Ovipositional and ovicidal effects of the microbial agent *Bacillus thuringiensis israelensis* on *Culex quinquefasciatus* Say (Diptera: Culicidae)

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ABSTRACT: The magnitude of oviposition as well as the size, shape and the number of eggs per of egg rafts egg raft were determined after gravid *Culex quinquefasciatus* Say oviposited on water treated with water dispersible granules (WDG) of *Bacillus thuringiensis* ssp. *israelensis* (Bti) and on untreated water. The mean number of eggs/raft was lower in the treated than in the untreated water. Bti concentrations from 0.5 to 2.0mg/L affected the shape of egg rafts and number of eggs in each raft. As the concentration of Bti increased from 0.5 to 2.0 mg/L the shape of egg rafts became more irregular with fewer eggs in each raft. Exposure to Bti at 2- and 26-h reduced the hatching rates, and fewer eggs hatched at 26-h of exposure to Bti. As the concentration of Bti WDG increased from 0.5 to 2.0 mg/L, the hatching rate decreased. Eggs exposed for 2-h to 2.0mg/L Bti had a hatch of 30% after 24 h, the rate increasing to 57% after 72 h. In contrast, in 26-h exposed eggs to 2.0 mg/L Bti, the hatching rate after 24 h was only 12% and this rate increased to 39% after 72 h. In larvae from eggs exposed for 2 h, the survival rate was 40% at 2.0 mg/L Bti and 87% in untreated controls. In contrast, the survival rates of larvae from 26-h exposed eggs was 91% in controls while it was 30% at 2.0 mg/L Bti. As the concentration of Bti increased from 0.5 to 2.0 mg/l the survival rates of larvae decreased. The combined effects of reductions of egg rafts, low number of eggs per egg raft, and reduced hatching and survival rates could have significant cumulative effects on the yield of adult mosquitoes, and this could result in a greater control potential of this microbial agent. ***Journal of Vector Ecology* 31 (1): 29-34. 2006.**

Keyword Index: Cx. quinquefasciatus, Bti, ovicidal effects, decrease size and shape of egg rafts.

INTRODUCTION

Since the discovery of the mosquitocidal properties of *Bacillus thuringiensis* ssp. *israelensis* de Barjac (Bti), various commercial formulations have been evaluated and developed for the control of mosquito larvae. Bti is an aerobic, Gram positive, spore-forming bacterium commonly found in the environment. It produces crystalline protein toxins during sporulation, which constitute the principal active ingredients in Bti formulations (de Barjac and Sutherland 1990, Gill et al. 1992). Upon ingestion by a susceptible species, the proteins in the crystals are solubilized in the midgut by a combination of alkaline pH and proteolysis (Aronson et al. 1986). This bacterium has been used operationally for the control of mosquitoes and black flies since 1980 in the United States. The crystalline endotoxins of Bti contain lethal stomach toxins. When formulated and used against mosquito larvae, they yield a high level of control of larvae of *Aedes*, *Anopheles*, *Culex*, *Psorophora*, and other mosquitoes (Mulla 1985, Lacey and Undeen 1986, Yap 1990). At the present time Bti is one of the most effective and dependable microbial larvicides available for use in area-wide mosquito and black fly control programs.

