Comparitve susceptibility of *Aedes aegypti* larvae against different mixtures of bacterial toxins of *Bacillus thuringiesis israelensis* and *Bacillus sphaericus*

Najat A. Khatter

Biology Department, Faculty of Science for Girls-King Abdulaziz University, Jeddah, Saudi Arabia
najat.khatter4@gmail.com

Abstract: The present study deals with the evaluation of efficiency of bacterial mosquito larvicides against *Aedes aegypti* when used in combinations with each other under laboratory conditions. Synergistic interactions among the multiple endotoxins of *Bacillus thuringiesis* Subsp. *israelensis* de Barjac (*Bti*) play an important role with high toxicity to mosquito larvae also the absence of insecticide resistance in populations treated with this bacterium. A lake of toxin complexity and synergism are the appartent causes of resistance to (*Bti*) in particular *Aedes* field populations. To identify endotoxins of the bacterium that might improve insecticidal activity and manage mosquito resistance, we tested their toxins alone and in combination. Most combinations of *Bacillus sphaericus* and *Bti* toxins were synergistic and enhanced toxicity relative to *B. sphaericus*, particularly against *Ae. aegypti*, when Cyr1Aa toxin from *Bti* was added to Ctty11A toxins of *B. sphaericus,* synergism value as high as 966-fold was observed and combinations were 5-86,000 fold more active than *B. sphaericus*. These data and previous studies using Cytolytic toxins, intiate proposed strategies for improving bacterial larvicides by combining *B. sphaericus* with *Bti*. These combinations increase both endotoxin complexity and synergistic interactions to enhance activity and help avoid insecticide resistance.
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1. Introduction

During the past decade, two bacterial mosquito pathogens were used as larvicides in mosquito control programs, namely *Bacillus thuringiesis* serotype H.14 and *Bacillus sphaericus* 1543-4. In spite of their relatively high larvicideal activity, yet, the application of the developed commercial formulations under field conditions are still needs further investigations.

Two bacteria, *Bacillus sphaericus* and *Bacillus thuringiesis israelensis*, that produce insecticidal protein endotoxins are used for mosquito control. Although both are highly toxic to mosquito larvae, there are fundamental differences in their toxin composition, mode of action, and relative risk for selecting insecticide resistance. The toxicity of *B. sphaericus* is due primarily to binary toxin (Bin) that binds to a specific receptor on the midgut microvilli of susceptible mosquitoes (Davidson, 1995, Charls *et al.,* 1996; Darboux *et al.,* 2001). In addition to high toxicity, *B. sphaericus* have residual toxicity against *Aedes* species in polluted water.

B. sphaericus has a narrow host range and targets a single receptor type in the midgut of susceptible mosquitoes, the latter characteristic places make high risk for selecting insecticide resistance, several cases of resistance have already been reported, (Yuan *et al.,* 2000; Mulla *et al.,* 2003). Alternatively, *Bti* produces four major endotoxins, Cry4A, Cry4B, Cry11A and Cyt1Aa (Delecluse *et al.,* 2000). These bacterial toxins has much insecticidal spectrum than *B. sphaericus* causing high toxicity against many mosquitoes. To overcome spectrum of activity limitations of *B. sphaericus* as well as to improve toxicity of these species and *Bti* attempts have been made to construct recombinant bacteria that combine the endotoxins of both species (Bourgouin *et al.,* 1990, Pocet *et al.,* 1997, Thiery *et al.,* 1998, Servant *et al.,* 1999, Li *et al.,* 2000; Sun *et al.,* 2001).

The production and application of *B.t* has been developed quickly. These *B.t.* toxins are effective against dipterous pests. A general accepted mode of action for Cry and Cty toxins describes the sequential steps of protoxins, activation, specific binding and cell toxicity, (Soberon *et al.,* 2007). Both the required activation and more importantly binding steps confer remarkable pest specificity to Cry proteins (Piolt and Ellar, 2007). Ingested insecticidal crystal proteins are activated to a toxic form by proteinses from the digestive insect gut fluids. After crossing the pertrophic matrix activated toxins bind to specific receptor proteins on the mid gut microvilli (Hura *et al.,* 2003). Increased toxicity is not the only goal for recombinant microbial strains refraction to selecting insecticide resistance in mosquitoes is also important. Computer models that stimulate the evolution of resistance demonstrated that under certain conditions mixtures of insecticides that act at different targets in the insect are beneficial in retarding resistance development, particularly if those insecticides interact synergistically (Curtis, 1985, Mani, 1985;Tabashnik, 1989). Those models may explain the lake of insecticide resistance to *Bti* which naturally expresses a complex mixtures of

