Pesticide Biochemistry and Physiology 96 (2010) 8–13

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Mechanism of resistance to spinosyn in the tobacco budworm, *Heliothis virescens*

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ARTICLE INFO

Article history:
Received 17 November 2008
Accepted 22 August 2009
Available online 1 September 2009

Keywords:
Tobacco budworm
Heliothis virescens
Spinosyn
Permethrin
Profenofos
Emamectin benzoate
Indoxacarb
Acetamiprid
Resistance
Insecticide

ABSTRACT

Topical laboratory selection of tobacco budworm larvae, *Heliothis virescens*, with technical spinosad for multiple generations resulted in larvae 1068-fold resistant to topical applications of the insecticide and 316.6-fold resistant to insecticide treated diet as compared to the parental strain. The penetration of 2′-O-methyl[^{14}C]spinosyn A across the cuticle of the susceptible (parental) and selected (resistant) tobacco budworms increased with time 3–12 h after application. A trend of reduced penetration in the resistant strain was found but the differences were not statistically significant. 2′-O-methyl[^{14}C]spinosyn A when injected into the hemocoel was not metabolized 96 h after treatment in both the susceptible and resistant strain, suggesting that a change in metabolism was not the mechanism of resistance. Electrophysiological studies indicated that dose-dependent spinosyn A-induced currents occurred in neurons from spinosyn resistant and susceptible (adult) tobacco budworms. At both 10 and 100 nM spinosyn A, however, the amplitude of these currents in the resistant insects was significantly smaller than the amplitude of currents observed from neurons from susceptible tobacco budworm adults. This suggests that neurons from resistant insects have decreased sensitivity to spinosyn A. However, the reduced inward currents in the resistant strain may or may not be related to the mode of action of the spinosyns. No statistically significant cross-resistance was noted for the spinosad resistant tobacco budworms for topical applications of permethrin (Pounce®), profenofos (Curacron®), emamectin benzoate (Denim®), or indoxacarb (Steward®). A statistically significant reduction in susceptibility to acetamiprid (Mospilan®) in artificial diet as determined from a resistance ratio of 0.482 was found.

1. Introduction

The availability and use of many of our major insecticide classes such as DDT, cyclodienes, organophosphates, carbamates, pyrethroids, and Bt toxins have been largely sequential. One consequence of this practice has been the development of pest resistance to one intensively-used chemistry after another [1–3]. History has shown that the heliothine complex is one of the more problematic pest groups in this regard [1–4], and studies of the potential for resistance and cross-resistance development and mechanism of resistance are needed to better design resistance management programs.

The spinosyns are a family of novel insecticidal natural products produced by the Actinomycete, *Saccharopolyspora spinosa* Mertz & Yao [5,6]. Spinosad is a naturally occurring mixture of spinosyns from *S. spinosa*, consisting primarily of two of the most insecticidal spinosyns, A and D [6,7]. Although highly insecticidal, spinosad has a very favorable mammalian and environmental toxicological profile [6,8–10]. Available data indicates that the spinosyns are nicotinic acetylcholine receptor activators that act at a site distinct from the target site of the neo-nicotinic insecticides [10–12].

Previous studies have described the selection of a laboratory strain of the tobacco budworm, *Heliothis virescens* (F.), for resistance to spinosyn [13–16]. After 14 generations, the topical LD_{50} of the selected strain was 1068-fold greater than the parental generation. Reciprocal single pair matings between the resistant and the parental strain and backcrosses of F_1 (R × S) females with resistant males indicated that a non-sex linked, (partially) recessive single gene was responsible for resistance to spinosyn.

Several studies have investigated the cross-resistance of spinosad in insect strains resistant to spinosyn as well as other

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doi:10.1016/j.pestbp.2009.08.009

insecticides. Wang et al. [17] found no cross-resistance in a spinosad resistant lab strain of *Spodoptera exigua* to fenvalerate, phoxim, methomyl, abamectim and cyfluthrin. No cross-resistance was found to spinosad for an abamectin resistant field population of *Liriomyza trifolii* [18] or for lab populations of *Musca domestica* resistant to lindane, tetrachlorovinphos, permethrin or DDT [19]. In some contrast, a field population of the diamondback moth, *Plutella xylostella*, was found to possess resistance to spinosad as well as abamectin, several Bt's and to a lesser degree fipronil [20]. Mota-Sanchez et al. [21] found varying degrees of cross-resistance among a variety of neo-nicotinoids for *Leptinotarsa decemlineata* but concluded that cross-resistance to spinosad was unlikely. Sayyed and Wright [22] found varying levels of cross-resistance between fipronil, indoxacarb and spinosyn. Thus, while cross-resistance between spinosad and other insecticides has been investigated in a variety of insect species (see also Salgado and Sparks [10]), cross-resistance of the spinosad-resistant *H. virescens* to other insecticides has not been investigated, nor has the mechanism of spinosad resistance.