Recently, Zahiri and Mulla (2004) showed ovipositional effects of Bti and another microbial larvicide, *Bacillus sphaericus*, in *Culex quinquefasciatus* (Say). They noted that Bti suspensions received lower number of egg rafts than the controls. It was further noted that there was an inverse relationship between the number of egg rafts and concentrations of Bti. As the concentrations increased the number of egg rafts oviposited in the treated water decreased. In addition to negative effects on oviposition, both male and female mosquitoes suffered high mortality on visiting or landing on aqueous suspensions of Bti and *B. sphaericus* (Zahiri and Mulla 2004). Adult mortality indicated a direct relationship with concentrations of Bti. This new discovery relating to extra larvicidal effects of Bti ingestion *per os* on mosquito oviposition and its toxic activity against adult mosquitoes point to the additional advantages of this agent in mosquito control. There are only a few studies on the effects of Bti on adult mortality, such as *Aedes aegypti* (Klowden et al. 1983), *Simulium vittatum* (Klowden et al. 1985), and house flies, stable flies, and green lacewings (Wilton and Klowden 1985). Similar extra larvicidal effects have been noted in some botanical larvicides that affect oviposition of mosquitoes. Su and Mulla (1998, 1999) investigated the ovicidal effects of two neem formulations against *Culex* mosquitoes. They showed that the ovicidal effect was influenced by neem concentration, age of the egg rafts, and age of the neem preparations. In our previous studies (Zahiri and Mulla 2004) negative effects of Bti on oviposition were noted. Not only were the number of egg rafts deposited on Bti suspensions lower, but also the sizes of the rafts were irregular and smaller than rafts collected from untreated water. An average-sized *Culex* raft contains about 220 eggs (Weber et al. 1991), but in our previous studies the number of eggs per raft from untreated waters was also lower than the average number of eggs laid by this mosquito, although egg rafts laid on Bti were fewer than controls. In view of these extra larvicidal effects of Bti it became necessary to investigate some other important biological effects other than larvicidal for this microbial agent. The objective of this research was to determine the ovipositional pattern of *Cx. quinquefasciatus*, size of egg rafts, and number of eggs per raft oviposited by *Cx. quinquefasciatus* on water suspensions prepared with Bti. We also evaluated the ovicidal activity of Bti suspensions against this mosquito and the survival rate of larvae hatching from eggs laid in Bti suspensions and controls.

MATERIALS AND METHODS

Mosquito colonies

A colony of *Cx. quinquefasciatus* was established at the National Institute of Health (NIH), Thailand, from egg rafts collected from Sirachai District, Northaburi Province, Thailand in 2001. From this parental colony, a sub-colony was established for oviposition bioassays in our laboratory. Egg rafts (8-10) were placed in an enamel pan (50 x 25 x 7 cm) containing 2 L of tap water and 2 g of rabbit pellets (Brookhurst Mill, Riverside, CA) as larval food. Larvae were reared under a photoperiod of 14:10 (L:D) at 27±2 ºC. The larvae were reared to the 4^{th} instar and allowed to pupate. The pupae were removed into cups containing tap water and placed in screen cages (23 x 30 x 23 cm) where the adults emerged. The adults were maintained under the same temperature and light conditions as above and provided continuously with a 10% sucrose solution. On day 5 after emergence, females were allowed to feed on restrained 2-5 day-old chicks overnight (Animal Use Protocol Number A-S0205015-2), University of California, Riverside, CA. Adults from this Thai sub-colony were also used in oviposition and hatching studies on ovicidal effects of Bti.

Figure 1. Egg rafts deposited by *Cx. quinquefasciatus* in water cup treated with Bti suspension (2.0 mg/l) and distilled water control. Note the high number of dead adults in Bti in the top picture. The cups below show the number of egg rafts with the dead adults removed.

Test materials

The material used for oviposition and ovicidal activity was a water dispersible granule (WDG) formulation of Bti (VectoBac ABG-6511 Lot# 60-070-BR). The potency for Bti (WDG) was 3,000 international toxic units (ITU/mg) as reported by manufacturer and was received on August 20, 2002, from Valent BioSciences Corporation, North Chicago, IL, and kept at 4°C. In all tests freshly prepared suspensions in distilled water were prepared during 2004 and 2005.

