Evaluation of a new formulation of *Bacillus thuringiensis israelensis*

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Abstract

The aim of this study was to determine the potency (ITU) and efficacy of a liquid formulation of *Bacillus thuringiensis israelensis* developed by the State University of Londrina named BioUel, against early fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*. The ITU/mg of BioUel was 960, the LC_{50} was of 0.271 (± 0.39) ppm, and the LC_{95} was 0.634 (± 0.099) ppm, in larvae of *C. quinquefasciatus*. In *A. aegypti* larvae, LC_{50} was 0.332 (± 0.042) ppm and LC_{95} was 0.694 (± 0.073) ppm. The ITU level of BioUel and its control results were similar to most commercial products tested. Stability was of approximately 90 days, which allows for local production.

Keywords: Aedes aegypti, biological control, bio-insecticide, *Culex quinquefasciatus*.

Avaliação de um novo formulado de *Bacillus thuringiensis israelensis*

Resumo

O trabalho determinou a potência (UTI) e a eficácia de um formulado líquido de *Bacillus thuringiensis israelensis* desenvolvido pela Universidade Estadual de Londrina, designado como BioUel, em testes com larvas de quarto instar inicial de *Aedes aegypti* e *Culex quinquefasciatus*. A UTI/mg do Biouel foi de 960, a CL_{50} foi de 0,271 (± 0,39) ppm e a CL_{95}, de 0,634 (± 0,099) ppm, em larvas de *C. quinquefasciatus*. Para larvas de *A. aegypti*, a CL_{50} foi de 0,332 (± 0,042) ppm e a CL_{95}, de 0,694 (± 0,073) ppm. A potência do BioUel e os resultados de controle foram comparáveis à maioria dos produtos comerciais testados. A estabilidade foi de aproximadamente 90 dias, o que permite uma produção local.

Palavras-chave: Aedes aegypti, controle biológico, bioinseticida, *Culex quinquefasciatus*.

1. Introduction

According to Forattini (2002), mosquito control is carried out mainly with chemical products, such as organophosphates, carbamates and pyrethroids. The utilisation of these products, however, has been limited because they are non-specific, pollute the environment, and their target insects have high rates of resistance. This situation has led to the development of biological insecticides using bacteria, fungi, viruses, and other alternative organisms (Consoli et al., 1997; Regis et al., 2001; Arantes et al., 2002; Tauil, 2002; Zequi and Lopes, 2007; Russell and Kay, 2008). The emphasis of this study is on the use of entomopathogenic bacteria of the genus *Bacillus* because of its high degree of specificity and environmental safety, thus particularly suitable and environmentally friendly for use in the control of insect pests.

In 1975, a new line of *Bacillus thuringiensis* (Bt) known as the variety *israelensis* (Bti) was identified and classified as serotype H-14 (Goldberg and Margalit, 1977), with high larvicidal activity for various genera of culicids and simulids. In Brazil, the use of commercial products based on Bt is burdensome because it must be imported and distributed. Local production would reduce costs and make it more affordable for use in the control of disease vectors; it would also promote the development of the local fermentation industry. The development of new bio-insecticide products and comparative studies with the commercially available formulations will determine the most efficient formulations and application strategies against insects.

The Londrina State University (Universidade Estadual de Londrina; UEL), in Paraná, southern Brazil, has been developing a product based on Bti, called BioUel. The research is currently at the formulation stage, and experiments

must be conducted to determine the formulation potency in international toxic units (ITU), as well as its efficacy and shelf life.

The ITU for the liquid formulation of BioUel was calculated by bioassays using larvae of *Aedes* (*Stegomyia) aegypti* (Linnaeus, 1762) and its efficiency and stability was compared with commercial products in the laboratory, with bioassays using larvae of *A. aegypti* and *Culex quinquefasciatus* Say, 1823.

2. Material and Methods

2.1. Production of the bio-insecticide

Fermentation was carried out in a 5 litre-capacity Discovery fermenter, MRV, Incelth, France. The composition of the culture medium was as follows: ($g.L^{-1}$) yeast extract, 4.0; glucose, 8.0; $MgSO_4$-$7H_2O$, 0.1; KH_2PO_4, 0.1; $MnSO_4$. $4H_2O$, 0.03; $CaCl_2$. $2H_2O$, 0.04; $(NH_4)_2SO_4$, 0.1. The medium was sterilised in autoclave at 120 °C for 20 minutes. Glucose and mineral salts were sterilised separately. The pH was adjusted to 7.0 before sterilisation. Fermentation occurred at 30 °C, 400 rpm and aeration rate of 1.5 vvm. After sporulation, the crystal-spore mixture was filtered in a tangential filter, 40-fold concentrated (Microdyn Filter/Module MD 020 / Frings Mycrodyn) and then dried in a forced-air oven.

The liquid formulation as concentrated suspension was produced by mixing Bti primary powder with detergent, emulsifier, sun block and dispersal agents for easy scattering and slow sedimentation.

