Leaf Dip Bioassay to Determine Susceptibility of Tobacco Hornworm (Lepidoptera: Sphingidae) to Acephate, Methomyl and Spinosad[1]

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Abstract The tobacco hornworm, *Manduca sexta* (L.), is an annual economic pest of fluecured tobacco in Georgia and throughout the southeastern U.S. Recent concerns about controlling hornworms with standard insecticides have been reported by producers and the Cooperative Extension Service. Therefore, a tobacco leaf dip assay was developed to determine the dosage-mortality responses of tobacco hornworms to three standard tobacco insecticides: acephate, methomyl, and spinosad. Larvae, 4 to 5 days old (second instar) and weighing 20 to 40 mg, were reared from eggs collected on field-grown tobacco. Serial concentrations of selected insecticides were prepared in 3.8-L containers, and untreated tobacco leaves were immersed in the solution for 5 s, then air dried. Larvae were placed directly on the treated foliage and examined for mortality after 24, 48 and 72 h of exposure. Hornworm larvae were highly susceptible to foliage dipped in spinosad, with LC50's of 4.3×10^{-4} at 24 h, 1.0×10^{-6} at 48 h, and 5.7×10^{-7} at 72 h (ml/ml). Hornworms were moderately susceptible to both methomyl and acephate, with 72 h LC_{50}'s of 1.0×10^{-4} (ml/ml) and 1.2×10^{-4} (mg/ml), respectively. Although the LC_{50}'s for spinosad continued to drop from 24 to 72 h, the LC_{50}'s for methomyl and acephate remained relatively constant from 24 to 72 h. Hornworm larval feeding was disrupted within 1 h of exposure to the higher concentrations of spinosad and methomyl, with cessation of feeding accompanied by larvae moving off the treated foliage. Feeding disruption on acephate foliage was not as evident during the initial 4 h of exposure. Baseline results from leaf dip assays are now established for three commonly used tobacco hornworm controls and can be used to document insecticide resistance.

Key Words *Manduca sexta*, tobacco hornworm, dosage-mortality responses, insecticide resistance, acephate, methomyl, spinosad

The tobacco hornworm, *Manduca sexta* (L.), causes annual losses of around $1 million to Georgia's tobacco crop due to control costs and crop damage (McPherson and Jones 2002). This pest consumes large amounts of tobacco foliage during its larval stages (McPherson et al. 1997). Tobacco is a high-value crop, thus producers need to protect the foliage from insect-induced plant injury during the entire growing season. As many as three insecticide applications are made each season specifically for hornworm control (Jones and McPherson 1997), which places this pest, and other

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arthropods as well, under heavy pressure for the development of insecticide resistance.

Insecticide resistance of *M. sexta* was reported for endrin and endosulfan in the early 1960's (Rabb and Guthrie 1964). Although this pest is controlled effectively with low rates of many commonly-used insecticides, including acephate, methomyl, spinosad and commercial formulations of *Bacillus thuringiensis* Berliner (Taylor et al. 1996, McPherson et al. 1996, 2001, Semtner et al. 1999), the potential for insecticide resistance remains a concern. In 1998, tobacco producers reported that some of the currently recommended tobacco insecticides had become less effective in controlling hornworms. As a result to this concern, Herzog et al. (2002) conducted baseline studies of susceptibility of tobacco hornworms to topical applications of selected insecticides. They reported that hornworms are highly susceptible to direct contact with acephate, methomyl, and spinosad. The study reported herein was conducted to expand upon the results reported by Herzog et al. (2002), by exposing tobacco hornworm larvae to tobacco foliage treated with these same three recommended insecticidal controls. Incorporating the findings of hornworm susceptibility from a leaf dip bioassay along with the results already reported on topical applications would help strengthen the tobacco insecticide resistance monitoring program that is being developed in Georgia to document tobacco hornworm resistance on flue-cured tobacco.

