Evaluation of an experimental product based on *Bacillus thuringiensis* sorovar. *israelensis* against *Aedes aegypti* larvae (Diptera:Culicidae)

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Received 4 September 2006; accepted 6 March 2007

Available online 14 March 2007

Abstract

The larvicidal activity of an experimental formulation of *Bacillus thuringiensis israelensis* (Bti) against *Aedes aegypti* larvae was evaluated under laboratory and simulated field conditions (SFC). Samples of technical powder (TP) were assayed to establish the LC50 and the potency of the product. The larvicidal activity of the TP and the tablet (T) were evaluated under SFC to assess the efficacy and the residual activity, measured against *Ae. aegypti* larvae. Either a T or 250 mg of TP were added to 50 L of water in plastic containers. Containers were exposed to sunlight or kept in the shade. Results showed a LC50 of 0.26 mg/L and a potency of 750 ITU/mg. In spite of differences in the toxicity amongst TP and T samples, all of them killed 98–100% of the larvae and the mortality remained high for six months, in the shade. The replacement of 20% or 60% of the water volume did not affect the activity of the product. Seasonal differences influenced the persistence of the product in containers exposed to sunlight. Both formulations showed an excellent performance, especially when kept in the shade. The Bti tablet evaluated in this study is potentially very useful in programs to control dengue vectors.

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Keywords: Entomopathogenic bacteria; Bti; Tablet formulation; Semi field test; Mosquito control

1. Introduction

About two hundred microbial products for insect control are currently on the market, 50% of them based on *Bacillus* entomopathogenic bacteria (Alves et al., 1998). These microbial agents are the most widely used around the world for integrated pest management (IPM), especially to control the lepidopterans and coleopterans species. Effective control of aquatic larvae such as those of mosquitoes and black flies, have been achieved using *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis israelensis* (Bti) (Ruas Neto, 1984; Hougard et al., 1997; Mardini et al., 2000; Regis et al., 2001). During sporulation, Bti produces protein crystals with insecticide activity (delta-endotoxins), composed of four major protoxins Cry4A, Cry4B, Cry11A and CryA. Its mode of action involves ingestion and solubilization of crystals followed by the cleavage of protoxins, activation of toxins and interaction with the cells of the midgut epithelium of susceptible larvae (Gill et al., 1992). These toxins act synergistically to produce full toxicity, thereby making it difficult to select insect populations resistant to this entomopathogen. This fact has been demonstrated in a program to control floodwater and snowmelt species of mosquito in Germany, a country that has been using Bti since 1980 (Becker and Ludwig, 1993). Bti is a highly selective insecticide for controlling culicidae and simulidae larvae. This offers the additional advantage of not affecting non-target species of vertebrates and invertebrates, thereby ensuring the safety of its prolonged use on a large scale, without damaging the environment (Guillet* Corresponding author. Fax: +81 3453 2449.
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1049-9644/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2007.03.002
et al., 1990; Mardini et al., 2000; Regis et al., 2000; Regis et al., 2001; Lima et al., 2003). Since 2001, larvicides based on Bti have been used in Brazil, as part of the National Program of Dengue Control, as an alternative for management of resistance to temephos, an organophosphate insecticide, in *Aedes aegypti* (Linnaeus) populations. Temephos has been continuously used in Brazil for mosquito control since 1986 (Macoris et al., 1999; Fundação Nacional de Saúde, 2002; Macoris et al., 2003).

The production and formulation of Bti have been improved to attain the desired effect of larvae control in a variety of breeding sites. Apart from factors related to maintenance of the viability of the microorganism during fermentation, biomass concentration and recovery of the phases, the final processing of the formulation is extremely important for obtaining an efficient and viable product as far as the cost of production is concerned. Although liquid suspension is the least expensive formulation, it is also relatively unstable compared to dry formulations, because the active ingredient is very vulnerable to environmental adversities (Couch, 2000). In addition, recent researches have demonstrated that solid products with slow release of the active ingredient are more suitable for *Ae. aegypti* control (Becker, 2000; Melo-Santos et al., 2001).