toxins that synergize one another (Georghou and Wirth, 1997). The interaction between Cyt I Aa and *B. sphaericus* suggests that combinations of toxins from both *Bti* and *B. sphaericus* might help slow the evolution of resistance because of the lack of cross – resistance between the endo toxins (Rodcharoen and Mulla, 1996 and Wirth *et al.,* 2000A) and potentially, any synergistic interactions among them. We tested a variety of *Bti* toxins, alone and in combination with *B. sphaericus* , with the goal of identifying mixtures that are synergistic and might improve activity and avoid resistance to *B. sphaericus* . Here, we look for that *Bti* Cry toxins and *B. sphaericus* interact synergistically and that most combinations were more toxic against susceptible and *B. sphaericus* – resistant *Ae. aegypti* than *B. sphaericus* . These combinations also were synergistic and highly active against *Ae. aegypti*. *B. sphaericus* binary toxin is more specific than the *Bti* toxins, being principally active against mosquitoes. The range of mosquito species that are affected by *B. sphaericus* is also narrower than of *Bti* . For example, the effect of *B. sphaericus* toxin on *Ae. aegypti* larvae is low to negligible for most isolates (Davidson, 1981, Wraight *et al.,* 1987, Lacey *et al.,* 1988b, Tiery and de Barjac 1989, Berry *et al.,* 1993, Davidson 1988, 1995; Monnerat *et al.,* 2004). On other hand, several *Aedes* species are moderately susceptible to the bacterium (Lacey *et al.,* 1988b, Mulla *et al.,* 1988b; Siegel *et al.,* 1996; 2001). The bacterial genetic determinants of the host ranges of *B. sphaericus* mosquito larvicidal toxins was reviewed by (Berry *et al.,* 1993). Minor variations in the toxicity among strains of 5a5b serovarieties are likely due to the presence of other toxins in addition to the binary toxin (Berry *et al.,* 1993, Wirth *et al.,* 2000a, 2001, 2004), who demonstrated that the Cyt toxins from *Bti* and *Bt* serovar *medellin* synergize the larvicidal activity of *B. sphaericus* to Cyt 1A was 3600 times more toxic to *Ae. Aegypti* larvae than *B. sphaericus* alone (Wirth *et al.,* 2000a).

2. Materials and Methods:

a. Bacterial strains and growth conditions:

Bt strain YBT-226 was identified in *Aedes aegypti* screen and is the property of E.1 Dupont de Nemours. *B. sphaericus* was obtained from H. D. Burges, Institute for Horticultural Research, Little Hamoton, MK, The conditions for growth and sporulation on CCY medium were as described for (Tailor *et al.,* 1992)

b. Purification of protein inclusions:

Protein inclusions were purified from spore / crystal mixtures by centrifugation through discontinuous sucrose gradients, (Thomas and Ellar, 1993). Protein yield was determined by the method of (Lowry *et al.,* 1951).

c. **Differential solubilization and activation of crystal proteins:**

Protein inclusions were incubated at 37°C for 60 min. at the concentration of 2 mg/ml in 50 mM Na2Co3.Hcl buffer at pH 4.5, 10.5 H.5 and in the presence or absence of 10 mM dithiathreitol. Insoluble material was pelleted by centrifugation at 10000 xg for 10 min. Soluble proteins were precipitated by adding 12% (w/v) critic acid until the solution reached pH 4.5 then incubating at – 20°C for at least 3 hrs. These precipitates were centrifuged at 1000 x g for 15 min. and the pellets washed in 2 mM sodium citrate p-H 4.8 before toxins by gut extracts chymotrypsin, and trypsin were as described by Nicholls *et al.* (1984).

d. Bioassays:

Aedes aegypti

For each test assay, larval feed consisted of 3g wheat bran and 0.4g yeast extract throughly mixed and auto cleaved and 1.3 ml *Bt* Crystal suspension were mixed thoroughly into the feed, then added to 250 ml distilled water, and 20 one-day-old larvae added. Dead larvae were counted after 5-days, during which time normal healthy larvae grow and pupate the concentrations at which 50% of larvae were killed (LC50) were determined by measuring in triplicate the death rate at different toxin concentrations.

Bioassays.