Materials and Methods

In 2001, tobacco hornworm eggs were collected in May-July from field-grown flue-cured tobacco produced on the Coastal Plain Experiment Station in Tift Co., GA. These field-collected eggs were placed into 3.8 L (30 × 18 × 11.5 cm) clear plastic containers along with untreated tobacco foliage and returned to the laboratory. Containers were checked daily and fresh foliage added as needed. When larvae reached 4 to 5 days old and were second instars weighing between 20 to 40 mg, 10 larvae were placed into a new container that held a single tobacco leaf (400 to 500 cm^2) that had been dipped into a desired concentration of insecticide. A single white paper towel without moisture was added to the containers because preliminary observations revealed that this procedure was adequate for hornworm survival through the 72 h observation period.

Individual untreated tobacco leaves were obtained from field plots and dipped into 3.8 L plastic buckets containing a serially diluted concentration of either the O-P insecticide acephate (Orthene 97 PE, Valent USA Corp, Walnut Creek, CA), the carbamate insecticide methomyl (Lannate LV, DuPont, E. I. de Nemours Inc., Wilmington, DE), or spinosad (Tracer 4 SC, Dow Agrosciences, Indianapolis, IN), a toxin derived from the fermentation products of an actinomycete bacterium. Tap water was used to dilute each concentration and contained 0.0 ppm chlorine, 120 ppm alkalinity ($CaCO_3$), and a pH of 7.5. Each leaf was completely submerged in the bucket containing the desired insecticide concentration and swirled for 5 s to assure complete and thorough coverage of the leaf surface. Then the leaf was removed and clipped to a string line to dry thoroughly before placing it into the plastic holding container. Six to eight concentrations were used to establish log-dose-probit lines, the median lethal dose, and associated parameters. A total of 40 to 60 larvae was used to establish each point (4 to 6 reps of 10 larvae each). Each 3.8 L container holding 10 second-instar larvae was held in an enclosed rearing room at 25 ± 3°C, 14:10 L:D, and 40-50% relative humidity. Mortality was examined 24, 48 and 72 h after exposure.

Criterion of death of a larva was failure to respond when touched with a probe. A moribund larva would move slightly when touched but appeared to be dying, thus it was counted as dead. Percent mortality was corrected for natural mortality (which was less than 3%) in water-dipped controls (Abbott 1925). To observe feeding behavior on the treated foliage, 3.8 L containers with 10 hornworm larvae each were prepared that included a high, moderate, and low mortality concentration of each material plus an untreated control (see Table 4 for concentrations). These containers were placed in an environmental control chamber set at 28 ± 2°C, continuous light, and around 50% RH. Larvae were observed at 1, 2, 3, 4 and 20 h after feeding exposure to record the number feeding (>2 mm fresh feeding site), when feeding ceased, number moving off foliage, and mortality. From the data that were collected, LC_{50}'s, 95% confidence limits, slopes, and regression analyses were obtained through probit analysis (Daum 1970). The concentration-mortality response lines were generated using non-linear regression analyses (SAS Institute 1990).

Results and Discussion

The susceptibility of second-instar tobacco hornworm larvae to leaf dip assays of acephate are presented in Table 1. The LC_{50} at 24 h was 1.8×10^{-4} (mg/ml), slightly higher than the LC_{50} values at 48 and 72 h. Larval mortality was identical for acephate at 48 and 72 h with an LC_{50} of 1.2×10^{-4} (mg/ml). The LC_{50} values (ml/ml) for methomyl were similar at 24, 48 and 72 h, although slightly higher at 24 h (1.2×10^{-4}) than at 48 and 72 h (1.0×10^{-4}) (Table 2). The slopes of the concentration-mortality lines were very similar for tobacco hornworm larvae examined with leaf dip assays of acephate and methomyl (Fig. 1).

