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EVALUATION OF POLYMER-BASED GRANULAR FORMULATIONS OF *BACILLUS THURINGIENSIS ISRAELENSIS* AGAINST LARVAL *AEDES AEGYPTI* IN THE LABORATORY

MARÍA GUADALUPE MALDONADO BLANCO,[1] LUIS JESÚS GALÁN WONG,[1] CRISTINA RODRÍGUEZ PADILLA[1] AND HUMBERTO QUIROZ MARTÍNEZ[2,3]

ABSTRACT. A strategy to increase residual activity of *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) extract through slow-release formulations and protection from solar radiation was studied. The median lethal concentration (LC_{50}) and 90% lethal concentration (LC_{90}) levels of laboratory-reared early 4th-stage larval *Aedes aegypti* after exposure to *Bti* extract were determined. Formulations with 4 polymers and 1 solar protectant were prepared, and their shelf lives were evaluated for year-long storage at 20–35°C and 50–80% relative humidity. Also, the effect of ultraviolet light on unformulated (extract) and formulated *Bti* larvicidal activity persistence was determined. Laboratory bioassays were conducted with larval *Ae. aegypti* introduced into treated and control containers at 1, 2, 7, 14, 21, 28, 35, and 42 days after treatment, and larval mortalities were checked 24 h after introduction. Probit analysis of *Bti* extract showed LC_{50} and LC_{90} values of 0.016 and 0.051 mg/liter, respectively. The polymer-based *Bti* formulations showed no significant loss of insecticidal activity after 8 months of storage. Ultraviolet irradiation reduced activity of unformulated *Bti* extract after different exposure times, up to 40–46%, whereas *Bti* formulated with gelatin or acacia gum showed lower variation in larvicidal activity than formulations with sodium alginate and paraffin for protecting the activity of *Bti* toxin. Residual activity against 4th-stage *Ae. aegypti* in the laboratory for the formulation containing acacia gum at 10% (w/w) was 80% mortality at 14 days after treatment, whereas the *Bti* formulation containing gelatin (10%, w/w) caused 65% mortality. In addition, *Bti* formulations made with paraffin at 5% (w/w) sustained up to 60% mortality for 21 days. Unformulated *Bti* showed only 2.6% mortality, and a commercial preparation maintained 37% mortality, both at 14 days after treatment.

KEY WORDS *Bacillus thuringiensis* serovar. *israelensis, Aedes aegypti,* polymer-based formulations, residual activity, microbial control

INTRODUCTION

Bacillus thuringiensis serovar. *israelensis* (*Bti*) has been used since 1980 for the control of mosquito and black fly larval populations because of its attributes such as high efficacy, specificity, relatively low risk of resistance development, environmental safety, large-scale production, ease of handling, and storage stability (Ali et al. 1994, Su and Mulla 1999). A wide variety of formulations of *Bti,* such as liquid, powder, granule, pellet, micropellet, and microgranule, have been used for field application to breeding sites, depending on the mosquito species (Thiéry et al. 1996).

Persistence and resuspension of the *Bti* toxins and spores in the feeding zone of mosquito larvae are some of the major considerations for efficacy of *Bti* formulations. To overcome some of these problems, efforts are being made to improve the effectiveness of *Bti* by prolonging its activity, as well as delivery by targeting the active ingredient in the larval feeding zone. These improvements are primarily based on development of a variety of formulations of microbial insecticides through the use of biopolymers such as sodium alginate (Murat-Elcin 1995), gelatin (Morales-Ramos et al. 1998), and lignin (Tamez-Guerra et al. 2000). These materials can be combined with solar protectors, such as melanin (Liu et al. 1993), congo red (Shapiro 1989), and malachite green (Bohm and Friend 1988).

In this report, we describe the preparation, storage, and residual activity of an unformulated and a formulated *Bti* extract. The formulated preparations were developed as granules for slow-release purpose with 4 polymers and 1 solar protectant under laboratory conditions.

