Spore-Toxin Interactions and Sublethal Effects of *Bacillus thuringiensis* in *Spodoptera frugiperda* and *Pseudoplusia includens* (Lepidoptera: Noctuidae)[1]

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ABSTRACT Interactions between the spores and δ-endotoxins of *Bacillus thuringiensis* Berliner were tested in a low-susceptibility insect, *Spodoptera frugiperda* (J. E. Smith). The spores (formulation MYD™) and one δ-endotoxin (formulation MVP™) had additive effects on mortality at a MVP™ dose of 117,500 µg/g of diet ($P < 0.01$) and synergistic effects at a MVP™ dose of 235,000 µg/g diet ($P < 0.01$). The spores and another δ-endotoxin (formulation MYX™) were antagonistic at a MYX™ dose of 117,500 µg/g diet ($P < 0.01$) and additive at a MYX™ dose of 235,000 µg/g diet ($P < 0.01$). The two δ-endotoxin formulations were additive with one another ($P < 0.01$). Sublethal concentrations of MVP™ fed to larvae retarded the development of larvae and pupae for 5 d ($P < 0.01$) and 1.2 d ($P < 0.01$), respectively, and decreased pupal weight by 48 mg ($P < 0.01$). The spore formulation did not affect ($P > 0.05$) pupal weight or the life span of larvae or pupae. Median lethal concentrations of MVP™ and MYX™ were 6,904 and 7,561 × greater, respectively, in *S. frugiperda* than in *Pseudoplusia includens* (Walker). In *P. includens*, sublethal concentrations of MVP™, MYX™, and Dipel™ fed to larvae significantly ($P < 0.05$) reduced pupal weight and increased pupal life span compared to control insects. MVP™ and Dipel™ increased larval life span significantly ($P < 0.05$), but MYD™ did not.

KEY WORDS *Bacillus thuringiensis, Spodoptera frugiperda, Pseudoplusia includens, Bacillus thuringiensis* spore, *Bacillus thuringiensis* δ-endotoxin, *Bacillus thuringiensis* toxin-spore interaction.

Bacillus thuringiensis Berliner is an insect pathogen active mainly against Lepidoptera, Diptera, and Coleoptera (Beegle and Yamamoto 1992). The insecticidal property of *B. thuringiensis* is due mainly to proteins referred to as δ-endotoxins. Heimpel and Angus (1959) distinguished types of Lepidoptera susceptible to *B. thuringiensis* based on their susceptibility to the δ-endotoxins and spores of the bacterium. Certain lepidoptera are highly susceptible and

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are killed by δ-endotoxin alone, whereas other Lepidoptera are less susceptible and require the simultaneous presence of the toxin and the spore (Heimpel and Angus 1959).

Recent techniques have increased the ease of studying separate effects of spores and δ-endotoxins of *B. thuringiensis*. Three known crystal protein genes produce different molecules of δ-endotoxin in *B. thuringiensis* subsp. *kurstaki* (Höfte and Whiteley 1989). Toxins of four of these genes, CryIA(a), CryIA(b), CryIA(c), and CryIIA, are present in the formulation Dipel™ (Abbott Laboratories, North Chicago, IL) based on *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Marrone and MacIntosh 1993). The formulations MVP™ and MYX™ (Mycogen Corp., San Diego, CA) are based on CryIA(c) and CryIA(b), respectively, of *B. thuringiensis* subsp. *kurstaki* inserted into *Pseudomonas fluorescens* Migula, and the recombinant bacteria are then killed by heat and iodine (Gelernter 1990). Thus, each of these formulations is comprised of one δ-endotoxin and no spores. Many agronomically important lepidopterous pests are more sensitive to CryIA(c) than CryIA(a) or CryIA(b) δ-endotoxins (MacIntosh et al. 1990, Padidam 1992). The formulation MYD™ (Mycogen Corp., San Diego, CA) is a δ-endotoxin-minus mutant of *B. thuringiensis* (Gelernter W., pers. commun.); thus, it is comprised of spores with no δ-endotoxins.