Tobacco hornworm larvae were highly susceptible to leaf dip residues of spinosad (Table 3). The LC_{50} at 24 h was 4.3×10^{-6} (ml/ml) which was 42× lower than the LC_{50} for acephate and 28× lower than the LC_{50} for methomyl at 24 h. At 72 h, the LC_{50} for spinosad was 5.7×10^{-7} (ml/ml), which was 210 and 175× lower than the corresponding LC_{50} values for acephate and methomyl, respectively (Table 3). The concentration-mortality lines for spinosad at 24, 48 and 72 h are presented in Fig. 2. These lines illustrate the continued mortality of hornworms being fed spinosad-treated foliage for up to 72 h. This naturally-derived insecticide from an actinomycete bacte-

Table 1. Concentration-mortality response of second-instar tobacco hornworm larvae to acephate using a leaf-dip bioassay

Observation period	LC_{50} (mg/ml) (95% CI)*	LC_{95} (mg/ml) (95% CI)*	Slope ±SE	F values	df
24 h	1.8×10^{-4} ($2.2 \times 10^{-4} - 1.5 \times 10^{-4}$)	6.1×10^{-4} ($1.0 \times 10^{-3} - 4.4 \times 10^{-4}$)	3.10 ±0.39	45.2**	1, 5
48 & 72 h	1.2×10^{-4} ($1.4 \times 10^{-4} - 1.0 \times 10^{-4}$)	4.5×10^{-4} ($7.1 \times 10^{-4} - 3.3 \times 10^{-4}$)	2.87 ±0.35	37.1	1, 5

* Concentration of acephate (mg/ml) that kills 50% of the second-instar hornworm larvae, calculated by non-linear regression fitted to a probit model with 95% confidence intervals. Mortality at 48 h and 72 h were identical. N = 205 larvae examined in this bioassay.

** All F values for regression were highly significant ($P < 0.01$).

Table 2. Concentration-mortality response of second-instar tobacco hornworm larvae to methomyl using a leaf-dip bioassay

Observation period	LC_{50} (ml/ml) (95% CI)*	LC_{95} (ml/ml) (95% CI)*	Slope ±SE	F values	df
24 h	1.2×10^{-4} ($1.6 \times 10^{-4} - 8.0 \times 10^{-5}$)	4.9×10^{-4} ($1.8 \times 10^{-3} - 3.0 \times 10^{-4}$)	2.65 ±0.53	24.9**	1, 7
48 & 72 h	1.0×10^{-4} ($1.4 \times 10^{-4} - 8.0 \times 10^{-5}$)	4.3×10^{-4} ($1.1 \times 10^{-3} - 2.8 \times 10^{-4}$)	2.76 ±0.49	31.6**	1, 7

* Concentration of methomyl (ml/ml) that kills 50% of the second-instar hornworm larvae, calculated by non-linear regression fitted to a probit model with 95% confidence intervals. Mortality at 48 h and 72 h were identical. N = 280 larvae examined in this bioassay.