MATERIALS AND METHODS

Production of the insecticidal extract of *Bti*

The strain T14 225 of *Bti* obtained from the International Entomopathogenic *Bacillus* Center Collection, held by the Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, México (FCB-UANL), donated by the Unité de Bactéries Entomophatogénes at Instituto Pasteur, Paris, France, and the fermentation media (Maldonado-Blanco et al. 1998) were selected previously in this study. The strain was grown in a medium based on agroindustrial by-products for 72 h in a rotatory shaker (New Brunswick Scientific Co., Edison, NJ) at 200 rpm and 30°C. At the end of fermentation, spores and crystals were harvested by following the methodology described by Dulmage et al. (1970).

[1] Departamento de Microbiología e Inmunología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (UANL), C.P. 66450, A.P. 105-F, San Nicolás de los Garza, Nuevo León, México.

[2] Laboratorio de Entomología, Departamento de Zoología de Invertebrados, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (UANL), CP 66450, AP 105-F, San Nicolás de los Garza, Nuevo León, México.

[3] To whom correspondence should be addressed.

The final product, the *Bti* extract, was evaluated for larvicidal activity and formulated for shelf life and residual activity tests under laboratory conditions.

The potency of these bacterial preparations was evaluated and compared by titrating them against reference standard powders. The International Standard powder for *Bti* preparations is IPS-82 (de Barjac and Larget-Thiéry 1984). The potency of a *Bti* product is defined in International Toxic Units (ITU)/mg on *Aedes aegypti* (L.) (Bora-Bora strain) young 4th-stage larvae according to the formula: LC_{50} (IPS-82) × 15,000/LC_{50} (*Bti* product), in which LC_{50} represents the lethal concentration resulting in 50% larval mortality after exposure of the larval population for 24 h.

Mosquito species tested

For laboratory bioassays, *Ae. aegypti* San Nicolás strain was used. This strain has been reared for several years at the Entomology Laboratory at FCB-UANL, at 25 ± 3°C, 14:10 h light:dark photoperiod, and 70–80% relative humidity.

Laboratory tests

Titration of the mosquitocidal extract: The *Bti* extract was evaluated against early 4th-stage larval *Ae. aegypti* according to the methodology of de Barjac and Larget-Thiéry (1984), and compared for potency with the IPS-82 standard. For the experiment, we used a stock solution of 50 mg/liter of the *Bti* extract. Ten glass beads were added to the stock solution, and each batch was shaken for 2 min. After that, serial dilutions in a range of 0.008 to 0.06 mg of extract per liter were used (between 6 and 7 doses). Each dose was applied to groups of 25 larvae placed in plastic cups containing 150 ml of water with 3 replications per dose. Four cups with only distilled water and 25 larval *Ae. aegypti* each were used as untreated controls.

The bioassays were carried out in a holding room maintained at 25 ± 3°C and 14:10 h light:dark photoperiod. The mortality data were recorded after 24 h. The tests were repeated 3 times on different days. If mortality in the control exceeded 5%, the test was discarded. Larval response to exposed dosages of powder was calculated by a log/dose/probit regression analysis (United States Applied and Environmental Health 1989). The LC_{50} obtained for the powder was compared with that of the standard IPS-82 and the potency was calculated by the formula mentioned above.

Preparation of formulations: Four components were used to prepare the test: the spore–crystal complex of *Bti* (active ingredient), 4 polymers at different concentrations (gelatin [food grade], acacia gum [Desarrollo de Especialidades Químicas, S. A. de C. V., Monterrey, Nuevo León, México], sodium alginate [Sigma Chemical Co., St. Louis, MO], and paraffin [domestic use]), a solar protectant (malachite green, Merck Products, Darmstadt, Germany), and ground cork (Intergasket S. A. de C. V. San Nicolás de los Garza, Nuevo León, México) passed through a no. 10 sieve (U.S. Standard Sieve Series, Dual Manufacturing Co., Chicago, IL).

The formulations were prepared by dissolving each polymer in an adequate amount of distilled water (except paraffin) after the active ingredient was added and homogenized with agitation. Next, the malachite green was added and mixed completely. Finally, cork was added to the mixture.