Spodoptera frugiperda (J. E. Smith) is a polyphagous insect that ranks among the major pests of cereal and forage crops (Luginbill 1928). *Bacillus thuringiensis* has been evaluated for control of this insect, but *S. frugiperda* generally is not very susceptible to *B. thuringiensis* strains including *B. thuringiensis* subsp. *kurstaki* (Hernandez 1988), the strain used in most *B. thuringiensis* commercial products (Beegle and Yamamoto 1992).

The purpose of the current study was to take advantage of these spore and endotoxin formulations to assess the relative roles of *B. thuringiensis* spores and toxins in the mortality of the low-susceptibility insect *S. frugiperda*. Additionally, lethal and sublethal effects of *B. thuringiensis* were compared in *S. frugiperda* versus the highly susceptible *Pseudoplusia includens* (Walker).

Materials and Methods

Third instars of *S. frugiperda*, collected originally from rice, were used in bioassays. Until they pupated, larvae were reared individually in 30-ml plastic cups containing velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) artificial diet (Greene et al. 1976). Pupae were sexed, and 20 individuals of each sex were placed in 3.78-liter paper containers, covered with cheesecloth as an oviposition substrate. Adults of *S. frugiperda* were provided with a solution of 150 ml honey, 355 ml beer, 12 g ascorbic acid, and 150 ml distilled water. These insects were reared at approximately 27°C, 50-70% relative humidity (RH), and a photoperiod of 14:10 (L:D) h. Third instars of *P. includens*, a species susceptible to *B. thuringiensis*, also were bioassayed. These insects, originally collected from soybean, were reared similarly to *S. frugiperda*.

Bioassays. Dipel 2X™ (*B. thuringiensis* subsp. *kurstaki*), MVP™, MYX™, and MYD™ (δ-endotoxin-free spore formulation) were tested in the bioassays. Concentrations of toxins in MVP™ and MYX™ were not available for publication. Appropriate dilutions of each formulation in sterile distilled water

were added to cooling *A. gemmatalis* artificial diet. The treated diet then was poured into 30-ml plastic cups and allowed to dry for 24 h. One third instar of *S. frugiperda* or *P. includens* then was added to each cup. Controls were treated similarly except that the diet had no *B. thuringiensis* spores or toxins. The larvae that did not eat within 2 d were discarded. Insects were incubated at 27°C and checked daily for mortality for 14 d.

A preliminary experiment was established to estimate median lethal concentrations (LC_{50}) of the four formulations based on *B. thuringiensis* with the above bioassay method. *Spodoptera frugiperda* or *P. includens* were tested, with 27 to 51 third instars per dose per replication. Dipel™ was included in the preliminary assay to directly compare the susceptibility of *S. frugiperda* and *P. includens.*

There were two experiments to test the interactions between spores and δ-endotoxins. The first interactions experiment included single dosages of MVP™ MYX™ (selected at slightly below the LC_{50} in order to maximize the opportunity to detect synergism), two concentrations of MYD™, and combinations of these treatments. All nine treatments and a control were replicated three times, with 30 larvae per treatment per replication. The second interaction experiment was identical to the first except that there was only one concentration of MYD™, the concentrations of MVP™ and MYX™ were twice as high as in the first experiment (due to unexpectedly low mortality in the Experiment 1), and sublethal effects of the treatments were observed. Higher concentrations of MVP™ and MYX™ were tested in Experiment 2 than in Experiment 1 in order to determine interactions at higher mortality levels. In other words, the two experiments combined allowed for detection of all three interactions (antagonism, additivity, or synergism) between spores and δ-endotoxin. The six treatments and control in Experiment 2 were replicated four times with 30 larvae per treatment per replication.