Early evaluations of Bti products indicated variations of persistence of larvicidal activity between 1 and 3 weeks, depending on the environmental conditions where the product was applied, suggesting the need for frequent applications (Hougard et al., 1997; Mulla, 1990; Becnel et al., 1996; Batista Filho et al., 1998). New formulations more appropriate for typical breeding sites and the feeding behavior of the larvae have achieved increased persistence, resulting in economic, environmental and operational advantages (Becker and Rettich, 1994; Melo-Santos et al., 2001). For better field performance of the Bti, new products are continually being developed, in the search for greater stability, storage time, ease of handling, and operational use without loss of quality (Couch, 2000).

The present study reports the evaluation of an experimental tablet formulation of Bti for *Ae. aegypti* larvae control under laboratory and simulated field conditions.

2. Materials and methods

2.1. Mosquito

*Aedes aegypti* larvae from a Recife-Lab colony kept in the Department of Entomology CPqAM/FIOCRUZ since 1996 were used. The breeding conditions were 26 ± 2 °C, RH from 65% to 85%, photoperiod 12/12 (L/D), larvae were fed daily with sterilized, macerated cat food (Whiskas®).

2.2. Bti experimental product

For this study, samples of technical powder (TP) and a tablet (T) containing 15% (w/w) of active ingredient (AI) from different production lots of *Bacillus thuringiensis israeli-

dens* (Bti) strain IPS82, were provided by Biotecnológica Indústria e Comércio Ltda (BIOTICOM). The production process involved the use of bacterial growth medium containing mineral salts, glucose and corn steep liquor, and the discontinued immersed fermentation (batch). The culture was produced in a fermentator type agitator and the biomass was recovered using the flocculation/sedimentation technique to obtain the AI (spores and crystals) according to the methodology described by Luna et al. (2002), with some modifications. The fermentation beer obtained was centrifuged and dried at 35 °C to obtain the primary powder (PP), comprising the AI and medium solids. Adjuvant ingredients were added, including a sunscreen agent to obtain the TP. The same procedure was repeated to produce 10 batches of the product. Two hundred and fifty milligrams of TP were compressed to make each tablet.

2.3. Laboratory bioassay

The TP of each lot was evaluated regarding the toxic activity in *vivo* bioassays against 4th instar larvae (L4) of *Ae. aegypti*. The bioassays were performed according to the standard protocol for Bti preparations described by de Barjac and Larget-Thiery (1984). Homogenous groups of twenty L4 were exposed to seven different concentrations of the product in triplicates, for a period of 24 h. Three cups remained untreated for control purposes. The lethal concentrations for 50% (*LC*50) and 90% (*LC*90) of the larvae were estimated on the basis of mortality data, using linear log-probit regression, on the SPSS 8.0 for Windows (1997) program. Bioassays were carried out in two steps. The first was a primary screening consisting of one assay performed with all TP lots and the second consisted of three replicate assays with three lots that were chosen from the first experiment, based on their different activity levels. The *LC*50 median served as parameter for determining the potency of the experimental product, expressed in terms of international toxic units (ITU/mg), compared to IPS82 lyophilized standard from the Pasteur Institute. By way of an Exploratory Analysis of Data Test, an average LC50 was calculated and a 95% interval confidence was determined for the different batches.

2.4. Viable spores quantification

The concentration of viable spores was estimated through bacterial suspension of TP (10 mg/4.5 ml of sterile water) by counting colonies after a thermal shock that kills vegetative cells (kept at a temperature of 80 °C for 12 min). After being sequentially diluted, 5 μl samples were placed in five points on petri dishes containing nutrient agar and incubated at 28 °C for 18–20 h. The colonies formed were counted and expressed as c.f.u. ml.

2.5. Trials under simulated field conditions (SFC)

These tests were carried out to evaluate the performance of the TP and T formulations from different batches under
simulated field conditions. The experiments were conducted using the methodology described by Melo-Santos et al. (2001). The doses tested were: 250 mg of the TP or one tablet for every 50 L of water from a well. The experiments were performed in transparent plastic containers (56.4 × 38.5 × 37.1 cm) covered with screened lids and that were placed in a greenhouse (96 m²) in shelves permitting direct exposure to sunlight, or in shelves under the shade (Fig. 1). Each experiment was carried out in triplicate and three containers were left untreated as a control. The temperature and the pH of the water were checked three times a week.