Effect of storage on insecticidal activity: Before preparing the test, shelf-life tests were conducted to evaluate the effect of storage at room temperature (20–35°C) on the 4 different combinations of the active ingredient and polymers (gelatin, acacia gum, sodium alginate, and paraffin). Samples were prepared by mixing 150 mg of each polymer with 50 mg of the active ingredient and 10 mg of the solar protectant in 5–8 ml of distilled water. These mixtures were dried at room temperature for 7 days. Seven batches of each mixture (the active ingredient and polymer) were prepared in petri dishes, sealed with paraffin film, and stored at room temperature for 1 year. The moisture percentage of samples ranged between 5.3 and 6.7%, as analyzed by an infrared moisture balance (A&D Co. Ltd., Japan). One batch of each polymer was evaluated at 0, 30, 60, 90, 120, 240, and 365 days of storage. After that, the active ingredient (*Bti* extract) was released from the polymer matrix by using different procedures, depending on the polymer: by dissolving the sample in water (acacia gum), by dissolving in water and heating at 40°C (gelatin), by dissolving in KH_2PO_4 1.64 M solution (Murat-Elcin 1995) (sodium alginate), and by dissolving 1st in 5 ml of chloroform and then in water (paraffin). The released active ingredient was evaluated against early 4th-stage larval *Ae. aegypti* at 0.9 mg/liter. At the same time, an unformulated extract also was evaluated under the same conditions.

Effect of ultraviolet (UV) light on insecticidal activity of formulated Bti: To test the effect of UV light on larvicidal activity of unformulated and formulated *Bti* extract, the unformulated *Bti* extract was irradiated for 24 h with a Spectroline UV light lamp of 75 mW intensity (Model ENF-280 C, Spectronics Corp., Westbury, NY) at 254 nm λ. After that, sample was tested against early 4th-stage larval *Ae. aegypti* at 1, 0.1, and 0.05 mg/liter concentrations, by using 3 cups containing 25 larvae each per dose and 1 cup as untreated control. Afterwards, the extract was UV irradiated for 48 and 72 h, and tested under the same conditions as used before. The mortality data were expressed as percentage of mortality and analyzed by Student's *t*-test for each sample before and after UV irradiation. Next, the dose was selected at which the effect of UV light on insecticidal activity of unformulated extract could be observed to compare the effect on

Table 1. Polymer-based granular formulations of *Bacillus thuringiensis* serovar. *israelensis* prepared and evaluated for residual activity in laboratory tests at 13.7 mg/liter dose against laboratory-reared 3rd- or 4th-stage larval *Aedes aegypti.*

	Amount (mg)			
Formulation	Polymer[1]	Floating material	Active ingredient	Solar protectant (malachite green)
F (2%)	27	1,269	69	13
F (3%)	41	1,255	69	13
F (5%)	69	1,227	69	13
F (10%)	138	1,158	69	13
F (15%)	207	1,089	69	13
F (20%)	276	1,020	69	13

[1] Four polymers (gelatin, acacia gum, sodium alginate, and paraffin) were used. In the case of gelatin and paraffin, all doses were used; in the case of alginate, only 2, 3, and 5% (w/w) were used; and with acacia gum only 2, 10, 15, and 20% (w/w) were used. The same dose of malachite green was used in all formulations.

Bti formulation. New mixtures (active ingredient and polymer) were prepared by mixing 300 mg of each polymer with 100 mg of the *Bti* extract and 20 mg of the solar protector in an adequate amount of distilled water to have a well-homogenized mixture. These active ingredient–polymer combinations were dried at room temperature for 7 days. One half of each mixture was tested against larval *Ae. aegypti* as follows. In the nonirradiated test, the active ingredient was released by using the same procedure as described above, and tested at 0.05 mg/liter, by using plastic cups containing 25 larvae each, with 3 replications for each treatment and 4 as untreated controls. Treatments were maintained at 25 ± 3°C and 14:10 h light:dark photoperiod. The other half of the sample mixture was irradiated for 72 h at 254 nm λ, and the larvicidal activity was determined as mentioned above for nonirradiated *Bti* formulations. Mean larval mortality was determined at 24 h after treatment and expressed as percent mortality. Data obtained were analyzed with a Student's *t*-test for 2 separate samples, before and after irradiation (of each polymer). These bioassays were repeated twice.

Residual activity of formulated Bti *under laboratory conditions:* Formulations prepared with the active ingredient (*Bti* extract) and the solar protectant, both at constant amounts in all formulations, and different doses of 4 polymers and floating material (cork), were prepared as shown in Table 1.

