TOXICITY OF BACILLUS THURINGIENSIS VAR. TENEBRIONIS AND CA-THURINGIENSIN AGAINST THE BOLL WEEVIL ANTHONOMUS GRANDIS (BOH.) [COLEOPTERA: CURCULIONIDAE]

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Abstract

A lyophilized powder spore - crystal complex of a native Bacillus thuringiensis and an aqueous preparation of killed genetically-engineered cells of the bacterium Pseudomonus fluonescens, which contained B. thuringiensis var. tenebrionis endotoxin crystal toxin was tested for their toxicity to larvae and adults of boll weevil, Anthonomus grandis (Boheman). When the two preparations were incorporated into the diet, their toxicity to larvae was similar. The LC_{50} value for the powder was 2.1 times greater for adults than for first stage larvae, but when this powder was distributed on the surface of the diet, the LC_{50} values were lower for adults than larvae. When weevils were dipped into the powder, LC_{50} values were less than those of the diet surface treatment. Regressions were non-significant for mortalities of adults fed on transgenic preparation in diet. Powder preparation showed LC_{50} of 6,290 mg per ml. diet after 120 h. Slope values for both preparations to adults were flat. An exotoxin of B. thuringiensis, called thuringiensin, was more toxic to adults than the transgenic endotoxin or a mixture of the two. From a cage test foliar sprays of the transgenic preparation showed an inverse relationship of mortality and rates tested, suggesting that as rates increase the boll weevil ingests less. In a field test yields of cotton treated with transgenic preparation were equal to yields of untreated cotton following application of season-long sprays.

Introduction

The boll weevil, Anthonomus grandis (Boheman), is a major pest of cotton in subtropical and tropical areas of the Americas. Presently, only broad spectrum insecticides are used to control this insect in these areas. If any efficacious biological agent became available which avoided the upsets in natural control typically associated with broad spectrum insecticides it could become an important component of cotton IPM programs throughout the Americas. Krige et al. (1987) and Herrnstandt et al. (1986, 1988 and 1989) showed that the bacterium, Bacillus thuringiensis var. tenebrionis was toxic to this insect and called this bacterium B. thuringiensis var. San Diego. It is called B. thuringiensis var. tenebrionis today.

Laboratory and field studies were conducted to determine (1) the toxicity of a native B. thuringiensis var. tenebrionis spore-crystal complex (= endotoxin) powder formulation and a formulation of killed transgenic Pseudomonus fluonescens cells containing B. thuringiensis var. tenebrionis crystal toxin against the boll weevil by three different bioassay procedures; (2) the toxicity of Ca-thuringiensin, an exotoxin of B. thuringiensis, and a mixture of the transgenic formulation of endotoxin and exotoxin against the adult by one bioassay procedure and; (3) the toxicity of the transgenic formulation as a foliar spray to cotton against adult boll weevils in a cage and a field test.

Materials and Methods

First and third stage larvae and adults of "ebony" strain boll weevil were obtained from USDA-ARS, Boll Weevil Research Laboratory, Mississippi State, MS. Larvae were reared on artificial diet until the bioassay. Weevils were allowed to feed on larval diet three to seven days prior to each bioassay.

The lyophilized spore-crystal complex powder (hereafter referred to as powder formulation) (Lot 123-35) of a native B. thuringiensis var. tenebrionis isolate contained 2130 g toxin per g of preparation (Herrnstadt and Wilcox 1989a). The engineered transgenic formulation (hereafter called the "transgenic" preparation) (CellcapÔ, Lot 4566760) consisted of killed Pseudomonus fluorescence (strain MB 101) cells containing B. thuringiensis var. tenebrous (MYX 1806) (Barnes & Cummings (1987), Herrnstadt and Wilcox (1988) and Gaentner (1990)) and contained 13100 mg delta endotoxin per ml formulation (Herrnstadt and Wilcox 1989a). Both preparations and the estimates of toxin concentrations were obtained from Mycogen Corporation, San Diego, CA. Technical Ca-thuringiensin (44% w/w) concentrated on calcium, (lot no. 25-252-BD) was obtained from Abbott Laboratories, North Chicago Ill.