2.6. Experimental treatments

The effects of two variables on the residual activity of the product were evaluated: direct exposure to sunlight (Fig. 1) and degrees of water replenishment according to the experimental design shown in Table 1.

2.7. Determination of residual activity

The following parameters were used to evaluate the performance of the product. The first was the initial efficacy, measured using the mortality rate of fifty L4 per container recorded 48 h after application of the product. The second parameter was the persistence or residual larvicidal activity, assessed by counting live pupae resulting from weekly introduction of fifty L1, observed until larvae mortality dropped to 80% or less. Larvae cadavers were not removed from the containers. Persistence was compared using a Mann–Whitney test for two samples, at a 5% significance level, on the SPSS version 8.0 for Windows (1997) statistics program.

2.8. Determination of product shelf life

The stability of the product in terms of shelf life was investigated at three-month intervals for a total period of two years. Tablets from batches 1 and 2 were tested. Eight individual packages containing 10 tablets each were stored in a dry place, away from light, at temperatures varying from 25 to 27 °C and relative humidity (RH) ranging from 60% to 80%. Three out of the 10 tablets from a package were randomly chosen for evaluation of the initial efficacy against L4 in the SFC test. At the beginning and at the end of the study period the TP activity was also evaluated by bioassays.

3. Results

3.1. Evaluation of toxic activity and concentration of viable spores

The values of LC₅₀ and LC₉₀ from the 10 TP lots are shown in Table 2. The lots show differences in toxicity levels and were grouped according to the value of LC₅₀ at the respective confidence intervals, namely: (1) 0.133–0.166 mg/L; (2) 0.184–0.279 mg/L; (3) 0.390–0.497 mg/L; and (4) >1.2 mg/L. Lot 3 showed the lowest toxicity compared to the others, and was therefore considered an “outlier” (Fig. 2A) and excluded from subsequent evaluations.

The analysis of the confidence intervals (95%) indicated an average LC₅₀ of 0.26 ± 0.10 mg/L for the product, and its potency, based on an LC₅₀ of 0.013 mg/L of the IPS82, was 750 ITU/mg of product. Lots 1 and 7 showed greater larvicidal activity and lots 4 and 5 showed slightly less activity than the average (Fig. 2B). The microbiological viability of TP lots 1, 5, and 10, estimated by the number of colonies per milliliter, were similar, although their LC₅₀s differed up to 3.7 times (Table 3). Tablets made with TP lots 1, 5 or 10, representatives of each confidence interval were tested under SFC in order to detect any performance differences.

3.2. Product performance under simulated field conditions

On SFC, the initial efficacy of both TP and T from lot 1 was 100%. The efficacy of lot 5 and lot 10 tablets was 98%. In control recipients the mortality varied from 0 to 16%.
The results relating to persistence of larvicidal activity in recipients kept away from sunlight (Table 4) revealed that all TP and T persisted to eliminate all larvae during the 180-day experiment period, after a single treatment, with no meaningful differences between them (p = 1). In the last evaluation period, a batch of L4 was added to the containers in order to find out whether the product still remained toxic for larvae in advanced stages, and a mortality rate of 99.1% was observed, demonstrating the toxicity of the product even after 180 days. The larvicidal activity of lot 1 remained unaltered in spite of the periodic replenishment of up to 60% of the water level in containers, showing that even with water renewal, both TP and T went on to eliminate all larvae within a 180-day period.

When exposed to sunlight, residual larvicidal activity in recipients treated with TP and T from lot 1 continued for 11 weeks (February–May 2005), with larval mortality rate fluctuating between 55% and 100% (Fig. 3). The lowest mortality rate was registered one week after treatment, which represented the period with the largest number of hours of sunshine during the experiment (9.2 h/day). With regard to TP and T from lot 10 tested during September–October 2005, a progressive decrease in the mortality rate was recorded after the second week, with complete loss of larvicidal activity after four weeks (Fig. 4). The highest number of hours of sunlight per day was observed in this period. When the same products were tested during November/2005 to March/2006, larval mortality rates ranged from 66% to 97% for 17 weeks (Fig. 5). In all tests carried out under sunlight, a 100% mortality rate was observed only during the first week of treatment.