The formulations had a granule size ranging from 1- to 2-mm diameter and 1 g contained 95–120 granules. These formulations were tested in the laboratory for evaluation of active ingredient release by using polystyrene containers (15.6 × 13.8 cm and 9 cm high) filled with 1 liter of dechlorinated water. The bioassay method was similar to that reported by Margalit et al. (1984) and modified as follows: 25 3rd- or 4th-stage larval *Ae. aegypti* were placed into each container. Treatment consisted of the *Bti* formulations (4 different polymers × 3–6 concentrations; Table 1) applied at 13.7 mg each, a *Bti* commercial product (Bactimos® briquets, lot 50831; Summit Chemical Co., Baltimore, MD), an unformulated *Bti* extract (tested at the same dose as formulations), and an untreated control. The bioassay was done 3 times (as replication) for each treatment. Larval mortality in each treatment was recorded after 24 h, and all living or dead larvae from each treatment were carefully removed with a dropper, counted, and discarded. A new batch of 25 larvae (3rd or 4th instars) were introduced into each treatment container at 1, 2, 7, 14, 21, 28, 35, and 42 days after treatment, and mortality checks were made 24 h after introduction in each case and the data were recorded.

RESULTS

Insecticidal activity of the *Bti* extract

Laboratory activity of the *Bti* extract produced in the flasks and the standard IPS-82 against early 4th-stage *Ae. aegypti* is shown in Table 2. Based on these data, the calculated experimental potency for *Bti* extract was 5,906 ITU/mg, and thus the formulations prepared with this extract had an arbitrary potency of 295.7 ITU/mg.

Effect of storage

The mixture of the *Bti* active ingredient with various polymers, evaluated against early 4th-stage larval *Ae. aegypti* at 0.9 mg/liter of the active ingredient, did not lose significant larvicidal activity

Table 2. Comparison of larvicidal activity of IPS-82 and an experimental *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) extract (produced in flask and used as active ingredient in polymer-based formulations) against early 4th-stage larval *Aedes aegypti* in the laboratory.

Material	LC_{50}[1]	LC_{90}	Potency[2]
Experimental *Bti*	0.016 ± 0.0025	0.051 ± 0.0021	5,906
IPS-82	0.0063 ± 0.0086	0.016 ± 0.007	15,000

[1] LC_{50}, 24-h median lethal concentration, values in mg/liter are means ± SE of 3 experiments; LC_{90}, 90% lethal concentration at 24 h after treatment.

[2] Potency is expressed in international toxic units (ITU/mg) with IPS-82 as the reference for activity titration against *Ae. aegypti.*

Table 3. Laboratory activity of unformulated experimental *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) extract and mixtures of this extract with various polymers (gelatin, acacia gum, sodium alginate, and paraffin) added at 15% (w/w) plus 1% malachite green, stored for various periods under variable temperature (20–35°C) and 50–80% relative humidity, tested at 0.9 mg/liter against laboratory-reared early 4th-stage larval *Aedes aegypti.*

Bti extract alone or with polymer mixture	Mean % larval mortality[1] at various times of storage (days)					
	7	25	60	120	240	365
Bti extract only	100	100	100	100	100	100a
AI[2] + gelatin	100	100	100	100a	100a	100a
AI + acacia gum	100	100	100	100a	100a	98a
AI + sodium alginate	100	100	100	92a	98a	95a
AI + paraffin	100	100	100	98a	100a	81b

[1] Average of 4 replications. Means followed by the same letter are not significantly different (ANOVA, least significant difference; $P \leq 0.05$).

[2] AI, active ingredient (*Bti* extract).

during 8 months of light variations (Table 3). However, at 365 d of storage, the *Bti* with paraffin mixture showed a loss of activity of 15–19% compared with the other 3 mixtures and the extract alone (without polymer) ($F = 26$, df = 3, $P < 0.001$; Table 3).

Effect of UV light on mosquito larvicidal activity of the *Bti* extract and the mixtures of the active ingredient with various polymers

To determine the effect of UV light on the *Bti* mosquito larvicidal activity, the *Bti* extract was tested before (nonirradiated) and after (24 h irradiation) UV irradiation at 1, 0.1, and 0.05 mg/liter against laboratory-reared early 4th-stage larval *Ae. aegypti.* Analysis of the data revealed a reduction of mortality of larval *Ae. aegypti* of nearly 28%, compared with the nonirradiated extract, only at the 0.05 mg/liter concentration, but this difference was not statistically significant (obtained Student's $t = 2.2942$, $t = 2.77$, $P > 0.05$; Table 4).