Oviposition pattern

To study the size and number of egg rafts deposited in Bti suspensions, two experiments were carried out, paired tests and multiple concentrations of Bti. For both experiments 50 males and 50 females were separated in the pupal stage (by size of the pupae) and introduced into each screen cage (23 x 23 x 32 cm) set in a room at 27 ± 2 ºC and a photoperiod of 14:10 (L:D) h, the pupae were allowed to emerge into adults in the test cages. Adults were provided continuously with 10% sucrose solution in a plastic cup provided with a cotton wick. They were blood fed on day 5 after emergence. For the pair test, two waxed paper cups (120 ml) were placed in each cage, one treatment cup containing 100 ml of distilled water with 2.0 mg/L Bti and the other cup without Bti serving as a control. For the multiple concentration test, four cups per cage containing 100 ml distilled water, to which Bti was added at concentrations of 0.5, 1.0 and 2.0 mg/L in each cup and a control. The treatments were replicated four times, using four cages. The oviposition test was carried out in the cages on day four after blood feeding. The test was conducted in the late afternoon, and the egg rafts were collected the following morning (after 16 h). In both experiments, the size of five randomly selected egg rafts from each treatment and untreated cups was measured under a stereomicroscope, and the number of eggs in all egg rafts was counted. The mean number of egg rafts, the number of eggs per raft and size of the five randomly selected egg rafts were statistically analyzed using the paired *t*-test in the pair test and a one–way analysis of variance (ANOVA) (Scheffe-F-test) in the multiple concentration tests.

Ovicidal activity

In preliminary studies we noted that the hatching rates of eggs laid over 24 h by gravid females in Bti suspension were lower than those of the distilled water controls. On this basis we initiated further studies to determine non-larvicidal effects of Bti in pair and multiple concentration tests on ovicidal activity. To study this relationship, 50 males and 50 females were placed in each screen cage in the same manner as in the oviposition tests. Four days after blood feeding a pair of oviposition cups, one treated with 2.0 mg/L Bti and the other as a control, were placed in each of four mosquito cages before

Table 1. Size of egg rafts and number of eggs per raft deposited by gravid *Cx. quinquefasciatus* in water treated with Bti (2.0 mg/l) and distilled water.

Treatment	No. of egg rafts		No. of eggs/raft		Size of egg rafts
	Total	Mean ± SE	Total	Mean ± SE	mean ± SE (mm)
Control	77*	19.25 ± 1.89	5819*	75.57 ± 4.17	3.60* ± 0.12
Bti suspension	33	8.25 ± 0.55	1315	39.84 ± 1.13	2.68 ± 0.09

*Asterisks indicate significant differences between treatment (Bti) and control by *t*-test at the 0.05 level.

the start of the dusk period (5 p.m.) and were removed from the cages after 4 h at 9 p.m. during which period most of the egg rafts were laid. The eggs laid during this 4-h period were considered as 2-h exposed eggs. For hatching, five randomly selected egg rafts laid during this period were selected from each treatment and the eggs counted in each egg raft. The selected egg rafts were then transferred individually for hatching to untreated water cups holding 200 ml distilled water. The remaining egg rafts were left in the Bti suspension and control cups for another 24 h (exposed for a total of 26 h to Bti treatment) after which five egg rafts were randomly selected from the 4 cups of each treatment and individually transferred to untreated water cups holding 200 ml distilled water for hatch assessment after counting the eggs in each egg raft. The hatching rate of eggs was assessed three days after oviposition in both 2- and 26-h exposed eggs. According to Su and Mulla (1998), the hatching rates were calculated as a percentage of the eggs with open operculum compared to all the eggs in the five egg rafts examined.

