Effect of Host Diet and Insect Source on Synergy of Gypsy Moth (Lepidoptera: Lymantriidae) Mortality to *Bacillus thuringiensis* subsp. *kurstaki* by Zwittermicin A

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ABSTRACT Zwittermicin A acts synergistically with the insecticidal activity of *Bacillus thuringiensis* subsp. *kurstaki* Berliner against gypsy moth (*Lymantria dispar* (L.)) larvae. The objective of this study was to assess the influence of insect source and diet on this synergy. Zwittermicin A increased the mortality caused by *B. thuringiensis* subsp. *kurstaki* in gypsy moths collected from four population sources feeding on artificial diet, and on larvae feeding on four tree species, in a dose-dependent manner. Zwittermicin A did not cause mortality of *L. dispar* when applied alone. The ability of zwittermicin A to act synergistically with *B. thuringiensis* subsp. *kurstaki* did not differ greatly among these four populations, although mortality was slightly lower in a field-collected population from Michigan. Zwittermicin A increased the activity of *B. thuringiensis* subsp. *kurstaki* on *L. dispar* feeding on white oak, aspen, larch, and willow. Larval mortality was directly proportional to the concentration of zwittermicin A applied to foliage, although the synergistic effect of zwittermicin A differed among host species. These results suggest strategies for employing synergists in the application and resistance management of microbial pesticides.

KEY WORDS *Bacillus thuringiensis*, zwittermicin A, *Lymantria dispar*, gypsy moth, synergism, host plant effects

THE GYPSY MOTH, *Lymantria dispar* L., is an invasive species affecting deciduous forests of the eastern and north-central United States. It remains a major pest despite the implementation of extensive management programs throughout the region (Montgomery and Wallner 1989). *Bacillus thuringiensis* subsp. *kurstaki* Berliner, a bacterial insecticide, is one of the most commonly used agents for controlling gypsy moth populations (Reardon et al. 1994, Prieto-Samsonov et al. 1997, Liebhold and McManus 1999), especially in areas of new expansion that lack natural enemies and have larval densities too low for effective control with other pathogens (Frederici and Maddox 1996). One problem affecting the use of *B. thuringiensis* subsp. *kurstaki* is variation in field efficacy, which can be influenced by weather, application patterns, and the age of larvae. In addition, plant chemistry and larval genotypic variation may influence susceptibility of gypsy moth larvae to *B. thuringiensis* subsp. *kurstaki* (Rossiter et al. 1990, Farrar et al. 1996).

Plants produce many secondary compounds that confer protection against herbivore feeding (Karban and Baldwin 1997). In addition to their direct effects on herbivores, these compounds may interact with bacterial, fungal, and viral pathogens of herbivores. Secondary plant metabolites have shown both synergistic and antagonistic effects on toxicity of *B. thuringiensis* subsp. *kurstaki* against the gypsy moth. For example, oak (*Quercus* spp.) tannins, and conifer terpenoids can decrease toxicity of *B. thuringiensis* subsp. *kurstaki* against gypsy moths (Barbosa 1988, Appel and Schultz 1994, Farrar et al. 1996). In contrast, activity against larvae feeding on aspen (*Populus* spp.) increased with increasing foliar levels of phenolic glycosides (Hwang et al. 1995). Secondary metabolites have been hypothesized to be responsible for the relatively low activity of *B. thuringiensis* subsp. *kurstaki* against gypsy moth larvae on willow (*Salix* spp.) (Farrar et al. 1996).

We previously showed that zwittermicin A, an antibiotic produced by *Bacillus cereus* (French & French) (He et al. 1994, Stabb et al. 1994), is a synergist of *B. thuringiensis* subsp. *kurstaki* activity against gypsy moths (Broderick et al. 2000). Zwittermicin A causes no mortality to gypsy moth larvae alone, but increases mortality twofold when as little as 350 pg is applied with 0.65 IU of *B. thuringiensis* subsp. *kurstaki*. These observations may have implications for developing strategies to improve management of various forest and agricultural pests, identifying new targets for *B. thuringiensis* subsp. *kurstaki* toxicity, and designing resistance management strategies. However, the extent to which this laboratory-based synergy can

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be extrapolated to natural conditions has not been tested previously.

Although genetic variability affects *B. thuringiensis* subsp. *kurstaki* efficacy among other agricultural lepidopteran populations (Tabashnik 1994) and some forest Lepidoptera, such as the spruce budworm *Choristoneura fumiferana* (Clemens) (Tortricidae) (van Frankenhuyzen et al. 1995, Kouassi et al. 2001), few studies have examined variation in susceptibility among gypsy moth populations. Moreover, susceptibility of larvae raised in laboratory cultures may not be predictive of field-collected populations because of selection of suboptimal genotypes through inbreeding or other culture conditions (Rossiter et al. 1990).