For sublethal effects, larvae that survived beyond 14 d were checked daily until they died or pupated to determine their life span from the day of exposure to the *B. thuringiensis* formulations to their pupation. The insects that pupated were sexed and weighed on a Mettler H80 balance 5 d after pupation. The life span of pupae from day of pupation to adult emergence was recorded. Sublethal effects also were recorded for *B. thuringiensis*-based formulations in *P. includens.*

Statistical analysis. The preliminary dose-mortality results were analyzed with the probit option of POLO program (Russell et al. 1977) to determine the median lethal concentrations and associated statistics. The percentages of observed mortality were corrected with Abbott's (1925) formula. Replications at each dosage were treated as individual data points for preliminary analysis and then combined into single data points for the final estimate of the LC_{50}. No attempt was made to fit a log-dose-probit regression line to the preliminary data for MYD™ because it was clear that these data did not lend themselves to a dose-dependent regression analysis. Similarly, there were too few data points between 0 and 100% mortality to estimate a log-dose-probit regression line for Dipel™ against *S. frugiperda.*

The interactions among formulations based on *B. thuringiensis* were assessed based on the formula $E = O_a + O_b (1\text{-}O_a)$, where E is expected

proportion of mortality from combined agents and where O_a and O_b were the observed proportions of mortality due to each agent acting alone (Richter and Fuxa 1984). The expected percentage of mortality (E_1) and the observed percentage of mortality (O_1) for each combination of formulations were tested with the χ^2 statistic: the value of $(O_1\text{-}E_1)^2/E_1$ was compared with the χ^2 table value for df = 1 and $P < 0.05$ (McVay et al. 1977, Gardner et al. 1986).

The effects of *B. thuringiensis*-based formulations on larval life span, pupal weight, and pupal life span data were tested by analysis of variance (ANOVA), and comparisons among means were based on Tukey's Studentized Range (HSD) test (SAS Institute 1985).

Results

Preliminary tests. The preliminary results confirmed the relatively low susceptibility of *S. frugiperda* to certain formulations of *B. thuringiensis* compared with *P. includens* (Tables 1, 2). The LC_{50} of Dipel™ in *P. includens* was 42.7 µg/g diet, whereas 1,000 µg of Dipel™ per g diet killed only 12.5% of *S. frugiperda* larvae. The differences in susceptibility of the two species were even more pronounced with the MVP™ and MYX™ formulations (Table 2). The Dipel™ formulation was more virulent than MVP™ and MYX™ to third instars of *S. frugiperda* (Table 1). It was difficult to kill 50% of the *S. frugiperda* larvae with MVP™ and MYX™, and the spore formulation MYD™ did not kill more than 10% or give a dose response.

Non only did the *B. thuringiensis* formulations cause low rates of mortality in *S. frugiperda*, but the results also were inconsistent in this insect. For example, MYX™ applied at the lower concentrations of 422 and 633 µg/g diet killed 3% of the larvae, whereas none were killed at concentrations two to eight times higher (1,056 to 5,012 µg/g) (Table 1). The LC_{50} of MYX™ in *S. frugiperda* had to be extrapolated (Tables 1, 2).

Interaction experiments. The interaction between MYD™ and MYX™ was antagonistic at the MYX™ concentration of 117,500 µg/g diet (Experiment 1) and additive at the MYX™ concentration of 235,000 µg/g diet (Experiment 2) (Table 3). The combination of MVP™ + MYX™ was additive in both experiments. The combination of MVP™ + MYD™ was additive at the MVP™ concentration of 117,500 µg/g diet and synergistic at the MVP™ concentration of 235,000 µg/g diet (Table 3).

Susceptibility of *S. frugiperda* to the *B. thuringiensis* formulations was so low and variable that it proved difficult to kill approximately 40 to 50% of the larvae, the target level of mortality with single-agent treatments. In Experiment 1, mortality ranged from 3.4-13.5% for the spore and the two δ-endotoxin formulations tested individually (Table 3); therefore, dosages were increased in Experiment 2. Mortality was almost 100% with the δ-endotoxin formulation MYX™ in Experiment 2 but was still only 0.1-4.2% with MYD™ and MVP™, respectively.