Ovicidal (hatching rate) effects of Bti also were carried out at various concentrations of Bti. In this experiment the same number of males and females were placed in each screen cage as above and blood fed on day 5 after emergence. Four waxed paper cups were filled with 100 ml distilled water each, of which three were treated with Bti suspensions at 0.5, 1.0, and 2.0 mg/L, and one cup as a control were placed in each cage and replicated four times. The treated and control cups were placed in cages containing gravid mosquitoes on day four after a blood meal before the start of the dusk period. After 4 h of oviposition, when most of the egg rafts were laid, five of the egg rafts laid during this period (considered as 2 h exposed) were selected at random from each of the four treated and control cups. The remaining egg rafts were left in both Bti suspension and control cups for 24 h, after which five egg rafts were selected at random (considered as 26-h exposed) from each treatment and individually transferred to 200 ml untreated water cups for hatching after counting the eggs in each egg raft and measuring the size of the egg rafts under a stereomicroscope. The hatching rate of eggs was assessed three days after oviposition in both 2- and 26-h exposed eggs. The hatching rates were calculated as a percentage of the total number of eggs with open opercula compared to all the eggs in the five egg rafts examined.

Survival rates

Survival rates of larvae from 2- and 26-h exposures were studied three days after hatching for both one dosage and various concentrations of Bti. Hatched larvae in the cups after 72 h from the treatment and control cups were transferred to individual enamel pans (50 x 25 x 7 cm) containing 2 L of tap water and 2 g of rabbit pellets as larval food. The larvae were reared to late (3^{rd} to 4^{th}) instars, and the larval density in each pan was adjusted to 200-250 larvae. The percentage survival of 3^{rd} and 4^{th} instars was calculated on the basis of total number of hatched eggs at the start.

RESULTS AND DISCUSSION

Oviposition pattern

In oviposition pattern tests, gravid *Cx. quinquefasciatus* preferred to lay more egg rafts in the distilled water than the Bti suspension (Table 1). In addition, there was also a marked

Table 2. Size of egg rafts and number of the eggs per raft deposited by gravid *Cx. quinquefasciatus* in water treated with various concentrations of Bti suspensions*.

Treatment Bti (mg/l)	No. of egg rafts		No. of eggs		Size of egg rafts
	Total	Mean ± SE	Total	Mean ± SE	mean ± SE (mm)
Control	66 [a]	16.50 ± 0.66	5625 [a]	85.23 ± 1.56	3.71 ± 0.08 [a]
0.5	24 [b]	6.00 ± 0.65	1089 [b]	45.37 ± 5.08	2.86 ± 0.14 [b]
1.0	22 [b]	5.50 ± 0.69	889 [b]	40.40 ± 2.35	2.73 ± 0.11 [b]
2.0	16 [c]	4.00 ± 0.61	532 [c]	33.25 ± 3.55	2.52 ± 0.15 [b]

* Means followed by the same letter in a column are not significantly different by F-test at the 0.05 level.

Table 3. Ovicidal activity and survival rates of larvae from eggs deposited by gravid *Cx. quinquefasciatus* in Bti suspension at 2.0 mg/l and distilled water control.

Treatment	Hatching and survival rates (%) 2-h. old eggs 24h	48h	72h	Survival larvae	26-h. old eggs 24h	48h	72h	Survival larvae
Control	65*	95*	95*	80*	75*	93*	95*	83*
Bti suspension	22	67	72	53	10	49	56	36

*Asterisks indicate significant differences between treatment (Bti) and distilled water control by *t*-test at the 0.05 level.

difference in the size of the egg rafts (Figure 1). The treated cups received a mean of 8.25 egg rafts per cup while the control cups received a mean number of 19.25 egg rafts per cup. Paired *t*-tests confirmed that the water treated with Bti received significantly fewer egg rafts ($P<0.05$) than the distilled water control (Table 1). Egg rafts collected from control cups contained a mean of 75.57 eggs per raft while those from Bti suspensions had a mean of 39.84 eggs per raft (Table 1). Control cups had larger and more uniform egg rafts than Bti suspension cups. Egg rafts collected from Bti suspensions were smaller in size and irregular in shape. The mean size of egg rafts in the control and Bti cups was 3.60 mm and 2.68 mm respectively (Table 1). The size of the egg rafts had a positive relationship with the number of eggs in each raft; larger egg rafts had more eggs in each raft and smaller and irregular rafts had fewer eggs (Table 1).