The water temperature in the recipients varied from 26.5 °C to 31.3 °C in the sunlight and from 26.3 °C to 29.8 °C in the shade throughout the testing period. The water pH varied from 6.9 to 9.9 in the recipients kept in the sun and from 6.9 to 8.4 in those kept in the shade.

The stability tests of the tablets demonstrated no alteration of the initial efficacy of the product under SFC, throughout two years. The larval mortality rates caused by the product samples kept under normal storage conditions, remained above 97% over 24 months (Table 5). However, a reduction of the TP activity was detected through a bioassay performed after two years of storage. The LC90 value increased from 0.24 to 0.46 mg/L for lot 1 and from 0.42 to 0.79 mg/L for lot 2.

### Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Formulation</th>
<th>Experimental conditions</th>
<th>Evaluation period</th>
<th>Water replenishment (%)</th>
<th>Total of larvae b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot 1</td>
<td>Technical Power</td>
<td>Sun</td>
<td>February–May/2005</td>
<td>20</td>
<td>1650</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>February–August/2005</td>
<td>0 e 20</td>
<td>7200</td>
</tr>
<tr>
<td></td>
<td>Tablet c</td>
<td>Sun</td>
<td>February–May/2005</td>
<td>20</td>
<td>1650</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>February–August/2005</td>
<td>0, 20 e 60</td>
<td>10800</td>
</tr>
<tr>
<td>Control</td>
<td>Sun</td>
<td>February–May/05</td>
<td>20</td>
<td>1650</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shade</td>
<td>February–August/05</td>
<td>0, 20 e 60</td>
<td>3600</td>
<td></td>
</tr>
<tr>
<td>Lot 10</td>
<td>Technical Power</td>
<td>Sun</td>
<td>September–October/05</td>
<td>20</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>November/05–March/06</td>
<td>20</td>
<td>2550</td>
</tr>
<tr>
<td></td>
<td>Tablet</td>
<td>Sun</td>
<td>September–October/05</td>
<td>20</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>November/05–March/06</td>
<td>20</td>
<td>2550</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>September/05–March/06</td>
<td>20</td>
<td>3600</td>
</tr>
<tr>
<td>Lot 5</td>
<td>Tablet</td>
<td>Sun</td>
<td>September–October/05</td>
<td>20</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>November/05–March/06</td>
<td>20</td>
<td>2550</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>September/05–March/06</td>
<td>20</td>
<td>3600</td>
</tr>
<tr>
<td>Control</td>
<td>Sun</td>
<td>September–October/05</td>
<td>20</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shade</td>
<td>November/05–March/06</td>
<td>20</td>
<td>2550</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>September/05–March/06</td>
<td>20</td>
<td>3600</td>
</tr>
</tbody>
</table>

a Tablet containing 15% of active ingredient.
b 46,800 larvae represents weekly colonization with 50 L1/recipient.

### Table 2

<table>
<thead>
<tr>
<th>Technical Powder</th>
<th>P* Value</th>
<th>LC50 b (mg/L) (Confidence Interval 95%)</th>
<th>LC90 c (mg/L) (Confidence Interval 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>0.621</td>
<td>0.144(0.133–0.154) 0.204(0.191–0.222)</td>
<td></td>
</tr>
<tr>
<td>07</td>
<td>0.631</td>
<td>0.155(0.146–0.166) 0.237(0.219–0.263)</td>
<td></td>
</tr>
<tr>
<td>09</td>
<td>0.330</td>
<td>0.203(0.184–0.222) 0.372(0.340–0.416)</td>
<td></td>
</tr>
<tr>
<td>08</td>
<td>0.469</td>
<td>0.233(0.214–0.254) 0.387(0.356–0.427)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.961</td>
<td>0.237(0.213–0.265) 0.436(0.388–0.506)</td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>0.530</td>
<td>0.246(0.221–0.273) 0.415(0.377–0.465)</td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>0.722</td>
<td>0.254(0.231–0.279) 0.419(0.381–0.469)</td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>0.839</td>
<td>0.437(0.390–0.497) 0.786(0.694–0.919)</td>
<td></td>
</tr>
<tr>
<td>03 d</td>
<td>0.647</td>
<td>0.439(0.396–0.485) 0.799(0.728–0.893)</td>
<td></td>
</tr>
</tbody>
</table>

