COMPATIBILITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* WITH THE FUNGAL PATHOGEN *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES)

SERGIO ORDUZ[1] AND R. C. AXTELL

Department of Entomology, North Carolina State University, Raleigh, NC 27695-7613

ABSTRACT. Larvae of *Culex quinquefasciatus* were exposed to infection by *Lagenidium giganteum* and various concentrations of *B.t.i.* or *B. sphaericus*. The resulting larval mortalities, percentages of infected dead larvae and percentages of larval body regions containing the fungus were compared. Overall, the effectiveness of *Lagenidium giganteum* against the larvae was not significantly affected by the presence of *B.t.i.* or *B. sphaericus*, and the fungal and bacterial agents were compatible. In experiments using 3-day-old larvae, the extent of growth of the fungus in the infected larvae and the percentage of the larvae infected were related to the concentration of *B.t.i.* in the range of 0.057–0.456 ITU/ml tested but were not related to the concentration of *B. sphaericus* in the range of 0.6–4.8 $\times$ 10^4 spores/ml. With larvae of various ages treated with a low concentration of *B.t.i.* (0.114 ITU/ml), exposure to the fungus increased the mortality rate in early but not late instars. After single and multiple applications of *B.t.i.* and *B. sphaericus* in the presence of the fungus, followed by drying and reflooding, the fungus persisted and reinfected larvae while the *B. sphaericus* persisted but the *B.t.i.* did not.

INTRODUCTION

The bacterium *Bacillus thuringiensis* var. *israelensis* de Barjac (*B.t.i.*) is widely used as a mosquito larvicide and *B. sphaericus* Neide is being developed for similar uses (Davidson and Sweeney 1983, Chapman 1985, Lacey and Undeen 1986, Lacey and Lacey 1990, Pantuwatana and Sattabongkot 1990). Another microbial agent, the fungus *Lagenidium giganteum* Couch, is a promising agent for control of mosquito larvae (Chapman 1985, Lacey and Undeen 1986, Lacey and Lacey 1990, Guzman and Axtell 1987a, 1987b; Kerwin and Washino 1988). The compatibilities of these bacterial agents with the use of *L. giganteum* for mosquito control are unknown. With the potential for wider use of these biological control agents in integrated mosquito management programs (Axtell 1979), the likelihood of sequential treatments of mosquito-breeding habitats with these agents will increase. We evaluated the effects of varied combinations of treatments of *Culex quinquefasciatus* Say larvae with *B.t.i.*, *B. sphaericus* and *L. giganteum* on larval mortalities and growth of *L. giganteum* in larvae.

MATERIALS AND METHODS

Bacillus thuringiensis var. *israelensis* (*B.t.i.*) was formulated as Vectobac® AS (Lot no. 75-018-BA, Abbott Laboratories) and contained 600 International Toxic Units (ITU) per milligram (equivalent to 5.76 $\times$ 10^8 ITU per liter). *Bacillus sphaericus* strain 1593 (BS-1593, Lot No. 84-929 BD, Abbott Laboratories) was a technical powder containing 3 $\times$ 10^{10} spores per gram.

Lagenidium giganteum was the California (CA) isolate derived from a culture obtained from J. Kerwin, University of California, Davis. The isolate is also available from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852) as accession no. 52675. The fungus was routinely maintained on agar containing sunflower seed extract (SFE) as previously described (Jaronski and Axtell 1984, Guzman and Axtell 1986, Axtell and Guzman 1987).

The experiments were conducted at 25 ± 2°C using larvae of *Cx. quinquefasciatus* from a 2-year-old colony derived from egg rafts collected in the vicinity of Raleigh, NC. The mosquitoes were maintained at 27 ± 2°C under a 16:8 L:D photoperiod. Preliminary tests were conducted to determine the dose-response for larvae exposed to different concentrations of *B.t.i.* and *B. sphaericus* to select appropriate treatment rates to use in bioassays to give a range of mortalities. These tests were conducted with 100 ml of water and 20 3-day-old larvae in each bioassay dish (8 cm diam). There were 12 dishes per treatment (4 tests on 3 different days) and 5 concentrations of *B.t.i.* and 6 dishes per treatment (2 tests on 2 different days) and 4 concentrations of *B. sphaericus*. The pooled data on larval mortality 24 h after treatment were used in probit analysis (PROC PROBIT, SAS Institute 1982). Concentrations of *B.t.i.* were expressed in ITU to facil-

[1] Current address: Biological Control Section, Corporacion para Investigaciones Biologicas, Medellin, Colombia.

itate comparisons with other formulations having different potencies.