In Experiment 2, some larvae survived after 14 d of exposure to *B. thuringiensis* but died before or during pupation. Four treatments (Control, MVP™, MYD™, MVP™ + MYD™) had a sufficient number of survivors that became adults to monitor sublethal effects of *B. thuringiensis* formulations

Table 1. Virulence of DIPEL™ two formulations (MVP™, MYX™) based on δ-endotoxins of *Bacillus thuringiensis* subsp. *kurstaki*, and a δ-endotoxin-free mutant spore formulation (MYD™) of *Bacillus thuringiensis* in third instars of *Spodoptera frugiperda*.

Concentration (μg/of diet)	% Mortality (n)
Dipel™	
835	9.7 (41)
1,000	12.5 (40)
5,000	83.3 (36)
7,500	100.0 (39)
Control	0 (40)
MVP™	
21	0 (40)
42	2.5 (40)
64	2.5 (40)
85	0 (40)
107	0 (40)
50,000	18.4 (38)
50,000	29.7 (37)
75,000	27.5 (51)
75,188	34.3 (35)
100,000	33.3 (39)
100,000	41.5 (41)
125,000	55.0 (40)
Control	0 (40)
MYX™	
105	0 (28)
211	0 (30)
422	3.3 (30)
633	3.4 (29)
1,056	0 (33)
2,374	0 (27)
2,506	0 (27)
5,012	0 (40)
10,025	5.0 (40)
20,050	2.5 (40)
30,075	2.5 (40)
62,000	18.9 (37)
62,657	14.6 (41)
75,000	30.9 (42)
75,188	26.3 (38)
100,000	36.0 (47)
Control	0 (40)
MYD™	
100	10.0 (40)
1,000	10.0 (40)
1,000	5.0 (40)
3,000	0 (40)
5,000	0 (40)
7,500	2.5 (40)
10,000	0 (36)
Control	0 (37)

Table 2. Log-dose-probit parameters of *Bacillus thuringiensis* formulations in third instars of *Spodoptera frugiperda* and *Pseudoplusia includens*.

Formulation	Slope ± SE	LC_{50}*	95% FL*	χ^2
		S. frugiperda		
MVP™	0.64 ± 0.09	338,290	153,190-1,348,100	12.626
MYX™	1.01 ± 0.17	424,910	138,770-76,779,000	53.406
		P. includens		
Dipel™	1.53 ± 1.61	42.7	34.9-53.7	16.659
MVP™	1.74 ± 0.32	49.0	41.9-59.6	3.721
MYX™	2.53 ± 0.24	56.2	50.9-62.1	8.906

* µg of the formulation per g of velvetbean caterpillar diet.

(Table 4). The MVP™ and MVP™ + MYD™ treatments of larvae significantly ($P < 0.01$) increased larval and pupal life span and decreased pupal weight compared to controls. The MYD™ treatments did not significantly ($P < 0.01$) affect life span or weight. Sublethal effects in *P. includens* were similar to *S. frugiperda* (Table 4). The two toxins and Dipel™ fed to larvae decreased pupal weight and increased larval and pupal life spans ($P < 0.05$) compared to controls, except that the increase in larval life span in the MYX™ treatment was not significant.

Discussion

The interactions among *B. thuringiensis* toxins and spores in *S. frugiperda* were mixed and dose-dependent (Table 3). The MVP™ toxin, CryIA(c), was additive with spores at the low concentration and synergistic at the high concentration. The MYX™ toxin, CryIA(b), was antagonistic with spores at the low concentration and additive at the high concentration. The two toxins were

Table 3. Interactions of two δ-endotoxin formulations (MVP™, MYX™) of *Bacillus thuringiensis* subsp. *kurstaki* and spores (MYD™) of a toxin-free mutant in third instars of *Spodoptera frugiperda*.