Groups of 20 early fourth instars were treated in 100 ml of deionized water in 250-ml plastic cups. Eight or more concentrations of crystal/spore suspension producing mortality between 0 and 100% plus an untreated control were used for each dose-response test, and tests were replicated five times on five different days. Dead and moribund larvae were counted after 24 and 48 hrs. Data were subjected to probit analysis, (FInney 1971), by using a program written for the PC (Raymond et al. 1995). Lethal concentration values with overlapping fiducial limits were not considered to be significantly different. Toxin mixtures were prepared based on the weight of the crystal/spore powders. Interaction between toxins was evaluated by the method described by (Tabashnik, 1992) in which the theoretical toxicity of a toxin mixture was predicted from the toxicity of the individual components. The synergism factor (SF) at LC50 was calculated by dividing the predicted theoretical value for each toxin combination by the observed toxicity value. According to (Tabashnik, 1992), an SF ratio equal to 1 was additive, a ratio < 1 was antagonistic, and a ratio > 1 was synergistic. For this study, SF ratio of 1.5 or greater were classified as synergistic because they represented a 50% increase in toxicity, whereas SF values of 1.1-1.4 were classified as weakly synergistic. Five values fell into that latter classification and represented a single point at either 24 or 48 hrs, whereas the value for that same mixture at the alternative time (i.e., 24 or 48 hrs) fell into the synergistic class. To determine whether toxicity of the toxin mixtures was improved relative to the toxicity of

B. sphaericus against *Culex quinquefascitus*, its primary target, an improvement factor (IF50) was calculated by dividing the LC50 for *B. sphaericus* against Syn-P by the LC50 of each toxin mixture toward the various susceptible and resistant mosquitoes. IF50 values > 1 occur if a given toxin mixture is more toxic than *B. sphaericus* . For this study, an IF50 value of two-fold was used as threshold for improvement because it represented a two-fold improvement in toxicity.

3. Results and Discussion

Certain *Bt* strains are known to produce endotoxins, these are heat-stable adenine or uridine analogues, excreted from long phase cells which are through to inhibit DNA – dependent RNA polymerase and consequently have an indiscriminate toxicity spectrum. The ȝ - endotoxins, however, are heat – sensitive and highly specific, (**Levinson *et al.*, 1990**).

Results in table (1) indicated that the CryI protein mixtures had the expected toxicity to *Aedes aegypti* - CryIA proteins have been widely studied and only one has been shown to possess diptern toxicity (**Haider and Ellar, 1987**). Tough preliminary work suggests it many also be toxic to Coleoptera (**Bradley *et al.*, 1992**). Furthermore, it shares 62% amino acid identify with the Cryv endo toxin. It is toxic to *Coleoptera* and *Lepidoptera*. Thus, it is not clear whether the observed *Ae. aegypti* toxicity is due to an individual toxin or to some synergism between the toxins (**Filha *et al.*, 1999, Regis *et al.*, 2001, Yuan *et al.*, 2001; 2003; Wirth *et al.*, 2004**). When the number of toxins produced by *Bti* has been limited to less than the natural complement of 4 toxins, especially when populations are repeatedly challenged with single toxins, significant resistance has been induced (**Georghiou and Wirth, 1997, Wirth and Georghiou, 1997; Wirth *et al.*, 2003**). Repeated challenges of larvae with combinations of *Bti* Cry4 toxins in the absence of CytA toxins have also produced resistance, also the CytA enables Cry4 and Cry11A endotoxins to overcome or delay development of resistance in mosquitoes (**Rodchoem *et al.*, 1991, Yuan *et al.*, 2000; Wirth *et al.*, 2003**).

Results in table (2) indicate the interaction between Cry toxins and *B. sphaericus* varied depending on the toxin (s) and the mosquito colony tested. Only the combinations of *B. sphaericus* (Ctty11A) + Cry11A tested against susceptible strain of *Aedes aegypti* at 24 and 48 hrs, Ctty11A + (Cry4A + Cry4B) were antagonistic. While mixture of Ctty11A + (Cry4A + Cry4B + Cry11A) were weakly synergistic. All combinations, except Ctty11A + Cry4A tested against *Ae. aegypti* induce significant effect. No antagonistic interactions were observed but two interactions were additive or weakly synergistic. Ctty11A + *Bti* (5 : 1) against *Ae. aegypti* at 24 h and Ctty11A + *Bti* (10 : 1) against *Ae. aegypti* . Mixtures of *Bti* at different ratios were toxic than Ctty11A. Little difference in lethal concentration value was observed when the proportion of *Bti* was reduced. Data in table (3) and the obtained values of the Ctty11A + Cry toxins shows that lethal concentration values were positive for synergism and no interactions were antagonistic and only Ctty11A + (Cry4A+ Cry4B + Cry11A + Cyt1Aa) against susceptible strain of *Ae. aegypti* at 24 and 48 hrs were weakly synergistic. All combinations were as toxic, or more toxic than only Ctty11A. The broad spectrum of synergy that is now apparent suggests that complex interactions occur among most of the mayor toxins of *Bti* and Ctty11A and were responsible for increased toxicity. More importantly these interactions should provide some level of protection against insecticide resistance because they involve toxins that target different receptors in the mosquito mid gut. Although it is not clear whether the mechanism of synergism between *B. sphaericus* (Ctty11A) and Cyt1Aa is the same as those between