* P = Probability for 5% significance.
+ C50 = Concentration lethal for 50% larvae.
+ LC90 = Concentration lethal for 90% larvae.
d Lot not considered in evaluation, for presenting low activity.
4. Discussion

The product based on Bti evaluated in this work in technical powder and tablet form showed the desired toxic activity for *Aedes aegypti* larvae, stability under normal storage conditions and excellent persistence under simulated field conditions, mainly in tests performed in the shade.

The LC₅₀ for *Aedes aegypti* larvae of TP samples produced under standardized conditions, showed variations of up to 3.7-fold. Nine out of ten lots showed acceptable toxicity levels. Two of them showed larvicidal activity greater than the estimated average (LC₅₀ = 0.26 ± 0.1 mg/L, potency of 750 ITU/mg) and two had less activity than the average. The literature on the production of entomopathogenic bacterium registers variations in the toxicity, considered as inherent to the production process that results from several factors relating to temperature, dissolved oxygen, pH and sugar concentration during the fermentation process or to the loss of crystals during biomass recovery and formulation (Couch, 2000; Skovmand et al., 2000). According to Skovmand et al. (2000), there is not always a correlation between the concentration of spores of a given culture and the quantity of crystals produced during the fermentation process. In fact, this was the reason for replacement of concentration of spores as a parameter to measure toxicity (LC₅₀) when the product was standardized (Skovmand et al., 2000). Our results corroborate this observation, since there was no clear correlation between concentration of spores and the toxicity for the different lots of the product (Table 3). In the simulated field test, samples of TP and T from three different lots demonstrated similar performance in the shade, suggesting that the differences observed between the LC₅₀ are not enough to affect larvicidal activity in the field. Some studies suggest that the potency of a product may not be an accurate indicator of its performance in the field, so far as the persistence of the larvicidal activity is concerned (Vilarinhos and Monnerat, 2004). The potency of the product is, however, a very important parameter for the standardization of the production process, which must vary as little as possible (Skovmand et al., 1997; Habib et al., 1998; Skovmand et al., 2000).

![Graph A](image1.png)

**Fig. 2.** Values of LC₅₀ of the technical powders of the experimental product based on *Bacillus thuringiensis israelensis*, from different production lots, measured by bioassay against *Aedes aegypti* larvae. (A) Including all the lots. (B) Excluding lot 3, which was considered an outlier.

**Table 3**

Toxic activity of technical powder (TP) from different production lots of *Bacillus thuringiensis israelensis* (Bti), evaluated in bioassay against 4th instar *Aedes aegypti*-Recife-Lab larvae and viable spore concentration

<table>
<thead>
<tr>
<th>Products</th>
<th>LC₅₀ᵃ (mg/L)Average ± SD (Confidence Interval 95%)</th>
<th>LC₉₀ᵇ (mg/L)Average ± SD (Confidence Interval 95%)</th>
<th>Viable spores (c.f.u./ml)ᶜ⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-1</td>
<td>0.140 ± 0.004(0.126–0.154)</td>
<td>0.241 ± 0.040(0.220–0.269)</td>
<td>1.5 × 10⁷</td>
</tr>
<tr>
<td>TP-5</td>
<td>0.514 ± 0.097(0.465–0.573)</td>
<td>0.889 ± 0.161(0.797–1.02)</td>
<td>1.3 × 10⁷</td>
</tr>
<tr>
<td>TP-10</td>
<td>0.245 ± 0.014(0.207–0.298)</td>
<td>0.436 ± 0.002(0.373–0.575)</td>
<td>2.5 × 10⁷</td>
</tr>
<tr>
<td>Lyophilized-IPS82ᵉ⁻¹</td>
<td>0.013 ± 0.002(0.011–0.015)</td>
<td>0.026 ± 0.004(0.023–0.031)</td>
<td>4.2 × 10⁹</td>
</tr>
</tbody>
</table>