Treatment*	Observed % mortality (n) ± SE	Expected % mortality	χ^{2}**	Response
	Experiment 1†			
MVP™	9.0 (90) ± 1.3			
MYX™	13.5 (90) ± 1.4			
MYD^{TM}_{1}	3.4 (90) ± 1.6			
MYD^{TM}_{5}	4.5 (90) ± 2.1			
MVP™ + MYX™	23.6 (90) ± 0.5	21.3	0.25	additivity
MVP™ + MYD^{TM}_{1}	12.4 (90) ± 1.9	12.1	0.01	additivity
MVP™ + MYD^{TM}_{5}	16.9 (90) ± 3.3	13.1	1.10	additivity
MYX™ + MYD^{TM}_{1}	5.6 (90) ± 1.9	16.4	7.11	antagonism
MYX™ + MYD^{TM}_{5}	9.0 (90) ± 1.3	17.4	4.06	antagonism
Control	1.1 (90) ± 0.5			
	Experiment 2‡			
MVP™	4.2 (120) ± 0.4			
MYX™	98.3 (120) ± 0.4			
MYD^{TM}_{1}	0.1 (118) ± 0.4			
MVP™ + MYX™	99.2 (120) ± 0.4	98.4	0.01	additivity
MVP™ + MYD^{TM}_{1}	17.7 (120) ± 1.5	4.3	41.76	synergism
MYX™ + MYD^{TM}_{1}	79.0 (120) ± 6.0	98.3	3.79	additivity
Control	0.8 (120) ± 0.4			

* MYD^{TM}_{1} = 1,000 μg spore formulation per g artificial diet. MYD^{TM}_{5} = 5,000 μg spore/g.

** Calculated $\chi 2$ compared with table $\chi 2 = 3.84$ (df = 1; P = 0.05).

† Toxin (MVP™, MYX™) concentrations = 117,500 μg per g artificial diet. Three replications with 30 insects per treatment per replication.

‡ Toxin (MVP™, MYX™) concentration = 235,000 μg per g artificial diet. Four replications with 30 insects per treatment per replication.

additive with one another. In research of other insect species, *B. thuringiensis* toxins were synergistic in a mosquito but not in seven species of Lepidoptera (Tabashnik 1992). Thus, the tendency toward a positive interaction (additivity or synergism) between spores and toxin increased as dosage of the toxin increased. In Experiment 1, MVP™ was the more effective toxin with spores whereas MYX™ was the more effective toxin without spores; this is similar to research of *Choristoneura fumiferana* (Clemens), in which the presence or absence of spores changed relative toxicity of two δ-endotoxins (Milne et al. 1990).

Table 4. Effect of sublethal doses of δ-endotoxin (MVP™, MYX™), spores (MYD™), and Dipel™ on the development of larvae and pupae of *Spodoptera frugiperda* and *Pseudoplusia includens*.

Treatment*	Mean** (n) ± SE	
	S. frugiperda	*P. includens*
	Larval Life Span (days)†	
CONTROL	10.8 (118) ± 1.0 a	15.5 (49) ± 0.4 a
MYD™	12.0 (109) ± 1.0 a	
MVP™	15.8 (70) ± 2.2 b	18.3 (50) ± 0.2 b
MVP™ + MYD™	15.9 (54) ± 2.3 b	
MYX™		16.1 (49) ± 0.2 a
DIPEL™		18.4 (47) ± 0.3 b
	Pupal Life Span (days)‡	
CONTROL	11.5 (118) ± 0.8 a	7.4 (49) ± 0.2 a
MYD™	11.5 (109) ± 0.7 a	
MVP™	12.7 (70) ± 1.1 b	9.2 (48) ± 0.2 b
MVP™ + MYD™	12.8 (54) ± 1.2 b	
MYX™		9.6 (49) ± 0.1 b
DIPEL™		12.0 (47) ± 0.4 c
	Pupal Weight (mg)§	
CONTROL	202 (118) ± 20 a	244 (49) ± 7 c
MYD™	202 (109) ± 22 a	
MVP™	154 (70) ± 21 b	195 (50) ± 5 ab
MVP™ + MYD™	156 (54) ± 20 b	
MYX™		215 (49) ± 5 b
DIPEL™		176 (47) ± 6 a

* MYD™ = 1,000 µg spore formulation per g artificial diet; MVP™ (*S. frugiperda*) = 235,000 µg formulation per g diet; MVP™ (*S. includens*) = 49 µg formulation per g diet; MYX™ = 56 µg formulation per g diet; Dipel™ = 41 µg formulation per g diet.