Laboratory tests were conducted from January to June, 1986, at Mississippi State, MS, and from September through December, 1989, at Weslaco, TX. Three laboratory bioassay procedures were conducted: (1) incorporation of each preparation into artificial diet and fed to larvae and adults, (2) surface treatment of artificial diet with the transgenic formulation and fed to adults, and (3) dipping adults into solutions of the transgenic preparation. Three procedures were compared to determine if one was more toxic to weevils than the others.

Diet incorporation-larval treatment

Boll weevil diet containing 21, 64, 213, 639, 2130, 6390 and 19170 μg delta endotoxin of the powder formulation per ml distilled water was dispensed into 9 cm diameter

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plastic petri dishes (5-10 ml per dish). Five first stage larvae were then placed on the diet. Doses were replicated three times with 25 adults per replicate). Bollworm diet (Shaver & Raulston 1971) containing 459, 917, 1834, 3668, 7336 μg toxin of the transgenic preparations per ml was dispensed into 30 ml plastic cups (8 to 10 ml per cup) where one third stage boll weevil larva were placed. Development of boll weevil larvae on bollworm and boll weevil diet appeared to be equal in these studies. Concentrations were replicated three times and 30 were evaluated in the first replicate and 20 in the second and third replicate. LC_{50} values are expressed as mg toxin for respective preparation.

Diet incorporation-adult treatment

Boll weevil diet containing 11, 22, 44, 87, 175, 196 and 262 mg toxin of powder formulation ml per distilled water were placed in 9 cm dia plastic petri dishes (8 to 10 ml per dish) with 5 adults. Doses were replicated 4 times with 25 adults per replicate.

Bollworm diet containing 21, 64, 213, 639, 2130, 6390, 19170, and 42600 μg toxin of transgenic preparation per ml was dispensed into 30 ml plastic cups (8 - 10 ml per cup) and one adult was placed into each cup. Doses of each preparation were replicated three times with 20 adults per replicate. LC_{50} values are expressed as described for diet incorporation larval treatment.

Surface treatment

Doses of 655, 1965, 3275, 4585, 5895, 7205, 8515, 9825, 11135 and 12445 μg toxin of transgenic preparation in one ml distilled water were pipetted onto 6.15 cm^2 bollworm diet surface in 30 ml cups and allowed to dry. Each cup contained 8 to 10 ml of diet. Ca-thuringiensin was tested at 0.05, 0.39, 3.3, 6.25, 12.5, 25, 50, 100, 1000, 5000, 10000 μg/ml distilled water per 30 ml cup and was conducted as described for the transgenic preparation.

The mixture of transgenic preparation (as μg toxin) + Ca-thuringiensin (as μg Ca-thuringiensin) per ml distilled water was tested at 12.9 + 4.9, 25.7 + 9.8, 51.44 + 19.5, 102.9 + 39, 204.9 + 78, 409.9 + 156, 818.8 + 312.5, 1637.5 + 62.5, 3275 + 1250 and 6550 + 2500 (2.6: 1 ratio endotoxin to exotoxin) for a total quantity of 18, 35, 71, 141, 283, 566, 1131, 2262, 4525 and 9050 mg per cm^2 on the bollworm diet surface. Doses of both endotoxin and exotoxin and mixture were replicated five times and a total of 150 weevils with 30 weevils/dose were tested. Results of surface treatments are presented as μg (toxin + Ca-thuringiensin)/cm^2.

Dipping

Weevils were dipped for 30 seconds into distilled water suspensions of the same mg toxin per ml. Doses were the same as used for surface treatment of the transgenic preparation. Weevils were then placed individually in a cup with bollworm diet. Sixty weevils per dose and two replicates of 30 weevils per replicate were tested.

Insects of all bioassays were held in laboratory room for mortalities at 27 $\pm$ 3°C and 50-70% RH.

Data analysis

Cumulative mortalities (total) of larvae and adults for each concentration were determined for both endotoxin preparations and the exotoxin after 24, 48, 72, 96 and 120 h post - treatment in all tests and corrected by Abbott's formula (1925). In addition, mortalities of adult weevils for the exotoxin and the endotoxin + exotoxin mixture were determined after 168 hrs (7 days). Weevils were classed as dead (no movement of body or legs upon prodding), moribund (movement of legs or proboscis) or alive. Appropriate LC_{50}'s, were determined by SAS Probit (SAS 1988). Probit regressions with ratios of slope values/SE $\leq$ 1.96 were not significant at $P_{0.05}$.