Experiment 1: Bioassays were used to determine the effects of various concentrations of *B.t.i.* or *B. sphaericus* on 3-day-old larvae previously exposed to fungal cultures on SFE-agar discs. Twenty larvae were placed in 100 ml of deionized water in plastic dishes (8 cm diam) and 0.25 ml of a 3.5% (w/v) liver powder slurry was added as food. A 20-mm^2 SFE-agar disc from a fungus culture plate (5–7 days old) having uniform growth was placed in each dish as described by Guzman and Axtell (1986). Zoosporogenesis from these agar discs was observed to occur in 5–8 h after immersion in the water. Within 2 h after adding the fungus to the dishes, an appropriate amount (less than 1 ml) of the *B.t.i.* or *B. sphaericus* stock preparation (in water) was added to each dish to yield the desired final concentration.

There were 9 bioassay dishes per treatment (3 replicates each day; 3 different days) for *B.t.i.* and *B. sphaericus.* The treatments included combinations of one of the bacillus species with the fungus, the fungus alone, each bacillus species alone, and untreated controls (no fungus or bacteria). The percentage mortalities of the mosquito larvae were determined at 24 and 48 h after exposure. At 48 h, 10 dead larvae from each of the dishes inoculated with *L. giganteum* were examined microscopically to calculate the infection rate by the fungus. For each infected larva, the proportion of the body that was infected with the fungus was estimated by the presence or absence of mycelium in each of 5 regions of the body including the head, thorax, abdominal segments 1–5, abdominal segments 6–7 and the posterior abdominal segments including their associated structures (i.e., the respiratory tube and anal gills). The data on mortalities and infection rates were analyzed by ANOVA and means compared by Tukey's HSD test to determine significant differences ($P \leq 0.05$) using SAS procedures (PROC ANOVA, PROC MEANS/TUKEY, SAS Institute 1982).

Experiment 2: Larvae of different ages (1- to 5-day-old) were treated with a single concentration of *B.t.i.* (0.114 ITU/ml) with and without exposure to fungal cultures on SFE-agar discs. The procedures were the same as in experiment 1 with 9 dishes per treatment for each age group.

Experiment 3: The bioassay procedure was modified to evaluate the effects of *B.t.i.* on 3-day-old larvae that were previously infected by zoospores of the fungus rather than by the agar disc method used for experiments 1 and 2. There were 3 dishes (20 larvae/dish) for each treatment. Larvae were infected by exposing them to high concentrations of zoospores (ca. 1,200 zoospores/ml) for 1 h, removing and rinsing the larvae with deionized water and placing the larvae in the bioassay dishes. To achieve the high concentration of zoospores, two SFE-agar plates (10 cm diam) of the fungus were placed in 1 liter of deionized water. The water was monitored by examining aliquots microscopically. When abundant zoospores were detected, the plates were removed and all of the larvae to be used in the experiment were placed in the water.

Experiment 4: The effects of single and multiple field applications of *B.t.i.* (0.154 ITU/ml) or *B. sphaericus* (5.26×10^4 spores/ml) on the fungus were simulated using vessels (15 cm diam) containing 1 liter of deionized water and a 0.5-cm layer of sand on the bottom. Initially 60 larvae (20 each of 1-, 3- and 5-day-old) were added along with one SFE-agar disc (200 mm^2) of fungus culture. Subsequently, natural oviposition was simulated by adding 10 newly hatched larvae to each container each day for 15 days. To each container, 1 ml of a 3.5% (w/v) liver powder slurry was added every other day to provide food for the larvae. There were 3 containers per treatment. Treatments included containers with and without the fungus that also received either a single initial application of *B.t.i.* or *B. sphaericus*, or multiple applications (daily for 15 days) of *B.t.i.* or *B. sphaericus*, or were untreated controls (no *B.t.i.* or *B. sphaericus*). After 15 days, the water was decanted from all of the containers, leaving the sand and mosquito larvae cadavers to dry. After drying for 15 days, each container was reflooded with 1 liter of deionized water. Twenty newly hatched larvae were added daily for 15 days. During that time, 1 ml of 3.5% (w/v) liver powder slurry was added to each container every other day. The mosquito pupae were removed and counted each day. At 5, 10 and 15 days after reflooding, 10 dead larvae were removed from each container and examined microscopically to detect the presence of the fungus. The numbers of pupae at time intervals after reflooding, were analyzed by ANOVA and the means per treatment compared using Tukey's HSD test to determine significant differences ($P \leq 0.05$) (PROC ANOVA, PROC MEANS/TUKEY, SAS Institute 1982).