ᵃ LC₅₀ = Concentration lethal for 50% larvae.
ᵇ LC₉₀ = Concentration lethal for 90% larvae.
ᶜ Average of three bioassays ± standard deviation.
ᵈ c.f.u/ml = colony formed unit per milliliter.
ᵉ IPS82 = The international standard for *Bacillus thuringiensis israelensis*. 
In the course of the production process for an entomopathogenic agent such as Bti, it is expected that an increase in toxic activity will occur as a result of the harvest of the spores and δ-endotoxin at the end of fermentation. In further stages of the process, more specifically to those relating to drying and formulation, the toxicity of the product can be expected to decrease or remain the same (Couch, 2000). In our study, the compression of the technical powder to obtain the tablet does not seem to lead to a loss of larvicidal activity, indicating that this process does not damage or impede the release of the active ingredient. The product in the form of a tablet facilitates the operational work, as it does not require the use of measurement tools, reduces the risk of dosage failure and losses in the application. According to Becker (2003), the development of solid formulation in the form of granules, donuts, tablets, etc, as well as appropriate application methodologies

### Table 4
Persistence of the technical powder (TP) and tablet based on *Bacillus thuringiensis israelensis*, against *Aedes aegypti*-Recife-Lab larvae in containers in the shade from February/2005 to March/2006. Tests were carried out in triplicates

<table>
<thead>
<tr>
<th>Group</th>
<th>Evaluation period</th>
<th>Product</th>
<th>No. larvae</th>
<th>Evaluation period (days)</th>
<th>Mortality(^b) larvae (%) over the period</th>
<th>(X \pm DP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-1</td>
<td>February–August/05</td>
<td>TP</td>
<td>3.600</td>
<td>180</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Control(^d)</td>
<td>February–August/05</td>
<td>Tablet</td>
<td>3.600</td>
<td>180</td>
<td>4.6 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>TP-5</td>
<td>September/05–March/06</td>
<td>Tablet</td>
<td>3.600</td>
<td>180</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TP-10</td>
<td>September/05–March/06</td>
<td>Tablet</td>
<td>3.600</td>
<td>180</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>September/05–March/06</td>
<td>—</td>
<td>3.600</td>
<td>180</td>
<td>4.4 ± 0.41</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Period after single treatment with product, during which mortality of larvae exceeded 80%.

\(^b\) Mortality of larvae during the period.

\(^c\) Average ± standard deviation.

\(^d\) Without treatment with Bti.

In the course of the production process for an entomopathogenic agent such as Bti, it is expected that an increase in toxic activity will occur as a result of the harvest of the spores and δ-endotoxin at the end of fermentation. In further stages of the process, more specifically to those relating to drying and formulation, the toxicity of the product can be expected to decrease or remain the same (Couch, 2000). In our study, the compression of the technical powder to obtain the tablet does not seem to lead to a loss of larvicidal activity, indicating that this process does not damage or impede the release of the active ingredient. The product in the form of a tablet facilitates the operational work, as it does not require the use of measurement tools, reduces the risk of dosage failure and losses in the application. According to Becker (2003), the development of solid formulation in the form of granules, donuts, tablets, etc, as well as appropriate application methodologies

![Fig. 3. Residual activity of the technical powder and lot 1 tablet based on *Bacillus thuringiensis israelensis* against 1st instar larvae of *Aedes aegypti*, when exposed to sunlight. Mortality values compared with the level of weekly insolation, from February to May 2005. Source: National Institute of Meteorology (INMET), Station Curado/Recife-PE.](image)

![Fig. 4. Residual activity of the technical powder and lot 10 tablet based on *Bacillus thuringiensis israelensis* against 1st instar larvae *Aedes aegypti*, when exposed to sunlight. Mortality rate compared with level of weekly insolation, from September to October 2005. Source: National Institute of Meteorology (INMET), Station Curado/Recife-PE.](image)