Results obtained with the *Bti* irradiated extract for 48 and 72 h, tested at 0.05 mg/liter, showed reductions in larval mortality with the irradiated extract, compared with the nonirradiated extract, although only the 72-h irradiated extract showed significant differences ($t = 2.9002$, df = 4, $P < 0.05$; Table 4).

The mixtures of *Bti* active ingredient with various polymers and the solar protectant, tested at 0.05 mg/liter concentration, showed no significant differences in larval mortality before and after the 72 h of UV light irradiation, with the exception of the mixture with paraffin ($t = 8.7412$, df = 4, $P < 0.05$; Table 4).

Residual activity of the various polymer-based *Bti* formulations under laboratory conditions

The formulations prepared with gelatin at 6 different concentrations showed similar patterns of toxicity over time. For example, formulations containing gelatin concentrations of 10 and 15% caused almost 100% larval mortality at the begin-

Table 4. Laboratory effect of ultraviolet light at 254 nm λ on the mosquito larvicidal activity of an experimental *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) extract and its mixtures with various polymers (gelatin, acacia gum, sodium alginate, and paraffin) exposed for 24, 48, or 72 h, compared with the nonirradiated extract and mixtures of the *Bti* extract plus polymer formulations tested at different concentrations against laboratory-reared early 4th-stage larval *Aedes aegypti.*

Time exposure (h)	Concentration (mg/liter)	*Bti* extract alone or with polymer mixture	Mean % larval mortality[1] at 24 h after treatment	
			Irradiated	Nonirradiated
24	1	*Bti* extract only	100	100
24	0.1	*Bti* extract only	81.2	81.2
24	0.05	*Bti* extract only	12	38.4
48	0.05	*Bti* extract only	16	46.4
72	0.05	*Bti* extract only	6.4[2]	46.4
72	0.05	AI[3] + gelatin	77.3	86.4
72	0.05	AI + acacia gum	94.6	98.6
72	0.05	AI + sodium alginate	58.4	92
72	0.05	AI + paraffin	20[4]	74.6

[1] Average of 3 replicates.

[2] Significant difference between irradiated and nonirradiated *Bti* extract by *t*-test at the 0.05 level.

[3] AI, active ingredient (*Bti* extract).

[4] Highly significant difference between irradiated and nonirradiated *Bti* extract + paraffin by *t*-test at the 0.05 level.