RESULTS AND DISCUSSION

In the preliminary experiments with 3-day-old larvae, the LC_{50} and LC_{90} for *B.t.i.* were 0.139 and 0.317 ITU/ml, respectively. These values were within the range of values for 4 strains of *Cx. quinquefasciatus* reported by Boike et al. (1990). The LC_{50} and LC_{90} for *B. sphaericus* were 3.1×10^4 and 5.6×10^4, respectively. These values were used to select the concentrations for

use in the experiments in order to give a range of mortalities.

Experiment 1: Treatments with concentrations of *B.t.i.* ranging from 0.057 to 0.456 ITU/ml resulted in differences in the effects of the fungus that were related to the concentration of *B.t.i.* (Table 1). At the low concentrations of *B.t.i.* (0.057 and 0.114 ITU/ml), the fungus increased the larval mortality above that achieved with only *B.t.i.*. At higher concentrations of *B.t.i.* there were slight or no increases in larval mortality with the presence of the fungus due to the very high mortalities caused by the bacteria. High percentages of the dead larvae retrieved after 48 h from the treatments containing the fungus were infected by the fungus in the *B.t.i.* treatments of 0.057, 0.114 and 0.228 ITU/ml, but low percentages were infected at the 2 higher concentrations. The percentage of the body regions containing fungus in the dead larvae that were infected with the fungus declined with increasing concentrations of *B.t.i.* In treatments with high concentrations of *B.t.i.*, there was rapid death of the larvae before there was sufficient time for the fungal infection to develop.

There was no evidence that the *B. sphaericus* treatments ranging from 0.6 to 4.8 $\times$ 10^4 spores/ml interfered with the fungus (Table 1). The larval mortality rate increased in the presence of the fungus with all concentrations of *B. sphaericus* at 24 h after treatment and with all but the highest concentration at 48 h after treatment. Nearly all of the dead larvae retrieved after 48 h from the treatments containing the fungus as well as the bacteria were infected with the fungus. There was no relationship between the percentage of dead larvae infected with the fungus and the concentration of *B. sphaericus* in the treatments. Likewise, the percentage of infected body regions in the infected larvae was high in all cases, although there was a slight decrease at the highest concentration of *B. sphaericus*. This reflected the slower action of *B. sphaericus* (compared with *B.t.i.*) in causing larval mortality and, consequently, allowing more time for fungal growth.

Experiment 2: The age (and, consequently, the size and instar) of the larvae at the time of exposure to combinations of *B.t.i.* and the fungus affected the larval mortality, percentage of larvae infected and the growth of the fungus in the larvae (Table 2). The fungus alone caused high mortalities in the 1- and 2-day-old larvae, intermediate mortality in the 3-day-old larvae and low mortality in the 4- and 5-day-old larvae. Likewise, the single concentration of *B.t.i.* alone caused decreasing larval mortality with increasing larval age. With the combination of treatments, the presence of the fungus resulted in greater mortalities after 24 and 48 h than occurred with *B.t.i.* alone for treatments of the 3-day-old larvae and after 48 h for treatments of the 4-day-old larvae (late third instar). No increased mortalities due to the combination treatments were detected among the other age groups because of the high mortalities due to

Table 1. Mortality and fungal infection of *Culex quinquefasciatus* larvae (3-day-old) 24 and 48 h after treatment with *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) or *B. sphaericus* in the presence or absence of the fungus *Lagenidium giganteum* (on SFE-agar discs).

Treatment	Mean % larval mortality*				Fungus in dead larvae (48 h)	
	24 h		48 h			
	No fungus	Fungus	No fungus	Fungus	Mean % of larvae**	Mean % of body**
B.t.i. (ITU/ml)						
0.0	1.5 e	48.5 d	1.5 d	96.1 a	100 a	99.4 a
0.057	22.0 e	54.5 cd	22.0 c	97.5 a	99.0 a	87.4 a
0.114	59.5 c	71.0 bc	71.1 b	99.5 a	76.7 b	66.4 b
0.228	83.5 ab	88.5 ab	90.5 a	99.5 a	76.7 b	53.2 bc
0.342	97.5 a	97.5 a	97.5 a	100 a	55.6 c	40.8 cd
0.456	98.5 a	98.5 a	99.5 a	100 a	53.3 c	33.4 d
B. sphaericus (10^4 spores/ml)						
0.0	0.0 g	31.6 b	0.0 d	96.1 a	100 a	100 a
0.6	21.6 f	60.5 d	40.5 c	100 a	100 a	98.4 ab
1.2	46.6 e	77.2 bc	61.6 b	100 a	100 a	98.4 ab
2.4	67.2 cd	89.5 ab	97.2 a	100 a	100 a	95.5 b
4.8	87.2 b	98.5 a	100 c	100 a	95.6 a	89.0 c

* Within each time (24 or 48 h) and including both columns for "no fungus" and "fungus" for *B.t.i.* or *B. sphaericus*, means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

** Means for *B.t.i.* or *B. sphaericus* followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

Table 2. Mortality and fungal infection of *Culex quinquefasciatus* larvae of various ages (1- to 5-day-old) 24 and 48 h after treatment with *Bacillus thuringiensis* var. *israelensis* (*B.t.i*) in the presence or absence of the fungus *Lagenidium giganteum* (on SFE-agar discs).