2.2. Determination of potency

The experiments were conducted in the Laboratory of Entomology, Department of Animal and Plant Biology, UEL, using early fourth instar larvae of *A. aegypti* and *C. quinquefasciatus* obtained from eggs kept at the lab insectarium.

The BioUel potency (ITU/mg) was evaluated following procedures described by WHO (1999) comparing with the International Standard IPS-82, for titration, against laboratory-reared *A. aegypti* larvae. A battery of bioassays was performed with five repetitions for each of six different concentrations tested and one untreated control, performed at 25 ± 2 °C. For IPS-82 the dose consisted of 0.04, 0.03, 0.02, 0.01, 0.008, and 0.005 mg/L. Distilled water was added to reach 150 mL, where 25 *A. aegypti* larvae were added. For the BioUel suspension, doses were 0.5, 0.3, 0.23, 0.17, 0.10 and 0.06 mg/L. Initial bioassays were also conducted with the fermented solution for the determination of the adequate concentration of crystals.

Larval population densities were assessed 24 hours after treatment and data were submitted to Probit analysis (Micro Probit program) to determine the LC_{50} of BioUel. The potency in ITU of BioUel was calculated using the following Equation 1:

$$\frac{\text{ITU / mg}}{(\text{test product})} = \frac{15.000\,(\text{ITU / mg standard}) \times LC_{50}\,(\text{mg / L})\ \text{of standard}}{LC_{50}\,(\text{mg / L})\ \text{of test product}} \qquad (1)$$

BioUel stability was determined by placing 500 mL of the product in a white opaque bottle kept in a cabinet in the laboratory, in local conditions. The bioassays were performed following the same method used in test the efficacy of BioUel, at concentrations of 0.5, 0.29, 0.22, 0.18, 0.13, and 0.10 ppm immediately after the product was produced, and also after 30, 60, 90, 120, and 150 days. Larval population densities were assessed 24 hours after treatment. The results were submitted to Probit Analysis (Micro Probit program) to determine BioUel LC_{50} and LC_{95}. The Tukey test was applied to the results obtained in the stability study.

3. Results and Discussion

The ITU level obtained for the new Bti formulation identified as BioUel, with *A. aegypti* larvae, was 960 (960 ITU/mg). BioUel has an ITU level comparable to commercial products and to products cited in the literature (Sharma et al., 2008), as shown in Table 1. For a new formulation comprising Bti, Melo-Santos et al. (2001) obtained 1146 ITU/mg, and Rodriguez et al. (1991) found 109.1 ITU/mg.

Bioassays evaluating BioUel efficacy to control *C. quinquefasciatus* larvae had LC_{50} of 0.271 (± 0.39) ppm, and LC_{95} of 0.634 (± 0.099) ppm (Table 2). For *A. aegypti* larvae, LC_{50} was 0.332 (± 0.042) ppm and LC_{95} was 0.694 (± 0.073) ppm (Table 3). These results suggest higher sensitivity in *C. quinquefasciatus* larvae. It is known that the control efficiency of insect disease vectors depends on formulation type and feeding behaviour of the mosquito larvae, in addition to the species susceptibility to the *Bacillus* toxin. As we applied the same formulation on species *C. quinquelasciatus* and *A. aegypti* kept in a container with less feeding space, it is supposed the LC50 and LC95 results were different for each species, due to differences in each species susceptibility.

The fermentation product was evaluated in bioassays, and the mean LC_{50} for *A. aegypti* larvae was 4.81 ppm and 0.14 ppm, respectively, before and after being filtered. The mean of 0.33 ppm obtained for the BioUel (Table 3) shows a loss of potency of about 2.4 times, probably due to the interaction between formulation ingredients and protein crystals. A loss of 1.5 times was actually expected.

Studies to understand the lethal action of commercial or new formulations of bio-insecticides with Bti-based active principles are conducted regularly (Ibarra et al., 2003; Praça et al., 2004; Lima et al., 2005; Osborn et al., 2007). However, the target-species in experiments vary according to the mosquito species of major health concern in each region. The type of formulation used also depends on the species. This diversity makes comparison of results difficult. Rabinovitch et al. (1999) tested a new isolate of Bti, called LFB-Fiocruz 710, with an LC_{50} of 0.03 mg/L for *Aedes albopictus* (Skuse, 1894), whereas for *Anopheles darlingi*, Root, 1926 the LC_{50} was 4.8 mg/L. Nayar et al. (1999) tested Vectobac® against larvae of *Culex nigripalpus* (Theobald, 1901) and found 0.131 and 0.208 ppm for LC_{50}

Table 1. Products containing *Bacillus thuringiensis israelensis* and respective potencies.

Product	Declared formulation potency	
BioUel	Aqueous suspension	960 ITU/mg[(1)]
Teknar	Aqueous suspension	3000 AAU/mg[(2)]
Vectobac AS	Aqueous suspension	1200 ITU/mg
Bthorus	Aqueous suspension	1200 ITU/mg

[(1)]Aedes aegypti Units; [(2)]International toxic unit; Source: Vilarinhos et al., 1998 - Modified.