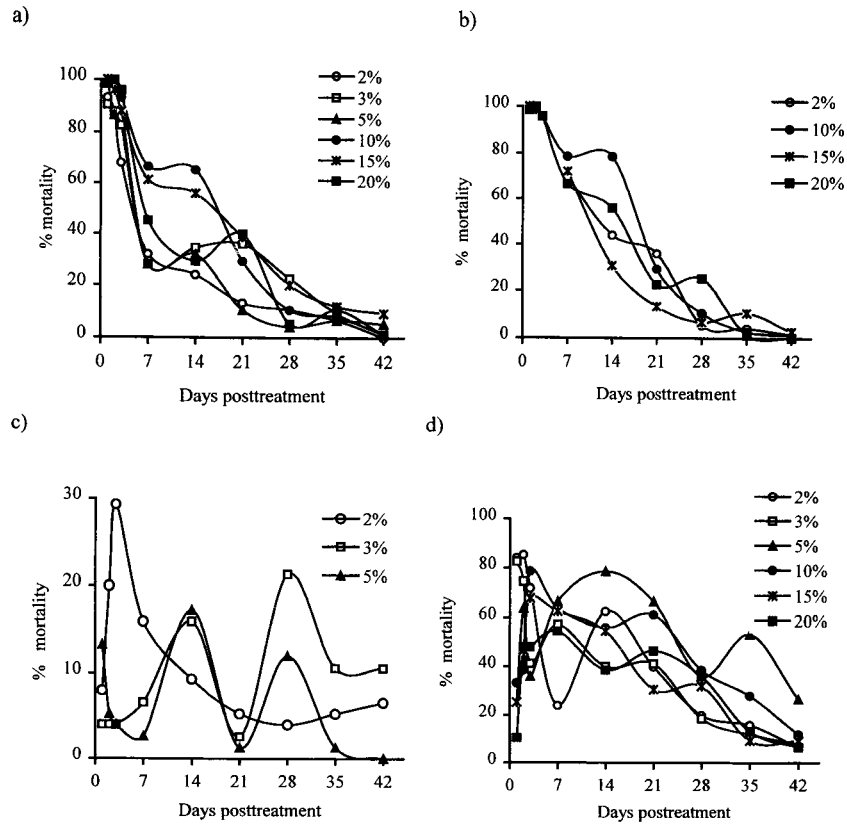

Fig. 1. Persistence of toxicity of polymer-based formulations of *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) prepared with *Bti* active ingredient and 4 polymers: (a) gelatin, (b) acacia gum, (c) sodium alginate, and (d) paraffin, at different concentrations (w/w) (Table 1), in addition to 1% malachite green and floating material, tested at 13.7 mg/liter against laboratory-reared early 4th-stage larval *Aedes aegypti* under laboratory conditions (25–28°C and 60–80% relative humidity).

ning of the test, and between 56 and 67% mortality after 7 and 14 days after treatment (Fig. 1a). Also, formulations containing 4 different concentrations of acacia gum showed a release pattern similar to the formulations with gelatin (Fig. 1b); however, the formulation containing 10% acacia gum maintained nearly 80% larvicidal activity at 14 days after treatment and thereafter decreased to the same level as that observed for the formulations with gelatin (Fig. 1b). The formulations containing sodium alginate showed relatively lower toxicity compared with the formulations containing other polymers, with larval mortalities <30% during the 6 wk of evaluation (Fig. 1c). The formulations containing 2 and 3% (w/w) paraffin initially resulted in nearly 80% larval mortality, whereas the higher concentrations of paraffin (5, 10, 15, and 20%) in the formulations resulted in lower, but better, sustained toxicity levels, as indicated by 5 and 10% paraffin polymer, which gave larval mortalities between 56 and 78% at 21 days after treatment. At 35 days after treatment, the formulation containing 5% paraffin produced >50% larval mortality, showing significantly the highest level of residual activity of the 4 polymers used (gelatin, acacia gum, sodium alginate, and paraffin) ($F = 25.41$, $P = 0.001$ and means comparison by Tukey at the 0.05 level, Tukey = 5.63; Fig. 1d).

The commercial preparation Bactimos briquets and the unformulated *Bti* extract were tested against larval *Ae. aegypti* under similar conditions as the prepared *Bti* formulations. Analysis of mortality data showed that the commercial formulation caused 72–90% larval mortalities in the 1st 3 days after treatment, with mortalities decreasing to 42, 37, and 1% after 7, 14, and 35 days after treatment, respectively (Fig. 2). Similarly, the unformulated *Bti* extract showed 81–100% larval mortalities at 3 days after treatment, decreasing to 28 and 2% at 7 and 14 days after treatment, respectively (Fig. 2), in which the polymer-based *Bti* formulations that resulted in the highest level of residual activity of each polymer (10% gelatin, 10% acacia gum, and 5% paraffin) also are shown for comparison.

DISCUSSION

The titer obtained for insecticidal extract of *Bti* in the flasks was 5,906 ITU/mg, so the formulations