Larval age (days)	*B.t.i.* (ITU/ml)	Mean % larval mortality*				Fungus in dead larvae (48 h)	
		24 h		48 h			
		No fungus	Fungus	No fungus	Fungus	Mean % of larvae**	Mean % of body**
1	0		88.9 bc		100 a	100 a	100 a
	0.114	92.8 ab	100 a	100 a	100 a	73.3 bc	59.4 b
2	0		76.1 d		100 a	100 a	100 a
	0.114	82.8 cd	96.1 ab	100 a	100 a	64.4 c	53.8 b
3	0		26.6 f		63.8 b	100 a	100 a
	0.114	22.8 f	47.2 e	44.4 c	96.1 a	82.2 b	62.5 b
4	0		3.3 g		7.2 e	14.4 e	14.2 d
	0.114	6.6 g	7.2 g	8.3 e	23.3 d	46.7 d	40.6 c
5	0		0.0 g		3.8 e	7.8 e	3.6 d
	0.114	0.0 g	0.5 g	1.6 e	3.3 e	6.7 e	6.2 d

* Within each time (24 or 48 h) and including both columns for "no fungus" and "fungus," means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

** Means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

Table 3. Mortlaity and fungal infection of *Culex quinquefasciatus* larvae (3-day-old) 24 and 48 h after treatment for 1 h with *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) following exposure or no exposure for 1 h to zoospores of the fungus *Lagenidium giganteum*.

Treatment	Mean % larval mortality*				Fungus in dead larvae (48 h)	
	24 h		48 h			
	No fungus	Fungus	No fungus	Fungus	Mean % of larvae**	Mean % of body**
*B.t.i.*ITU/ml)						
0.0	0.0 g	17.5 g	0.0 d	75.0 b	100 a	98.6 a
0.114	5.0 g	28.5 ef	7.5 d	82.5 ab	93.3 a	78.0 ab
0.228	8.5 g	32.5 ef	11.0 d	83.5 ab	100 a	91.4 a
0.456	42.5 cde	37.5 de	58.5 c	82.5 ab	100 a	90.0 a
0.684	56.0 bc	48.7 cd	58.5 c	86.0 ab	73.3 b	63.4 b
0.912	81.0 a	66.2 ab	85.0 ab	93.5 a	56.7 c	38.6 c

* Within each time (24 or 48 h) and including both columns for "no fungus" and "fungus," means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

** Means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

both pathogens in the 1- and 2-day-old larvae and the low mortalities in the 5-day-old larvae (fourth instar). The proportion of the dead larvae infected by the fungus and the percentage of the body regions infected among the larvae retrieved from the fungal treatments alone were significantly higher than for those from the combination treatments for the 1- to 3-day-old larvae while the opposite occurred among the 4-day-old larvae and no significant differences among the 5-day-old larvae.

Experiment 3: Treatment of 3-day-old larvae with *B.t.i.* at concentrations of 0.114–0.912 ITU/ml for 1 h after the larvae were already infected by exposure to fungal zoospores resulted in significant increases in larval mortalities after 48 h at all except the highest concentrations of *B.t.i.* in comparison with larvae that were not infected (Table 3). After 24 h there were significant increases only at the low concentrations of *B.t.i.* (0.114 and 0.228 ITU/ml). At 48 h after treatment, the percentage of dead infected larvae containing mycelia and the percentage of the body regions with mycelia were both high in the *B.t.i.* treatments of 0.114 to 0.456 ITU/ml, while there were significantly lower values for the larvae from the higher concentrations of *B.t.i.*. Those lower values reflected the rapid larval mortalities from the high concentrations of *B.t.i.*, which limited the amount of growth of the fungus in the larvae.