Cage test

Azinphosmethyl at 0.28 (A.I.)/ha and transgenic preparations of B. thuringiensis at 25, 50 and 100% concentration (v/v) were sprayed on cotton "DPL 90" in 1990. First, cotton was sprayed with B. thuringiensis var. tenebrionis with a backpack CO_2 power sprayer at 715 liters/ha. Weevils (20) were then placed on treated plants and a bag was placed over the plants for 24 hr. After 24 hr bags were removed and mortality was recorded. Surviving weevils were placed on artificial diet for further observations. Mortalities were adjusted for an untreated check (Abbott 1925). Five replicates of each treatment were made. Analysis of variance was determined and percentage mortalities were separated by student Newman Kuell's (SNK) test at $P_{0.05}$.

Field test

An untreated check and sprays of transgenic formulation at 2.3 and 28.3 (50% solution) liter/ha were evaluated for control of boll weevil on a "as needed" basis in Weslaco, Texas in 1990. Cotton was planted the third week of March and yields were taken at the end of the season. Cotton "DES-119" was irrigated as pre-plant and then once more at first square in plots 0.04 ha (12 rows wide (1 m apart) and 33 m long). Treatments were replicated 4 times. The same area of corn was planted between plots to minimize movement of weevil from untreated to treated plots.

Sprays were applied 16 times with a 12 row high clearance sprayer at 57 liters/ha at 21 kg/cm^2 through TX-3 nozzles spaced 50.8 cm (20 in) apart on the boom.

Undamaged and damaged (egg and feeding punctured) squares and bolls were counted on 5 to 10 whole plants selected at random in each plot once or twice weekly and averaged for the season. Plots were sampled 12 times during the season.

Seed cotton was harvested twice and totaled from four 3 m long rows in the center of the plot at the same time. Analysis of variance was applied to all data. Mean separations were made by Fishers Protected LSD at P=0.05 for yields of seed cotton and percent damage by the boll weevil to squares and bolls

Results and Discussion

Larval treatment

LC_{50} value for transgenic formulation was 2,800 μg toxin (120 h), when incorporated into the diet and bioassayed against third stage larvae (Table 1). Mortality of check larvae was 8% after 120 h. LC_{50} value of the powder formulation was 2470 mg/toxin (120) hr when incorporated into the diet and bioassayed against first stage larvae. Mortality of untreated larvae used in powder formulation bioassays was 15% after 120 h.

LC_{50} values of both formulations 96 to 120 h post - treatment were statistically similar. Therefore, the transgenic formulation was as toxic to third stage (and last) larvae as the powder formulation was against first stage larvae. Slopes of both formulations were similar and increased steadily from 24 to 120 h while standard error values steadily decreased.

Adult treatment

When the transgenic formulation was incorporated into the larval diet the ratio of slope/SE was $\leq$ 1.96. This means the probit regression was not significant all five times mortalities were taken (Table 2). After 120 h mortalities of untreated weevils were 18%. The LC_{50} for the powder formulation was reduced ca 75% after 120 h compared to the 24 h mortality. Mortality of check weevils was 13.8% after 120 h for the powder formulation. Slopes of transgenic and powder formulations were about equal 48 h to 96 h while standard errors of the slopes were variable.

As expected LC_{50}'s of the transgenic formulation for surface treatment of the diet were significantly less than the dip treatment of the weevils after 72 to 120 hr (Table 3). Mortality of check weevils after 120 h were 17 and 18% for surface treatment and dip treatment, respectively. These results suggest that some contact activity could have occurred with the dip treatment perhaps because the endotoxin of B. thuringiensis var. tenebrionis entered the oral cavity or other body openings.

Slopes of the regression for dipping weevils in concentrations of endotoxin remained about the same over the five time periods while slopes of transgenic formulation on surface treatment increased steadily during the five time periods (Table 3).