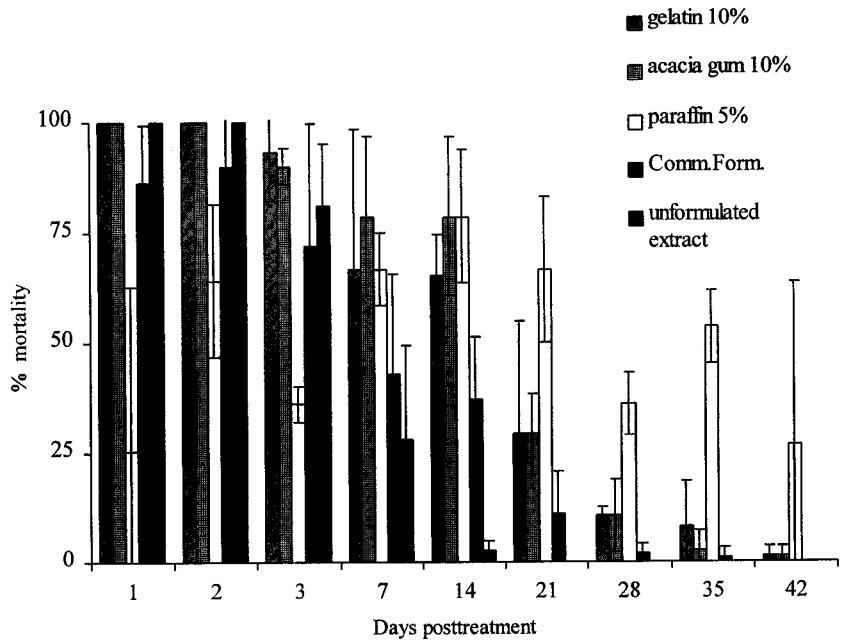

Fig. 2. Comparison of residual activity of polymer-based formulations of *Bacillus thuringiensis* serovar. *israelensis* (*Bti*) (gelatin, acacia gum, and paraffin), a commercial product, and unformulated *Bti* extract against 4th-stage larval *Aedes aegypti* under laboratory conditions (25–28°C and 60–80% relative humidity).

tested under laboratory conditions had an arbitrary potency of 295.7 ITU/mg. This value was similar to that reported by Ali et al (1994), containing 200 ITU/mg in their semifield work with various formulations from Abbott Laboratories (North Chicago, IL).

The laboratory bioassays in the present study to determine the effect of storage on the active ingredient when it was mixed with different polymers for purposes of slow release showed that the 4 different mixtures remained stable (maintained almost 100% activity) for up to 8 months under regular environmental conditions (temperature of 20–35°C and relative humidity of 60–80%). Similar results on the duration of laboratory stability of 3 commercial *Bti* formulations of Abbott Laboratories were reported by Nayar et al. (1999). Of the polymers tested in this study, only the mixture of active ingredient with paraffin showed any significant loss of activity that could have been caused by the chemical nature of the polymer used.

Photoinactivation is one of the major environmental factors affecting the stability of *B. thuringiensis* toxins (Leong et al. 1980, Molloy et al. 1981, Pozsgay et al. 1987). When observing the effect of UV light on mosquito larvicidal activity of *Bti* extract and various mixtures of active ingredient and polymers in the present study, it was evident that the extract alone was affected by UV light after different periods of exposure (40–46% reduced activity), even when the extract was exposed as a powder. Similar results were observed with the use of aqueous suspensions (Liu et al. 1993). However, several concentrations of the extract had to be tested on target larvae to observe that effect in the present study.

The mixtures of active ingredient with polymer and the photoprotectant after irradiation showed some differences in the activity against larval *Ae. aegypti,* with respect to the nonirradiated mixtures, in which the irradiated formulations with gelatin and acacia gum showed lower reductions in the toxicity against *Ae. aegypti* than the irradiated preparations containing sodium alginate and paraffin (although only this last mixture presented significant differences). This result probably was due to the chemical nature of the polymer used, which gave lower protection against UV light. However, an additional test was made with mixtures of active ingredient and polymer (without photoprotectant) in which the mixtures were irradiated under the same conditions as the mixtures containing photoprotectant. Analysis of the results showed relatively reduced larval mortality of *Ae. aegypti* with the mixtures that did not contain photoprotectant (in a range of 28–64%) compared with those mixtures that contained photoprotectant (4–54%) (data not included). Thus, the addition of substances that protect the activity of the *Bti* toxin from UV light could extend the residual activity of formulations in the field (Shapiro 1989, Morales Ramos et al. 1998, Tamez-Guerra et al. 2000).

The persistence of larvicidal activity under laboratory conditions of the 4 polymers used in the

formulations showed different release characteristics, maintaining between 60 and 80% mortality for 14 days; even at 35 days after treatment, the best formulation (containing 5% paraffin) caused more than 50% mortality of larval *Ae aegypti* in comparison with a commercial product (Bactimos briquets), which showed only 1% mortality, and the unformulated *Bti* extract, which lost all activity between 14 and 21 days after treatment. Therefore, these formulations extended the residual activity in laboratory up to 2–3 wk.

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