** All F values for regression were highly significant ($P < 0.01$).

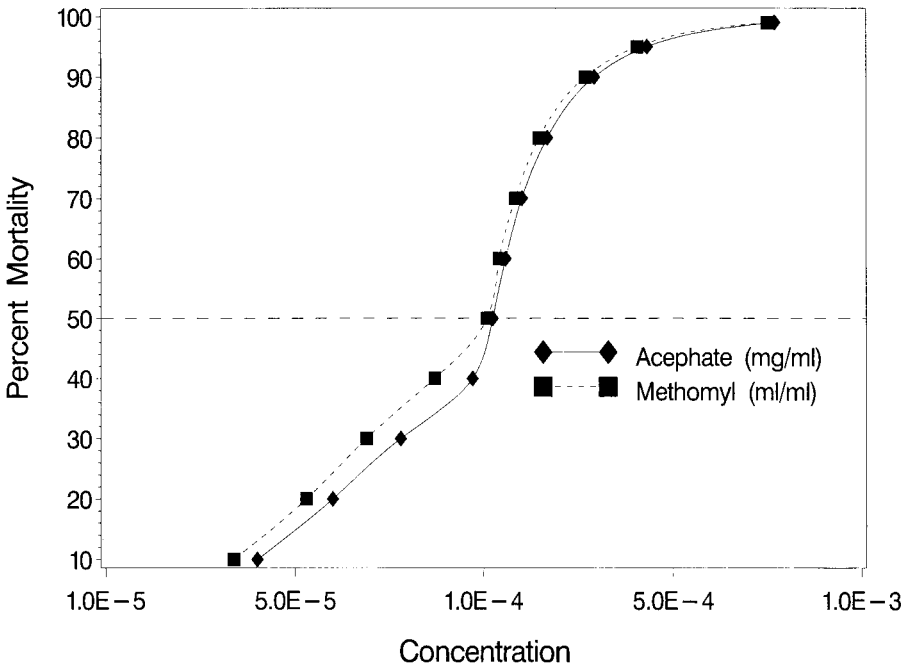

Fig. 1. Concentration-mortality lines showing responses of second-instar tobacco hornworm larvae to leaf-dip assays of acephate and methomyl 72 h after feeding exposure (concentration expressed in scientific notation, i.e., 1.0E-5 = 1.0×10^{-5}).

rium is primarily a stomach poison that disrupts the insect nervous system (Anonymous 1999). Thus, it is not surprising to observe continued mortality of hornworms for 72 h.

The LC_{50} values (ml/ml or mg/ml) for second-instar hornworm larvae reported in

Table 3. Concentration-mortality response of second-instar tobacco hornworm larvae to spinosad using a leaf-dip bioassay

Observation period	LC_{50} (ml/ml) (95% CI)*	LC_{95} (ml/ml) (95% CI)*	Slope ±SE	F values	df
24 h	4.3×10^{-6} ($7.3 \times 10^{-6} - 2.9 \times 10^{-6}$)	1.2×10^{-4} ($5.7 \times 10^{-4} - 4.8 \times 10^{-5}$)	1.13 ±0.14	103.5**	1, 5
48 h	1.0×10^{-6} ($1.3 \times 10^{-6} - 8.0 \times 10^{-7}$)	1.5×10^{-5} ($3.4 \times 10^{-5} - 9.0 \times 10^{-6}$)	1.41 ±0.16	61.5*	1, 5
72 h	5.7×10^{-7} ($6.8 \times 10^{-7} - 4.8 \times 10^{-7}$)	2.7×10^{-6} ($4.7 \times 10^{-6} - 1.9 \times 10^{-6}$)	2.43 ±0.31	109.4**	1, 5

* Concentration of spinosad (ml/ml) that kills 50% of the second-instar hornworm larvae, calculated by non-linear regression fitted to a probit model with 95% confidence intervals. N = 290 larvae examined in this bioassay.

** All F values for regression were highly significant ($P < 0.01$).

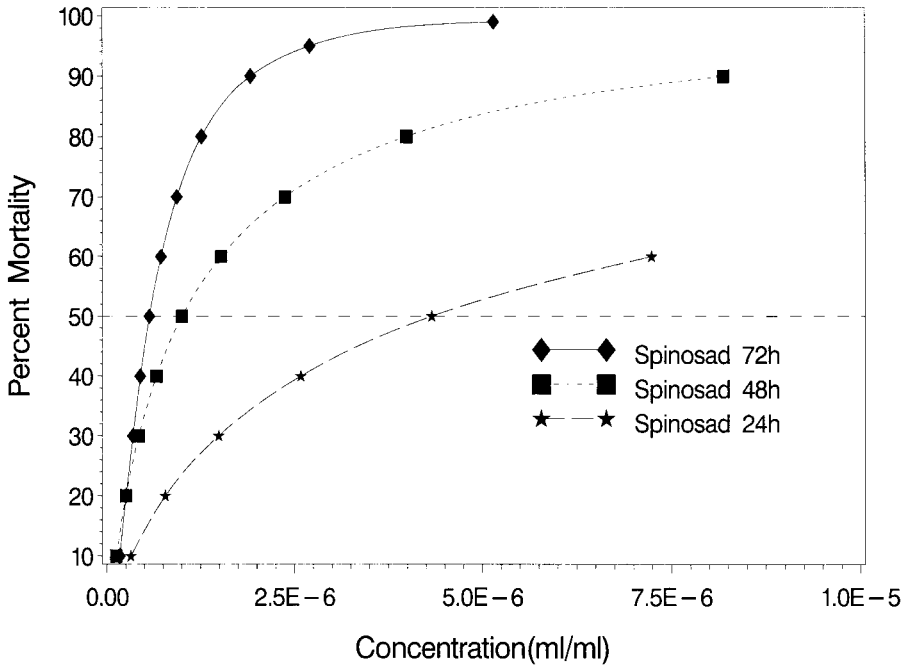

Fig. 2. Concentration-mortality lines showing responses of second-instar tobacco hornworm larvae to leaf-dip assays of spinosad 24, 48 and 72 h after feeding exposure (concentration expressed in scientific notation, i.e., 2.5E-6 = 2.5×10^{-6}).