The current study considered: 1) whether the synergy between zwittermicin A and *B. thuringiensis* subsp. *kurstaki* is effective with field populations of gypsy moths, and if so, if it varies among population sources? and 2) whether synergy occurs when larvae feed on host foliage, and if so, whether this varies among tree species?

Materials and Methods

Comparison of Populations. Gypsy moth egg masses were obtained from culture NJSS at the USDA-APHIS laboratory at the OTIS Air National Guard Base, Cape Cod, MA (MA Culture) and the Beneficial Insects Introduction and Research Laboratory, USDA-ARS, Newark, DE (DE Culture). Egg masses were collected from field sites in Wisconsin (WI Field) and Michigan (MI Field). Wisconsin egg masses were collected on 24 March 1999 from five trees (four oak (*Quercus* sp. L.) and one maple (*Acer* sp. L.)) on a rural property in York Township, Dane County, R11E, T9N. The Michigan egg masses were collected on 23 March 1999 from a newly established population in a mixed oak/aspen (*Quercus* sp./*Populus* sp.) stand in Lincoln Township, Clare County, R5W, T18N. Field-collected egg masses were kept in plastic bags at 4°C from the time of collection until use in assays. Assays were timed to coincide with larval emergence in Wisconsin (approximately 5 May 1999).

Gypsy moth larvae were reared as described in Broderick et al. (2000). Briefly, egg masses were surface-sterilized with a solution of Tween 80 (polyoxyethylene sorbitan monooleate), bleach, and distilled water. Larvae were reared in 17-cm Petri dishes on an artificial diet (USDA Formula; Bioserve, Frenchtown, NJ) under a 16:8 (L:D) photoperiod at 25°C in quarantine facilities at the University of Wisconsin-Madison. Larvae were provided with artificial diet, which was replaced every 48 h.

Larvae were starved for 24 h after they molted to the third instar. We placed single larvae (10–20 mg) in cells ($4 \times 2.5 \times 1.5$ cm) of rearing trays covered with mylar. A constant dose of 0.65 IU of *B. thuringiensis* subsp. *kurstaki* Foray 76B per larva (Valent Biosciences, Chicago, IL) was used, which was previously demonstrated to cause 25% mortality to third instar gypsy moths (Chenot and Raffa 1998, Broderick et al. 2000). Zwittermicin A was purified as described in Silo-Suh et al. (1998) and four doses of zwittermicin A (0.00095, 0.35, 1.4, and 5.0 ng) were tested on larvae from each population. These doses previously caused 25, 50, 75, and 95% mortality in third instar gypsy moths when combined with 0.65 IU of *B. thuringiensis* subsp. *kurstaki*, respectively (Broderick et al. 2000). Three control treatments accompanied these combinations: 0.65 IU *B. thuringiensis* subsp. *kurstaki* Foray 76B alone, 1.4 ng zwittermicin A alone, and a blank control. Each dose was tested in quadruplicate, with each sample consisting of 30 newly molted third instars. All treatments were applied in a volume of 1 μl to a standard diet disk (4-mm diameter, 1-mm height) and fed to the larvae on two consecutive days, after which larvae were fed untreated diet. Mortality was recorded every 24 h for 5 d.

Effects of Host Plant Species. Gypsy moth neonates from culture NJSS (MA Culture) were provided with an artificial diet for 24 h after emergence, and then divided into 40 groups of 200 larvae each. Ten groups of larvae were transferred to foliage from each of four tree species. Whole leaves, collected from 10 trees of each species, were placed in Petri dishes lined with a piece of moistened filter paper. The petioles were inserted in water-filled microcentrifuge tubes to prevent desiccation. Foliage was replaced every 48 h until the larvae were used for experiments.

Two-year-old white oak (*Quercus alba* L.), larch (*Larix laricina* (Du Roi) K. Koch), and quaking aspen (*Populus tremuloides* Michaux) trees were obtained from the Wisconsin Department of Natural Resources Nursery, Hayward, WI. Two-year-old scrub willow (*Salix fragilis* L.) trees were obtained from the Iowa State Nursery, Ames, IA. The trees were chilled at 4°C for 20 d to ensure good bud development. After the chilling period, the trees were planted in 12-l pots in Sunshine LC1 (Sungro Horticulture, Bellevue, WA) and flood irrigated. The trees were grown in a greenhouse at 25°C under a 16:8 (L:D) photoperiod. Trees were watered every 5 d until bud development, after which they were watered every 2–3 d.