** Means within each experimental category and insect species, followed by the same letter, are not significantly different ($P = 0.01$; Turkey's Studentized Range [HSD] test [SAS Institute 1985]).

† *S. frugiperda*: $F = 190.60$, df = 3, $P < 0.0001$; *P. includens*: $F = 31.20$, df = 3, $P < 0.0001$.

‡ S. frugiperda: $F = 67.57$, df = 3, $P < 0.0001$; *P includens:* $F = 65.45$, df = 3, $P < 0.0001$.

§ Weight of 5-day-old pupae; *S. frugiperda:* $F = 113.73$, df = 3, $P < 0.0001$; *P. includens:* $F = 23.71$, df = 3, $P < 0.0001$.

It is possible, though unlikely, that the mutant, toxin-free spores (MYD™ formulation) contained small amounts of δ-endotoxin in the spore coats. Even if this were the case, the amounts undoubtedly were too small to affect the interaction results, particularly in view of the lack of response to MYD™ and the large doses of δ-endotoxin necessary to affect *S. frugiperda* (Table 1).

The current results can be compared to those with *Galleria mellonella* (L.) (Burges et al. 1976). *Galleria mellonella* was 10 times more susceptible to a 1:1 combination of spores and δ-endotoxin than to spores alone; δ-endotoxin alone was 10^4 times less potent than the 1:1 spore-crystal complex (Burges et al. 1976). In another study, *G. mellonella* was sensitive to 1:1 mixtures of spores and δ-endotoxin, while spores coated with toxin or crystal toxins mixed with small amounts of spores were moderately potent (Li et al. 1987). As in the current study, there also have been inconsistent results with *G. mellonella*. Nishiitsutsuji-Uwo and Endo in 1981 (cited in Li et al. 1987) reported that purified δ-endotoxins were almost as potent in *G. mellonella* as a 1:1 spore-toxin mixture. Their purified toxin was derived from oligosporogenous mutants of *B. thuringiensis* and therefore carried few spores.

The δ-endotoxin (MVP™) and toxin plus spores (MVP™ + MYD™) but not spores alone (MYD™) inhibited the development of third instars of *S. frugiperda* (Table 4). Similarly, MVP™, MYX™, and Dipel™ inhibited the development of *P. includens* (Table 4). Thus, the low-susceptibility and high-susceptibility insects exhibited similar sublethal effects after exposure to *B. thuringiensis*. Retardation of larval development due to intoxication with *B. thuringiensis* can result from anorexia subsequent to the destruction of the midgut epithelium (Heimpel and Angus 1959). Retarded development of lepidopterous larvae fed *B. thuringiensis* toxins has been observed previously in *S. frugiperda* (Hernandez 1988) as well as other insects (Devriendt and Martouret 1976, Salama and Sharaby 1988, Ramachandran et al. 1993). Retarded development of pupae after larval ingestion of *B. thuringiensis* was reported in *Agrotis ypsilon* (Hüfnagel) (Salama and Sharaby 1988). The weight of pupae of *A. ypsilon* exposed as larvae to sublethal doses of *B. thuringiensis* was significantly decreased (Salama and Sharaby 1988). However, pupal *C. fumiferana* survivors of *B. thuringiensis* did not suffer weight loss even though larvae took longer to pupate than control insects (Ramachandran et al. 1993). Intoxicated larvae were able to recover quickly from gut damage and regain normal weight after a prolonged feeding period (Ramachandran et al. 1993).

Thus, *S. frugiperda* is not very susceptible to standard formulations of *B. thuringiensis* and is not the ideal insect to control with Dipel™ or, particularly, the products MVP™ or MYX™. The results indicated that at least one δ-endotoxin could be synergized by *B. thuringiensis* spores at certain dosages, but not to a degree that would be helpful in microbial control.

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