Table 2. Lethal concentrations (LC_{50} and LC_{95}) of BioUel using early fourth instar larvae of *Culex quinquefasciatus* at mean room temperature of 25 ± 2ºC and room photoperiod (n = 25 larvae).

	LC_{50} (ppm)	Lower limit	Upper limit	LC_{95} (ppm)	Lower limit	Upper limit
	0.2743	0.23	0.3741	0.6557	0.4774	1.4295
	0.2044	0.1792	0.2329	0.497	0.3959	0.7334
	0.2937	0.2591	0.3488	0.647	0.4902	1.1368
	0.2817	0.2489	0.3267	0.5985	0.4698	0.9487
	0.3015	0.2621	0.3668	0.7728	0.5597	1.4773
Mean	**0.27112**	**0.23586**	**0.32986**	**0.6342**	**0.4786**	**1.14514**
SD	**0.0387505**	**0.03407**	**0.05721**	**0.09992**	**0.05837**	**0.31598**

SD = Standard deviation.

Table 3. Lethal concentrations (LC_{50} and LC_{95}) of BioUel using early fourth instar larvae of *Aedes aegypti*, at a mean room temperature of 25 ± 2 °C and room photoperiod (n = 25 larvae).

	LC_{50} (ppm)	Lower limit	Upper limit	LC_{95} (ppm)	Lower limit	Upper limit
	0.3758	0.3292	0.4551	0.7527	0.578	1.2852
	0.3727	0.3287	0.4405	0.706	0.5637	1.0396
	0.3259	0.2843	0.3924	0.7345	0.5582	1.2114
	0.2823	0.2448	0.3454	0.7074	0.5159	1.3346
	0.301	0.2663	0.3541	0.5675	0.4601	0.7795
Mean	**0.33154**	**0.29066**	**0.3975**	**0.69362**	**0.53518**	**1.13006**
SD	**0.04196**	**0.03765**	**0.04947**	**0.07316**	**0.04792**	**0.22561**

SD = Standard deviation

and LC_{90}, respectively; for larvae of *Aedes taenorhynchus* (Wiedemann, 1821), LC_{50} and LC_{90} were respectively 0.208 and 0.374 ppm. According to these results, both species, *C. nigripalpus* and *A. taenorhynchus,* were more susceptible to the bio-insecticide than the two species used in our study, *C. quinquefasciatus* and *A. aegypti*. As they are classified in the same genera, are genetically close, and share the ability to colonise receptacles in urban areas, this comparative analysis can be allowed.

All the formulated commercial products should have a shelf life with no more than 20% potency loss (FAO and WHO, 2002). According to Thiery and Hamon (1998), the stability of IPS-82 has been regularly tested to evaluate its larvicidal activity and potency, and the yearly overall mean had a coefficient of variation lower than 20%. In the present study, BioUel lost potency against *C. quinquefasciatus* and *A. aegypti* by 8.7 and 17.6%, respectively, in the first month of storage. Five months later, the loss reached 35.4% and 26.3%, respectively (Table 4).

Significant mean difference was observed for LC_{50} in the following storage months, both for *A. aegypti* (ANOVA, $F = 285.6$, $p < 0.01$) and *C. quinquefasciatus* (ANOVA, $F = 125.4$, $p < 0.02$). There was no significant difference in LC_{50} means between the second and the third months for *A. aegypti* larvae, and between the fourth and the fifth storage months for *C. quinquefasciatus* larvae (Tukey, $p < 0,05$) (Table 4). Product stability was maintained for three months. In a large country like Brazil, a product with three months of stability can be used for regional but not large scale production, for economic reasons.

So, for three months of storage, the effectiveness of BioUel is comparable to products available on the market.

Table 4. Susceptibility of early fourth instar larvae of *Culex quinquefasciatus* and *Aedes aegypti* to BioUel stored for up to 150 days (LC_{50} in ppm).

Time (days)	LC_{50} mean/SD *C. quinquefasciatus*	% Loss	LC_{50} mean/SD *A. aegypti*	% Loss
1	0.21 ± 0.013^{a}		0.28 ± 0.014^{a}	
30	0.23 ± 0.007^{b}	8.7	0.34 ± 0.001^{b}	17.6
60	0.27 ± 0.009^{c}	22.2	0.35 ± 0.001^{c}	20.0
90	0.29 ± 0.006^{d}	27.6	0.35 ± 0.005^{c}	20.0
120	0.31 ± 0.006^{e}	32.3	0.36 ± 0.007^{d}	22.2
150	0.32 ± 0.005^{e}	35.4	0.38 ± 0.006^{e}	26.3
CV	**0.033209**		**0.0128**	

Means followed by the same letter in same column do not differ based on Tukey's test at 5% level of significance. CV = Coefficient of variation.

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