![Fig. 5. Residual activity of the technical powder and lot 10 tablet based on *Bacillus thuringiensis israelensis* against 1st instar larvae *Aedes aegypti*, when exposed to sunlight. Mortality rate compared with the level of weekly insolation. Source: National Institute of Meteorology (INMET), Station Curado/Recife-PE.](image)
gives rise to advantages in operational control programs, such as ease of storage, transportation and applicability in the field, or greater stability and sustained release of the active ingredient. Formulations of the tablet type may be more adequate for the particular characteristics of breeding sites of mosquito species such as *Ae. aegypti*, as they are more practical to apply and more accepted by the population, especially since they do not change the appearance of the water and do not generate solid residues in the recipient (Becker, 2000; Melo-Santos et al., 2001).

At the concentration used in the SFC tests, the tablet remained efficient after two years of storage, suggesting that the characteristics relating to the larvicidal activity of the pathogen, and the preservation and rate of release of the active ingredient remained stable enough to promote high larval mortality, a fact evidenced by the reproducibility of the results throughout 24 months of testing. However, these results indicated that the evaluation of initial activity under SFC using field dose (approximately 11 folds the LC₉₀ value) is not sensible enough to detect loss of the TP toxicity.

According to information from FUNASA (2002), the most common breeding sites for *Ae. aegypti* in Brazil are domestic containers for water storage. Common characteristics of most of these are to be partially or totally covered with lids, although they are not always mosquito-proof, and the water is constantly used and replaced. In the experimental design of the SFC we chose to simulate the replenishment of water in domestic containers and exposure to the sun. The colonization of containers treated with L1 is also a way to simulate conditions in the field, since the female mosquitoes lay their eggs in the treated breeding site. The use of L1 instead of older larvae increases the sensitivity of the test, as demonstrated by Melo-Santos et al. (2001).

In the case of Bti, whether exposed to sunlight or not, both the TP and the T had identical initial control efficacy (mortality rates from 98% to 100%), indicating that the product formulated releases a satisfactory amount of crystals in the trophic zone of the larvae within the first 48 h after application. This factor is extremely important to ensure efficient larvicidal activity after a few hours, since under natural conditions larvae from different stages may be present in the container at the time of application of the product. In the shade, the products tested remained 100% efficacious for 6 months. The replenishment of up to 60% of the treated water and the consequent dilution of the product could have a negative effect on the persistence of larvicidal action, although this did not occur during the period of observation (180 days). The tablet settles quickly and remains at the bottom of the container, suggesting that most of the active ingredient remains concentrated in that region. Thus, it is possible that the process of water refilling does not agitate the water sufficiently to cause crystals and spores to resurface, which would favor its elimination. Benjamin et al. (2005), testing VectoBac DT² tablet in earthen containers for drinking water with weekly 50% water replenishment, observed that the formulation sank to the bottom on introduction and the Bti toxin was concentrated along the sides and at the base of the treated containers, with the result that the treated domestic water used by people contained little or no Bti toxin. The authors reported a 166-day persistence of this product against *Aedes* spp. Long persistence of a tablet based on Bti, was also observed by Mulla et al. (2004), who registered excellent control of *Ae. aegypti* larvae in ceramic jars for a period of about 112 days. It is known that the persistence of the products depends as much on the characteristics of its own formulation as well as the environmental conditions of the breeding site. The continuous viability of the spores is another important factor, because under favorable conditions recycling may occur through the production of toxins in the midgut of larvae killed by Bti (Aly, 1985; Aly et al., 1985; Khawaled et al., 1988).

The results of this study confirm the importance of solar radiation as a negative factor in the persistence of Bti in the environment, as observed in previous studies (Obeta, 1996; Nayar et al., 1999; Thiéry et al., 1999; Melo-Santos et al., 2001; Vilarinhos and Monnerat, 2004). In the test performed during the period of higher incidence of solar radiation (October 2005), there was a rapid decrease in the mortality rate, reaching less than 70% in the second week and total loss of larvicidal activity in the 4th week after treatment. Similar results have been observed in previous studies using containers placed outdoors (Melo-Santos et al., 2001; Vilarinhos and Monnerat, 2004). In the containers exposed to sunlight, changes in some aspects regarding water quality were observed after the first week, such as micro algae growth and increased alkalinity. The presence of microorganisms and detritus in suspension in

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**Table 5**

Mortality of 4th instar *Aedes aegypti* larvae, according to the storage time of tablet based on *Bacillus thuringiensis israelensis*, from two different production lots, kept at ambient with temperature varying from 25 to 27 °C and relative humidity from 60% to 80%. The tests were carried out every three months for two years.