The exotoxin Ca-thuringiensin was significantly more toxic than the endotoxin B. thuringiensis var. tenebrionis (Table 4) but it was equally toxic to the mixture after 168 hr. Thus, most of the toxicity of the mixture was due to Ca-thuringiensin. Mortalities of check weevils were 16.8 and 21.5% after 168 hr for the exotoxin and the mixture of endotoxin and exotoxin, respectively. However, slopes of the exotoxin and the mixture of endotoxin and exotoxin were flatter than were the endotoxin. We suggest that more factors are involved in toxicity of the exotoxin than the endotoxin of this insect. No information has been found indicating that this exotoxin was equal or more or less toxic to the boll weevil than the endotoxin. Also no information has been found showing that the mixture of an endotoxin and exotoxin were equal, less or more toxic than either toxin alone.

Cage spray

Azinphosmethyl caused significantly greater mortalities of boll weevils than all three rates of B. thuringiensis var. tenebrionis (Table 5). The lowest rate of the endotoxin was more toxic to the caged weevils than the higher rates and was statistically comparable to azinphosmethyl at 96 h post - treatment. However, the variation among replicates was large. Higher mortality at lower concentration is unexplained but may be due to effects of high doses on weevil feeding behavior and/or application and spray deposit variables. After 3 days of exposure differences between mortalities by the rates of endotoxin were not significantly different but rates did differ after 4 days. Untreated mortalities did not exceed 10%.

Field Tests

Percentage damaged squares and bolls among both rates of the transgenic formulation were significantly lower than the untreated check when 16 applications were applied at 1-4 day intervals (Table 6). However, plots treated with the transgenic formulation produced yields statistically equal to the untreated plots. Thus the endotoxin was not effective against this insect at the rates tested although the greatest rate tested was equivalent to a 50% (v/v) solution.

If 11.2 μg endotoxin/cm^2 is equal to 147 mg toxin present on a horizontal plane when 1.12 kg/ha is sprayed on the plants then we cannot kill many adults of the boll weevil. This is far below any concentration indicated by the LC_{50} values when incorporated into the diet or placed on the surface of the diet. At 1.12 kg/ha, the endotoxin was shown to be ineffective based on the laboratory studies, a potted plant spray test and a field test against adults of the boll weevil.

Although our foliar applications of B. thuringiensis var. tenebrionis did not control boll weevils on cotton, we found the insect to be susceptible to endotoxin proteins. Expression of endotoxin mechanism to deliver these insecticidal protein toxins to diet-incorporation assays is indicated when LC_{50}'s to larvae of boll weevils are 2000-3000 £ μg/toxin. LC_{50}'s for B. thuringiensis var. kurstaki strains against tobacco budworm, Heliothis virescens, in similar diet incorporation assays are 1 to 10

μg/ml of diet. Therefore, effective control of the boll weevil, even in transgenic delivery systems, would require identification of new strains with increased activity and high expression in transgenic cotton plants.

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Table 1. Toxicity by transgenic [a/] and native [b/] preparations of Bacillus thuringiensis var. tenebrionis as μg toxin/ml. diet against larvae of the boll weevil.

Hrs	Slope ±SE	LC_{50}	(95% Confidence Interval)
		Transgenic	
24	1.1 ± 0.91[d/]		
48	1.2 ± 0.81[d/]		
72	1.3 ± 0.79[d/]		
96	1.5 ± 0.64	3535.3	(1682.5-5180.5)
120	1.6 ± 0.6281	800.4	(1185.4-7226767.7)
		Native	
24	0.9 ± 1.14[d/]		
48	1.0 ± 1.02[d/]		
72	1.2 ± 0.81[d/]		
96	1.5 ± 0.65	2600.6	(2137.6-3187.4)
120	1.6 ± 0.63	2469.7	(2031.9-3008.2)

[a/] Transgenic Pseudomonus flour preparation contains 13100 μg B. thuringiensis var. tenebrionis toxin/ml (Lot 4566750 of MYX 1806).
[b/] Native preparation contains 2130 μg toxin/g (Lot 123-35).
[c/] Third stage larvae were bioassayed with transgenic preparation and first stage larvae with native preparation.
[d/] Slope/SE ration of $\geq$ 1.96 indicating non significant regression.