Ctty11A and Cry toxins, similar patterns of interaction were observed. For example, synergism factor ratios were lower against susceptible mosquitoes that possess a normal Ctty11A – receptor, whereas much higher synergism factor ratios were observed. One explanation for these results is that in susceptible mosquitoes, Ctty11A binds to its receptors leading to low synergism factor Ctty11A, because of its high activity in polluted water and long residual activity, has an important role in mosquito larval control that is at risk because of its limited host range. *Bti* and Ctty11A provide effective alternatives to broad spectrum larvicides in many satuations with little or no environmental impact. Their compatibility with other biological control agents will enable a more sustainable approach to mosquito control than would be possible with conventional chemical larvicides. (**Pereira *et al.*, 2008, Tabashnik *et al.*, 2009, Ghahan *et al.*, 2010, Tabashink *et al.*, 2011, Suchada *et al.*, 2011, Bravo, 2011**).

In conclusion, our study of synergism between Cry, Cty toxins of *Bti* and *B. sphaericus* toxins, may give a good evidence that mixing toxins with different combinations may be a good candidate as part of a multiple – toxin strategy to control toxin resistance in insect pests. The development of insect resistance to toxins is the major threat to the widespread adoption of *Bacillus thuringiesis* for pest control. Multiple – toxin strategies may potentially delay insect resistance. One possible strategy to control potential resistance within insect populations is to use Cry toxins (which were with high toxicity) to improve using *B. sphaericus* by `developing other different mechanisms of binding toxins to avoid the development of resistance.

Table (1): Toxicity of *Bacillus thuringiesis israelensis* (*Bti*) and *Bacillus sphaericus* toxins against susceptible strain of *Aedes aegypti.*

Toxin	Time (h)	LC50 µg/ml	LC95 µg/ml
B. thuringiesis (*Bti*)	24 48	0.0371 0.0127	0.125 0.163
B. sphaericus	24 48	0.210 0.471	2.39 0.262
Cry4B + Cry1A + Cry11A	24 48	0.0172 0.0135	0.108 0.0664
Cry4B + Cry4A	24 48	0.0723 0.107	1.50 0.503
Cryt1Aa	24 48	213 206	430 490
Cry11A	24 48	2.31 1.06	99.2 21.5
Cry4A	24 48	25.8 13.6	250 165

Table (2): Toxicity values and evaluation of synergism between Cry – toxins of *Bti* and (Ctty11A) toxicity of *Bacillus sphaericus* against *Aedes aegypti.*

Toxin	Time	LC50 (µg/ml)	Synergism Factor LC50	Improvement Factor LC50
Ctty11A + Cry4A (5 : 1)	24 48	0.0866 0.00571	27.4 2.4	22.3 4.1
Ctty11A + Cry11A (10 : 1)	24 48	0.160 0.0253	71.6 16.1	39.7 9.6
Ctty11A + Cry4A + Cry4B (5 : 1)	24 48	0.174 0.0116	15.4 2.6	5.2 2.9
Ctty11A + Cry4B + Cry11A (5 : 1)	24 48	0.0579 0.0178	1.64 0.721	3.49 30.9

Table (3): Toxicity values and evaluation of synergism between (Ctty11A) toxicity of *Bacillus sphaericus* and *Bti* against susceptible strain of *Aedes aegypti.*

Toxin	Time	LC50 (µg/ml)	Synergism Factor LC50	Improvement Factor LC50
Ctty11A + *Bti* (3 : 1)	24 48	0.0169 0.0272	2.8 5.1	4.9 7.2
Ctty11A + *Bti* (10 : 1)	24 48	0.463 0.581	4.8 18.1	5.3 16.7
Ctty11A + *Bti* (30 : 1)	24 48	0.370 0.00310	1.4 6.6	4.0 2.6
Ctty11A + Cry11A + Cry4A + Cry1B (6 : 1 : 1)	24 48	0.0539 0.0216	2.6 4.1	2.6 3.7
Ctty11A + Cry4A + Cry4B + CytIAa (6 : 1 : 1)	24 48	0.148 0.0167	4.6 6.8	3.2 4.4
Ctty11A + Cry4A + CytIAa (6 : 1 : 1)	24 48	0.0693 0.0138	1.7 1.2	1.6 0.96
Ctty11A + Cry11A + CytIAa (6 : 1 : 1)	24 48	0.238 0.0634	1.9 1.3	1.2 0.90

Corresponding author
Najat A. Khatter
Biology Department, Faculty of Science for Girls-King Abdulaziz University, Jeddah, Saudi Arabia
najat.khatter4@gmail.com

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