality assay, portability to the field and simplicity of operation. The assay is non-lethal in some applications, permitting resistance assays for other insecticides.

The principle of the feeding disruption assay is monitoring the presence or absence of blue feces when the insect is placed on artificial diet containing a blue indicator dye and a diagnostic concentration of the insecticide. The production of blue feces indicates that the insect is resistant. Several diagnostic concentrations of formulated spinosad were tested. An optimum diagnostic dose was 1.6 μg of active ingredient (spinosad) per ml artificial diet (Fig. 3). At this dose, 100% of the susceptible Hv Franklin tobacco budworms produced 0-2 fecal pellets while 100% of the Hv selected insects produced >2 fecal pellets in 24 h. Only a small fraction of the resistant budworms (2.3%) produced 3-5 pellets (Fig. 3). These studies demonstrated that the feeding disruption assay can be used for monitoring spinosad resistance in larvae of the tobacco budworm.

Summary

A highly spinosad-resistant strain of the tobacco budworm was developed in the laboratory by the topical application of technical spinosad to larvae in successive generations. The susceptibility of the parental strain to spinosad was typical of other field collected budworms from the Southeastern U.S. The susceptibility of resistant budworms in the laboratory did not revert to that of the parental strain after 6 generations in the absence of spinosad treatments, immigration of new genes into the population and challenges to fitness particular to the field environment. Resistance occurred in both the larval and adult stages validating the use of adult vial tests for resistance detection. A novel feeding disruption assay was developed for monitoring spinosad resistance in neonates collected as eggs from the field. This assay has a number of advantages over the adult vial test.

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Table 1. Field-collected compared to parental and resistant strains, topically treated with technical spinosad (88% pure)[a].

Strain	LD_{50} (µg/larva)[b]	Slope ± SE	95% C.I.
Hv Parental	0.182 (6d)	3.24±0.58	0.043-1.368
Hv Parental	0.131 (15d)	2.66±0.47	0.028-0.778
Hv Selected (G14)	100[c] (15d)		
Hv Washington,MS	0.098 (6d)	1.40±0.14	0.043-0.224
Hv Franklin, LA	0.119 (6d)	1.55±0.11	0.069-0.212
Hv Johnston, NC	0.212 (6d)	1.28±0.31	0.024-18.15
Hv Quitman, GA	0.517 (6d)	1.33±0.04	0.383-0.712

[a]Abberviations: C.I.=confidence interval; G14=generation 14; Hv=*Heliothis virescens*; SE=1 standard error of the mean.
[b]Mortality was measured at six days (6d) and fifteen days (15d) after treatment with technical spinosad. See Materials and Methods for explanation.
[c]Highest dose tested of 100 µg of technical spinosad per larva produced 47.1% mortality.

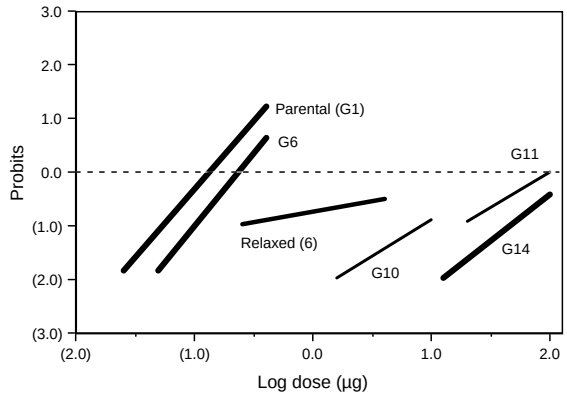

Figure 1. Log Dose-mortality (probits) data for the topical treatment of technical spinosad to parental (G1) and selected (G6 to G14) tobacco budworms. The mortality was determined six days after treatment except for G14 (measured at 15 days after treatment). The relaxed strain was reared in the laboratory in the absence of selection pressure with spinosad for six generations (G9-G14). The data plotted for relaxed is for G14 budworms. Dose is in µg of technical spinosad/insect.

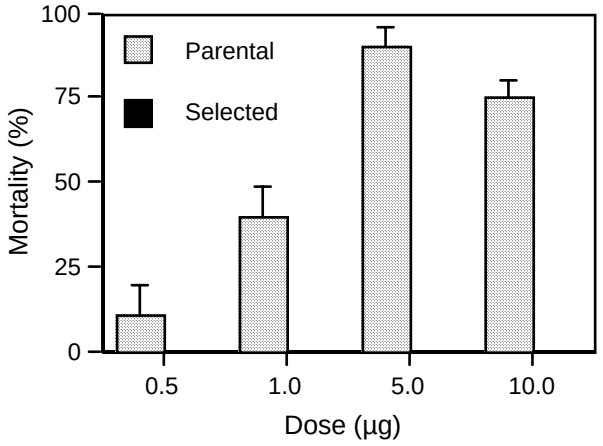

Figure 2. Percentage mortality for adult tobacco budworms from the parental and resistant (selected, G19) strains, topically treated with technical spinosad on the frons region of the head. Mortality was determined 24 h after treatment. The percentage mortality for the selected strain was zero for all doses tested. Dose is in µg of technical spinosad per insect. Error bars are one standard error of the mean.

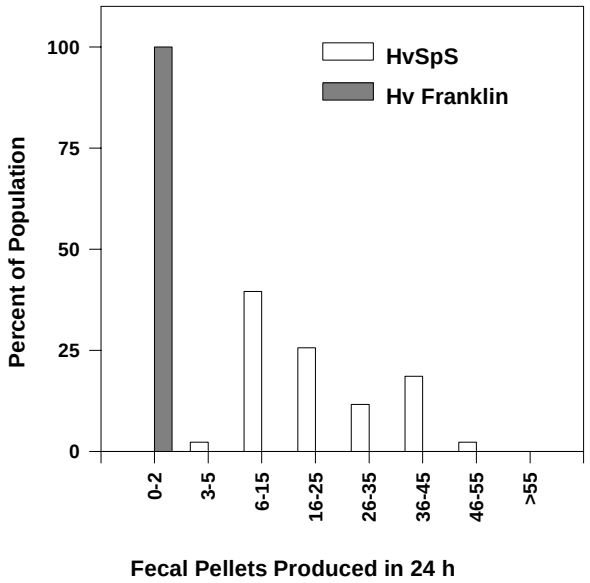

Figure 3. Feeding disruption assay for resistance detection in neonates of the tobacco budworm. Hv Franklin and Hv spinosad selected (HvSpS) neonates were placed on artificial diet containing 1.6 µg of spinosad (active ingredient) per ml diet and blue feces production measured after 24 h. Spinosad was added to the diet as a formulated material (Tracer).