<table>
<thead>
<tr>
<th>Tablet</th>
<th>Mortality of larvae (%)</th>
<th>every 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>01</td>
<td>98.7 ± 1.1</td>
<td>100.0</td>
</tr>
<tr>
<td>02</td>
<td>99.1 ± 0.5</td>
<td>98.7 ± 1.1</td>
</tr>
<tr>
<td>00 (control)</td>
<td>0</td>
<td>1.0 ± 1.4</td>
</tr>
</tbody>
</table>

* Average ± Standard deviation.
the aquatic environment may affect the availability of bioinsecticides due to the adsorption of Bti or Bs spores and crystals to particulates before they settle to the bottom of the breeding site (Ohana et al. 1987; Skovmand and Baudoinqu 1997; Sheeran and Fisher, 1992). Moreover, a very alkaline pH favors the solubility of the crystals, making pro-toxins more prone to degradation. The association of such factors would contribute to reducing the larvicidal activity of the product, especially when solar radiation reaches levels ≥ 9 h of continuous sunlight per day. On the other hand, the prolonged larval control observed under specific environmental situations, suggests that Bti recycling might compensate partial loss of toxic crystals caused by UV and others deleterious factors. In tests under exposure to sunlight performed at other times of the year, the persistence was surprisingly long: 77–119 days. Despite the decrease in levels of mortality below the limit previously established (80%), the evaluation was maintained on such tests in order to confirm the loss of activity of the product. It became evident that suspension of evaluation at that time would lead to an erroneous interpretation of the persistence time, since larvicidal activity was restored in the second week after treatment, keeping the mortality rate over 80% (Figs. 3 and 5).

Bacterial recycling may also play an important role in the length of persistence. In recent semi-field tests, we observed a higher concentration of Bti spores in plastic containers six months after a single treatment, providing observed a higher concentration of Bti spores in plastic of the persistence time, since larvicidal activity was restored previously established (80%), the evaluation was maintained under specific environmental situations, suggests that Bti recycling might compensate partial loss of toxic crystals caused by UV and others deleterious factors. In tests under exposure to sunlight performed at other times of the year, the persistence was surprisingly long: 77–119 days. Despite the decrease in levels of mortality below the limit previously established (80%), the evaluation was maintained on such tests in order to confirm the loss of activity of the product. It became evident that suspension of evaluation at that time would lead to an erroneous interpretation of the persistence time, since larvicidal activity was restored in the second week after treatment, keeping the mortality rate over 80% (Figs. 3 and 5).

Bacterial recycling may also play an important role in the length of persistence. In recent semi-field tests, we observed a higher concentration of Bti spores in plastic containers six months after a single treatment, providing a similar long-term control of Ae. aegypti larvae (Araújo et al., unpublished data), suggesting the occurrence of Bti recycling in this particular environment. It is important to highlight that, besides living in clean water, Aedes lar- vae, differently from Culex quinquefasciatus larvae, feed preferentially on the bottom and wall surfaces, which greatly favors a prolonged control resulting from Bti recycling.

The results confirm the efficacy of this product for Ae. aegypti larvae control. The tablet was shown to be the most appropriate formulation for use in domestic containers, to be easily applied, and to last for long periods without a reduction in excellent levels of activity.

Acknowledgments

We are grateful to CAPES and Fundação Oswaldo Cruz/PDTSP/Rede Dengue for the financial support, to the Institute Pasteur, for providing the Bti reference powder IPS82 and to Dr. André Freire Furtado and Dr. Tereza Magalhães for the valuable scientific contribution in the correction of this manuscript.

References