Foliage treatments were assayed using 10 trees for each species. Assays were performed as described above, except that two levels of zwittermicin A (0.35 and 1.4 ng) were applied to leaf disks (4-mm diameter) and larvae were transferred to untreated foliage on day 3. In addition, the initial level of 0.65 IU *B. thuringiensis* subsp. *kurstaki* Foray 76B resulted in 100% mortality for larvae feeding on aspen, so the dosage was reduced to 0.16 IU to provide a level of mortality similar to other foliage treatments ($\approx$25%). A piece of filter paper (2.5 cm^2) was placed in the bottom of each assay cell and remoistened daily to retain leaf disk moisture.

Statistical Analysis. A quadratic regression of percent mortality versus a natural log transformation of zwittermicin A (ng), was fit using Minitab (Minitab 1995), as in Broderick et al. (2000). An imputed value for zero of 10^{-4} was used for the *B. thuringiensis* subsp. *kurstaki* control treatment with no antibiotic added.

The LD_{50} and LD_{95} values denote the amount of purified zwittermicin A added to 0.65 IU *B. thurin-*

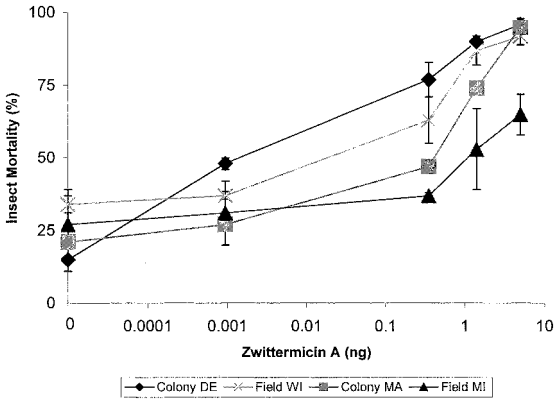

Fig. 1. Effect of zwittermicin A on *B. thuringiensis* subsp. *kurstaki* (Btk) toxicity to third instar *Lymantria dispar* from four insect sources. Each point represents the mean mortality ± SEM of a total of 120 larvae (four replications with 30 larvae each).

giensis subsp. *kurstaki* needed to cause 50 and 95% mortality, respectively. Standard error was determined using PROC MEANS (SAS Institute 1990).

Synergy ratios of larvae feeding on various foliage sources were calculated as:

$$\frac{\%\ \text{Mortality [Btk + 1.4 ng ZmA]}}{\%\ \text{Mortality (Btk)} + \%\ \text{Mortality (1.4 ng ZmA)}}$$

Larval mortalities were analyzed by PROC GLM using feeding substrate as a treatment. Where significant treatment effects were observed ($P < 0.05$), means were separated using Fisher protected least significant difference (LSD) (SAS Institute 1990). Mean synergy ratios were analyzed, and separated where appropriate, by the same procedures.

Results

Effects of Gypsy Moth Population Source on Mortality Caused by *B. thuringiensis* subsp. *kurstaki* and Zwittermicin A. The addition of zwittermicin A significantly increased the mortality associated with feeding on *B. thuringiensis* subsp. *kurstaki* for all four populations of gypsy moths (Fig. 1; Table 1). Larvae from colony populations (MA and DE) and the WI field population exhibited strong increases in mortality related to increasing doses of zwittermicin A. Mortality of these populations did not differ significantly at the highest dose of zwittermicin A (5 ng), although they differed across the intermediate zwittermicin A doses (Fig. 1). The MI field population exhibited less synergy by zwittermicin A of *B. thuringiensis* subsp. *kurstaki.* Zwittermicin A caused no mortality (range, 0–3.3%), relative to controls on any population.

Table 1. Regression analysis of mortality of third instar *Lymantria dispar* from field and colony sources

Population	*n*	F	df	*P*	LC_{50} (ng)[a]	LC_{95} (ng)[a]
Colony OT	120	138.69	19	<0.0001	0.2100	7.18
Colony DE	120	183.65	19	<0.0001	0.0014	3.69
Field WI	120	30.64	19	<0.0001	0.0380	5.20
Field MI	120	7.53	19	0.013	0.8550	251.40

[a] LC_{50} and LC_{95} values indicate the quantity of zwittermicin A required to increase *B. thuringiensis* subsp. *kurstaki* toxicity to 50 and 95%, respectively.