Results of multiple concentrations of Bti indicated the same trends as in the single concentration test. Oviposition cups control, 0.5, 1.0, and 2.0 mg/L Bti suspensions received a mean number of 16.50, 6.00, 5.50, and 4.00 egg rafts respectively (Table 2). As the concentration of Bti increased from 0.5 to 2.0 mg/L, the number of eggs per egg raft decreased. The mean number of eggs per raft collected from control cups was 85.23, and this number decreased to 33.25 at 2.0mg/L Bti suspension (Table 2). The average size of the randomly selected egg rafts in control, 0.5, 1.0, and 2.0 mg/l of Bti suspensions were 3.71, 2.86, 2.73, and 2.52 mm respectively (Table 2). These results show that the number of egg rafts in the control cups was greater than in each concentration of Bti. Irregularity in size of the egg rafts was directly related to the number of eggs in each raft. As in one dosage of Bti, smaller and irregular egg rafts had fewer eggs in each raft than larger egg rafts. The concentration of Bti had an inverse relationship not only to the size of egg rafts but also to the number of eggs rafts deposited and the eggs contained in each egg raft.

The usefulness of Bti as a larvicide has been shown in mosquito control programs (Mulla 1985, Lacey and Undeen 1986). Zahiri and Mulla (2004) have shown additional advantages of this product and its role in reducing oviposition and causing adult mortality. Irregular size of egg rafts and lower number of eggs in each raft deposited by gravid females on water treated with Bti offer additional advantages of using this product in mosquito control programs

Ovicidal activity

To study the ovicidal activity of Bti, five randomly selected 2-h exposed egg rafts deposited in treated and control water were transferred from the Bti suspension and control to untreated distilled water and the hatching rate recorded at 24, 48, and 72 h. The hatching rates of eggs exposed to 2.0 mg/L of Bti for 2 h were significantly lower from those in the control (Table 3). At 24 h after transfer, 65% of the eggs laid in the control hatched, and by 48 and 72 h after transfer most

Table 4. Ovicidal activities and survival rates of larvae from eggs deposited by gravid *Cx. quinquefasciatus* in water treated with various concentrations of Bti suspensions*.

Treatment Bti mg/l	Hatching and survival rates (%) 2-h old eggs 24h	48h	72h	Survival larvae	26-h old eggs 24h	48h	72h	Survival larvae
Control	69 a	83 a	97 a	87 a	69 a	77 a	98 a	91 a
0.5	53 a	66 a	80 a	62 b	51 b	57 b	62 b	55 b
1.0	44 b	53 b	59 b	50 c	49 b	59 b	61 b	45 b
2.0	30 c	47 b	57 b	49 c	12 c	30 c	39 c	30 c

* Means followed by the same letter in a column are not significantly different by a F-test at the 0.05 level.

of the eggs (95%) in untreated regimens hatched. Eggs deposited in Bti suspension and removed to distilled water had a hatching rate of 22, 67, and 72% at 24, 48 and 72 h, respectively (Table 3). Hatching rates recorded for the 26-h exposed egg rafts showed the same trends as the 2-h exposed eggs (Table 3). At 24 h 75% of the control eggs hatched, and most of the eggs (93-95%) were hatched out by 48 and 72 h (Table 3). Eggs deposited and exposed for 26 h to 2.0 mg/L Bti suspension had hatching rates of 10% at 24 h, increasing to 49% and 56% at 48 and 72 h respectively (Table 3).