Table 2. Toxicity by transgenic[a/] and native[b/] preparations of Bacillus thuringiensis var. tenebrionis as μgs toxin ml/diet against boll weevil.

Hours	Slope ±SE	LC_{50}	(95% Confidence Interval)
	Transgenic		
24	1.1 ± 0.59[d/]		
48	1.2 ± 0.67[d/]		
72	1.2 ± 0.64[d/]		
96	1.17 ± 0.61[d/]		
120	1.0 ± 0.53[c/]		
	Native		
24	1.8 ± 0.55	25606	(21067.8-32299.4
48	1.7 ± 0.59	15771	(∞ - ∞)
72	2.2 ± 0.48	8927	(∞ - ∞)
96	3.1 ± 0.32	7636	(6693.2 - 8705.0)
120	2.2 ± 0.46	6290	(0.0 - ∞)

[a/]See footnote [a/] of Table 1.
[b/]See footnote [b/] of Table 1.
[c/]See footnote [c/] of Table 1.
[d/]See footnote [d/]

Table 3. Toxicity of Transgenic Bacillus thuringiensis var. tenebrionis preparation against boll weevil by surface treatment of diet as μg toxin/cm^2 and dipping weevils into aqueous solutions as μg toxin/ml water.

Hours	Slope ± SE	LC_{50}	(95% Confidence Interval)
		Surface Treatement	
24	1.1 ± 0.98[b/]		
48	1.3 ± 0.77[b/]		
72	1.5 ± 0.67	1917.4	1364.8-3883.6
96	1.6 ± 0.65	1665.5	1230.4-2866.7
120	1.6 ± 0.64	1483.8	1103.1-2417.6
		Dipping	
24	1.8 ± 0.54	1369.9	920.2-2974.8
48	1.7 ± 0.6	916.1	554.1-1489.1
72	1.5 ± 0.8	672.2	593.0-1242.0
96	1.6 ± 0.6	554.3	135.0-966.8
120	1.6 ± 0.6	515.8	150.6-858.4

[a/]See footnote [a/] of Table 1. (Lot 4566750 MYX 1806). For and adults for surface treatment and dipping, respectively.
[b/]See footnote [c/] of Table 1.

Table 4. Toxicity of thuringiensin and thuringiensin (as μgs/cm^2) + Bacillus thuringiensis var. tenebrionis by surface treatment of the diet against adult boll weevils after 168 h as μg toxin.

Slope ± SE	LC_{50}	(95% Confidene Interval)
	thuringiensin + B.t. tenebrionis	
0.185 ± 0.046	0.2	(0.0008-155.8)
	thuringiensin	
0.09 ± 0.025	0.05	(0.0003-0.5)
	B.t. var tenebrionis	
1.45 ± 0.69	1161.6	(8677.5-1670.9)

Table 5. Residual toxicity of Bacillus thuringiensis var. tenebrionis to adults of boll weevil applied as sprays to cotton plants

Treatment	Concentration (Kg/ha or %/V)	Percentage Killed After (days) ± Standard Deviation			
		1	2	3	4
B.t. tenebrionis	100%	0	0	5 ± 8	11 ± 13
	50%	0	0	6 ± 5	13 ± 5
	25%	0	10	14 ± 3	80 ± 40
Azinphosmethyl	0.28	82 ± 19	91 ± 8	93 ± 6	95 ± 4
Check		1 ± 3	1 ± 3	1 ± 3	5 ± 6

Table 6. Effectsof aquous formulation of bacterium on boll weevil damage and yields of seed cotton in field plots at Weslaco, Texas, 1990.

Compound	Rate (Liter /ha)	% Damaged [a/] Squares	% Damaged [a/] Bolls	Yield (kg.seed cotton/ha[a/]
Bacillus thuringiensis	2.3	16.0	29.0	797.0
var. tenebrionis[1/]	28.2	16.0	24.0	556.0
Check		28.0	50.0	594.0
LSD		8.2	16.8	224.2

[a/] Applied on days 156, 157, 159, 163, 166, 170, 171, 173, 183, 187, 191, 194, 198, 201, 204, and 206.