Effects of Diet on Mortality by *B. thuringiensis subsp. kurstaki* and Zwittermicin A. Diet had a significant effect on susceptibility of larvae to *B. thuringiensis* subsp. *kurstaki* (Table 2). Mortality was highest among larvae feeding on aspen, with 0.65 IU *B. thuringiensis* subsp. *kurstaki* resulting in 100% mortality. Therefore we applied a lower dose of *B. thuringiensis* subsp. *kurstaki* (0.16 IU) to provide a level of mortality similar to the other diet treatments (≈20% mortality). The mortality caused by *B. thuringiensis* subsp. *kurstaki* on larvae feeding on artificial diet, white oak, and larch was similar (≈20%). Larvae feeding on willow exhibited the lowest mortality by *B. thuringiensis* subsp. *kurstaki* (10%) (Table 2).

Larval diet also had a significant effect on the synergy of *B. thuringiensis* subsp. *kurstaki* by zwittermicin A (Table 2). The synergy ratios were similar for larvae fed on larch, white oak, and artificial diet. The synergy ratio was significantly higher on larvae-fed willow and aspen. Zwittermicin A did not affect mortality in the absence of *B. thuringiensis* subsp. *kurstaki* (range, 0–2%) for any of the diet treatments.

Discussion

These results demonstrate synergy by zwittermicin A of the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* on gypsy moth larvae from different populations. This synergism is equivalent among larvae from field and colony populations fed an artificial diet. These results also demonstrate that zwittermicin A synergy of *B. thuringiensis* subsp. *kurstaki* varies when larvae feed on foliage of different host plants. To the best of our knowledge, this is the first description of a synergist's potentiation of a microbial pesticide being mediated by host plants.

These experiments suggest the potential for zwittermicin A or other synergists to increase *B. thuringiensis* subsp. *kurstaki* activity to desired levels of pest control. However, substantial testing on human health, environmental, and economic attributes are required before implementation can be assessed. We observed the highest synergy on the plant, willow, which yielded the highest insect tolerance to *B. thuringiensis* subsp. *kurstaki.* Similarly, synergists of synthetic pesticides typically yield the highest synergy ratios against the most resistant insect strains (Raffa and Priester 1985).

The high synergy observed on willow is of particular interest because of the relatively low toxicity of *B. thuringiensis* subsp. *kurstaki* against gypsy moth feeding on this species (Farrar et al. 1996). First, much of the emphasis in gypsy moth management is on slowing spread into new areas and persistence of local populations on willow is a significant impediment

Table 2. Effect of host diet on susceptibility of third instar gypsy moth to *B. thuringiensis* subsp. *kurstaki* (Btk) and zwittermicin A (ZmA)

Diet	Larval mortality (%):					Synergist ratio
	No treatment	1.4 ng ZmA	0.65 IU Btk	Btk + 0.35 ng ZmA	Btk + 1.4 ng Zm A	
Artificial diet	0a	0a	23b	50a	75b	3.27b
White Oak	0a	2a	19b	36b	60d	3.14b
Larch	0a	0a	20b	30c	65c	3.31b
Willow	0a	0a	10a	20d	40e	4.00a
Aspen	0a	1a	100c	–	–	–
Aspen	0a	1a	25b[a]	36b[a]	98a[a]	3.92a[a]
F	–	0.75	155.49	181.57	964.98	30.56
df	4	4	4	4	4	4
P	–	0.5632	<0.0001	<0.0001	<0.0001	<0.0001

Larval mortalities were analyzed by PROC GLM using feeding substrate as a treatment. Means for larval mortality and synergy ratios were separated using Fisher's protected LSD. Values followed by the same letter within a column do not differ significanlty ($P < 0.05$).

[a] Btk level used = 0.16 IU, to provide mortality similar to other diet treatments; 0.65 IU could not be used with aspen because this level resulted in 100% mortality.

(Sharov et al. 2002). Second, the gypsy moth exert most of its economic impact in residential areas in which the willow is an important ornamental tree. Third, willow has potential as a biofuel tree and already contributes to a large percentage of energy production in some European countries (Börjesson 1996). Using trees, such as the willow, as alternatives to fossil fuels requires the growth of trees in plantation settings in which damage by insect pests is especially problematic and often requires application of microbial pesticides for control (Anderson et al. 1983).

The relationship among *B. thuringiensis* subsp. *kurstaki*, zwittermicin A, insect herbivores, and host plants provides a useful model for studying events mediated through an animal gut, particularly those affected by microbial presence (Broderick 2001). The system offers an opportunity to study the interactions among gut microbiota, antibiotics, and host plant chemistry, which may have parallels with interactions that determine human intestinal health.

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