this leaf dip assay with acephate, methomyl and spinosad were much lower than the LC_{50} values (µg/insect) reported for second-instar tobacco hornworm exposed to topical applications of these same three insecticides (Herzog et al. 2002). However, both the leaf dip assay and the topical application revealed that spinosad is highly

toxic to second-instar tobacco hornworm larvae. Although methomyl and acephate are also very toxic to hornworms, their LC_{50} and LD_{50} values are considerably higher than corresponding values for spinosad.

Tobacco hornworm larvae exposed to the higher rates of spinosad produced mostly small pin-hole feeding sites at 1 and 2 h after exposure to treated tobacco foliage, and no new feeding signs were observed after 2 h, even though 7 larvae were still alive on the 1.0×10^{-6} concentration at 20 h (Table 4). This feeding response is typical for a stomach poison, like spinosad, which may disrupt or halt feeding activity but not cause mortality until 24 and 48 h later. Methomyl was very active as a contact insecticide at the two higher rates examined in the feeding trial. At 1 h after exposure, there were no feeding holes >2 mm and only some small pin-hole punctures observed on the tobacco foliage treated with the 1.5×10^{-4} and 1.3×10^{-4} concentrations (Table 4). Numerous larvae were observed off of the treated foliage at 3 and 4 h after exposure, and no feeding was observed after 2 h at these higher concentrations of methomyl. Acephate-treated foliage required a longer feeding exposure on the highest rate before feeding ceased and/or larvae moved off the treated surface (Table 4).

Development of these baseline susceptibility data for leaf dip assays for acephate, methomyl, and spinosad is an important additional step towards implementing an insecticide resistance monitoring program. These results, along with the reported LD_{50} values for these three commonly-used insecticides (Herzog et al. 2002), will be

Table 4. Tobacco hornworm larval feeding damage (>2 mm diam foliage removed) to tobacco foliage dipped in serial concentrations of selected insecticides

Concentration	Number of larvae feeding (N = 10)					Dead
	1 h	2 h	3 h	4 h	20 h	20 h
ml/ml			Spinosad*			
5.0×10^{-4}	3	3	0	0	0	8
1.0×10^{-6}	5	6	0	0	0	3
5.0×10^{-7}	5	9	10	9	4	2
Control	8	10	10	10	10	0
ml/ml			Methomyl**			
1.5×10^{-4}	0	0	0	0	0	6
1.3×10^{-4}	0	0	0	0	0	5
8.0×10^{-5}	5	5	7	7	7	3
Control	8	10	8	10	10	0
mg/ml			Acephate†			
2.6×10^{-4}	7	7	5	3	2	7
1.3×10^{-4}	8	8	7	7	5	3
6.6×10^{-5}	7	10	8	7	8	1
Control	9	10	10	9	10	0

* Numerous pin hole feeding signs on 5.0×10^{-4} at 1 and 2 h, no new feeding signs on 5.0×10^{-4} and 1.0×10^{-6} after 2 h.

** Some pin hole feeding on 1.5×10^{-4} and 1.3×10^{-4} at 1 and 2 h, 5 larvae off leaf surface at 3 and 4 h and no new feeding after 2 h on 1.5×10^{-4} and 1.3×10^{-4}.

† 3 larvae off leaf surface at 3 h and 5 larvae off leaf surface at 4 h on 2.6×10^{-4}.

useful for documenting tobacco hornworm resistance on flue-cured tobacco. If suspected resistant populations are present, then the leaf dip assay and/or topical application techniques can be utilized with those populations to obtain concentration-mortality or dosage-mortality response curves that can be compared to the baseline data reported herein.

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