Research presented in this paper compares resistance ratios of spinosad, permethrin, profenofos, emamectin benzoate, indoxacarb and acetamiprid between spinosyn resistant and susceptible stains of *H. virescens*. Also considered are the possible differences in the cuticular penetration, metabolism and electrophysiological responses to treatments with spinosyn A between the two strains, and a determination of the possible mechanism of spinosad resistance.

2. Materials and methods

2.1. Insects

Tobacco budworms, *H. virescens*, were raised on artificial heliothine diet [23] at 27 °C with 50% RH and a 14:10 L:D photo regime. The original selection of the spinosyn-resistant strain was made by topically applying technical spinosyn in 1 μL acetone to the dorsal thorax of third instars [13–16]. Mortality (lack of response to a blunt probe within 15 s) was assessed from 12 d after application to pupation or death of all larvae. After twelve rounds of selection, resistance was at high levels.

2.2. Insecticide penetration studies

Any difference in cuticular penetration between susceptible and resistant strains was tested by topical application of 2′-O-methyl[^{14}C]spinosyn A (provided by T.C. Sparks, Dow AgroSciences, Indianapolis, IN; 0.14 μg, 22,000 dpm, 51.6 mCi/mmol activity) in 1 μL acetone to the dorsal thorax of third stadium larvae. The experiment was replicated twice, with five third stadium larvae used for each time point after application. After treatment, larvae were incubated in 20 mL glass scintillation vials. After 3, 6 and 12 h, respectively, the larvae were removed from the incubation vial and externally washed twice with 1 mL acetone for 30 s 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; two aliquots of 100 μL each were removed and placed in a scintillation vial. Scintillation cocktail was then added and the mixture vortexed. The radioactivity in each vial was quantified by lsc to provide an estimate of the internal content of spinosyn A. The radioactivity remaining in the original holding vials was also quantified by lsc. Preliminary studies indicated that since the larvae were unfed during the course of the study, frass production was minimal and the amount of radioactivity present in the frass during the course of the study was negligible. As such any frass present was counted as part of the holding vial. Data were analyzed using PROC GLM in the computer program, SAS [24]. Based on plotting of the residuals (not shown), no additional data transformation was deemed necessary. In Fig. 1, data are presented as percentage of the label present internally ±1 standard error of the mean.

2.3. Electrophysiological studies

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. [25]. Ganglia were de-sheathed and incubated in 0.5 mg/mL collagenase (Type 1A; Sigma Chemical, St. Louis, MO) at 37 °C for 7 min, then washed three times with saline (as below) and dissociated by trituration with pipettes 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. [26]. 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 (provided by T.C. Sparks, DowAgroSciences) 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 three independent observations (±SEM). Statistical significance was assessed using a two-tailed unpaired *t*-test ($P < 0.05$).

2.4. In vivo metabolism of spinosyn A

To determine if a change in the rate of spinosyn metabolism occurs in the resistant strain, 5th instars of susceptible and resistant budworms were injected in the hemocoel with 2′-O-methyl[^{14}C]spinosyn A (provided by T.C. Sparks, Dow AgroSciences; 51.6 mCi/mmol activity) in 0.5 μL acetone with a 30 ga Hamilton syringe needle. Larvae ($n = 35$) of each strain were injected using 5 larvae per dose per incubation time. At 2, 4, 8, 16, 24, 48 and 96 h after injection, the larvae were placed in 3 mL of isopropanol, some of which was used to rinse the larval rearing chambers of any excreted material. Larvae and rinsate were homogenized for 10 s and the extract centrifuged for 15 min at

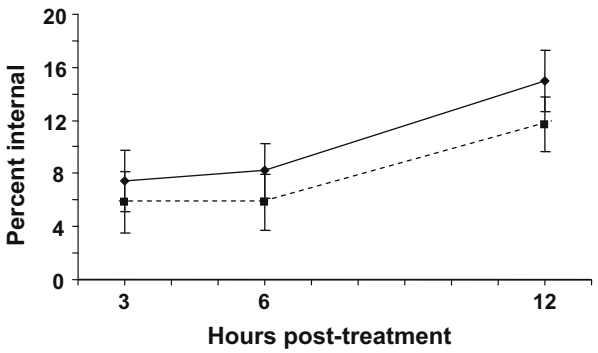

Fig. 1. Penetration of [^{14}C]spinosyn A through the dorsal cuticle of last stadium tobacco budworms. Data are the means of five larvae. Mean separation is based on least squares means ($P \leqslant 0.05$). Error bars are 1 SEM. — susceptible strain; – – – resistant strain. $P = 0.0534$, time = 0.0096, strain = 0.1905, strain * time = 0.9275.