The interactions between insect pathogens are sometimes categorized as additive, synergistic or antagonistic although different methods

of testing and data analysis may yield different results (McVay et al. 1977, Fuxa 1979, Jaques and Morris 1981, Cossentine and Lewis 1984, Richter and Fuxa 1984). The equations and chi-square ($P = 0.05$) procedures described by McVay et al. (1977) and Richter and Fuxa (1984) were applied to the data on 3-day-old-larvae (Tables 1 and 2) to compare the observed mortality with the expected mortality with the combinations of bacteria and fungus (on agar discs) for each concentration of bacteria and time period. All except one of the chi-square values for *B.t.i.* and *B. sphaericus* were not significant, indicating no evidence of synergism or antagonism with the combinations. Chi-square comparisons of the data on 3-day-old larvae infected by zoospores prior to exposure to *B.t.i.* (Table 3) indicated evidence of antagonism after 24 h, but not after 48 h. Thus, overall, the results of experiments 1–3 indicated that the bacteria-fungus interactions in 3-day-old larvae were additive within the range of bacteria concentrations that produced less than 100% larval mortality.

Experiment 4: The results of simulating single and multiple applications of *B.t.i.* and *B. sphaericus* to habitats containing the fungus are presented in Table 4. After drying and reflooding the containers that were previously treated with combinations of *B.t.i.* and the fungus, there were initially (days 8–9, 10–11) no significant differences in the production of pupae from all of the treatments after adding larvae daily. By days 14–15 after reflooding, however, there were significantly fewer pupae in the containers that initially contained the fungus whether or not there was treatment with *B.t.i.* The fungus was observed microscopically in dead larvae retrieved from the fungus-treated containers at 5, 10 and 15 days after reflooding. This was clear evidence of the persistence of the fungus in the habitat, most likely in the mosquito larvae cadavers. There was no evidence of any effects from the *B.t.i.* treatments after drying and reflooding.

With the single and multiple applications of *B. sphaericus* to containers with and without fungus, there were very few or no pupae produced by 12–15 days after reflooding and adding larvae daily. The fungus was observed microscopically in dead larvae retrieved from the fungus-treated containers. This was clear evidence of the persistence of the fungus in the habitat either in the presence or absence of *B. sphaericus*. The lack of pupae in the containers treated only with *B. sphaericus* indicated the persistence of *B. sphaericus* after drying and reflooding, most likely in the larvae cadavers.

These results indicate that *B.t.i.* and *B. sphaericus* are compatible with *L. giganteum* and applications of either bacterial agent in combination with the fungus are not likely to result in interactions that reduce the immediate effectiveness of the treatments for control of mosquito larvae. At low concentrations (insufficient to cause 100% control) of *B.t.i.* or *B. sphaericus*, treatments with the fungus increase larval mortality rates. Treatments with high concentrations of these bacterial agents may reduce recycling of the fungus from infected cadavers as a

Table 4. Effect of multiple (daily for 15 days) and single (on day 1) treatments with *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) (0.154 ITU/ml) or *B. sphaericus* (5.26×10^4 spores/ml) in the presence or absence of the fungus *Lagenidium giganteum* (on SFE-agar discs) on subsequent production of *Culex quinquefasciatus* pupae after drying, reflooding and adding newly hatched larvae daily for 15 days.

Treatment		Mean no. pupae per container at days after reflooding*			
Bacillus	Fungus	8–9	10–11	12–13	14–15
	Treated with *B.t.i.* before drying and reflooding				
Multiple	Present	27.7 a	10.7 a	2.7 a	0.3 c
Multiple	Absent	23.3 a	19.7 a	11.0 a	19.0 c
Single	Present	15.7 a	9.3 a	0.3 b	0.0 b
Single	Absent	21.3 a	14.0 a	8.3 ab	19.3 ab
None	Present	8.3 a	4.0 a	0.0 b	0.0 c
None	Absent	22.3 a	15.7 a	11.7 a	22.7 a
	Treated with *B. sphaericus* before drying and reflooding				
Multiple	Present	9.0 a	3.7 b	0.0 b	0.0 b
Multiple	Absent	14.0 a	6.7 b	3.0 b	0.7 b
Single	Present	13.3 a	6.0 b	0.0 b	0.0 b
Single	Absent	3.3 a	2.3 b	0.0 b	0.0 b
None	Present	10.7 a	3.0 b	0.0 b	0.0 b
None	Absent	15.7 a	25.3 a	27.3 a	20.3 a

* Within each time period for *B.t.i* or *B. sphaericus*, means followed by the same letter were not significantly different ($P \geq 0.05$, Tukey's HSD test).

consequence of limited growth of the fungus in the larvae due to rapid high larval mortality. This effect is more pronounced with *B.t.i.* treatments than with *B. sphaericus* due to the slower lethal effects of the latter. The fungus is capable of surviving in larval cadavers in the presence of *B.t.i.* or *B. sphaericus* and reinfecting larvae after drying and reflooding.

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