Ovicidal activity at various concentrations of Bti was determined in the same manner as for the one dosage of Bti. Five randomly selected egg rafts from 2-h exposed deposited eggs in each concentration of Bti were used, and hatching rates were recorded at 24, 48, and 72 h after transfer to distilled water. Hatching rate in the control ranged from 69% at 24 h to 97% at 72 h (Table 4). Hatching rate at 0.5 mg/L Bti was 53% at 24 h, increasing to 80% at 72 h. The same trends in time were shown for 1.0 and 2.0mg/L Bti, hatching rate increasing from 24 to 72 h from 44 to 59% at 1.0 mg/L Bti and from 30 to 57% at 2.0 mg/L Bti, respectively (Table 4). There was an inverse relationship between concentration and the magnitude of hatching rate. Hatching rates of 26-h exposed egg rafts from various concentrations of Bti were recorded for the same period of time. In the controls, the hatching rates were 69, 77, and 98% at 24, 48, and 72 h, respectively (Table 4). At the 0.5 mg/L Bti the hatching rate was 51, 57, and 62% at 24, 48, and 72 h, respectively (Table 4). At the 1.0mg/L Bti concentration, the hatching rate was 49, 59, and 61% at 24, 48, and 72 h respectively (Table 4). At the 2.0 mg/L Bti concentration, the hatching rate was 12, 30, and 39% at 24, 48, and 72 h, respectively (Table 4). As the concentration of Bti increased the hatching rates decreased from 62 (control) to 12% at 24 h, from 77 to 30% at 48 h and from 98 to 30% at 72 h. In general, hatching rates increased with time from 24 to 72 h at both durations, but the hatching rate decreased with an increase in Bti concentration.

Survival rate

The survival rates of larvae hatching from eggs deposited in control and Bti suspensions at 2.0 mg/L and removed to untreated tap water 2 h post oviposition were recorded up to late instars. The survival rates of larvae were 80% and 53% for eggs collected from control and Bti suspensions, respectively (Table 3). Survival rates of larvae from eggs that were exposed to Bti (2.0 mg/L) suspension for 26-h were significantly lower (36%) as compared to the control (83%) (Table 3). The survival rates at both exposure periods to Bti were significantly lower than that in the control (Table 3). However, the survival rate for the 26-h exposed eggs declined much more than the 2-h exposure period (Table 3).

The survival rate of larvae resulting from eggs exposed to multiple concentrations of Bti was also studied. As the concentration of Bti increased, the survival rate decreased. The survival rate of larvae (from eggs laid in multiple concentrations) was 87, 62, 50, and 49% for the control and 0.5, 1.0, and 2.0 mg/L of Bti suspensions respectively (Table 4). A similar pattern of survivorship was seen for the 26-h exposed egg rafts. The survival rates of larvae recorded were 91, 55, 45 and 30% from eggs laid in untreated control, 0.5, 1.0, and 2.0 mg/L Bti suspensions, respectively (Table 4). The highest reduction of survival rates was noted at 26 h exposed eggs to Bti at 2.0mg/L.

The results suggest that Bti has profound effects on the number and size of egg rafts. Drinking and pre-oviposition drinking (Weber and Tipping 1993) indicated that adults were more attracted to Bti suspensions, therefore more dead adults were seen on the surface of Bti suspensions than control cups (Zahiri and Mulla 2004). It seems that females die before they deposit the whole compliment of eggs. Zahiri and Mulla (2004) studied the reduction of *Cx. quinquefasciatus* egg rafts by this microbial agent; the reduction might be related to adult mortality. As mentioned above, egg rafts deposited in the highest concentration of Bti were more irregular and smaller in size. This agent also has a negative impact on the hatching of exposed eggs and survival rates of larvae resulting from exposed eggs. The hatching rates and survival rates of larvae had an inverse relationship with the concentrations of Bti. Although the concentrations used here were not higher than the normal application rates used in mosquito control programs, we still noted reductions in oviposition, number of eggs, hatching rates and survivorship. The usefulness of Bti as a larvicide in mosquito control programs has been shown for a long time. However, reductions of egg rafts and reduced hatching and survival rates suggest additional advantages of Bti when used in mosquito control programs. These findings open new areas of research on different mosquito species, other microbial agents, and the mechanism of adult mortality and ovicidal activity, to elucidate the potential extra larvicidal effects of this agent in mosquito control programs.

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