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Enduring toxicity of transgenic *Anabaena* PCC 7120 expressing mosquito larvicidal genes from *Bacillus thuringiensis* ssp. *israelensis*†

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Summary

Persistence of biological control agents against mosquito larvae was tested under simulated field conditions. Mosquito larvicidal activity of transgenic *Anabaena* PCC 7120 expressing *cry4Aa*, *cry11Aa* and *p20* from *Bacillus thuringiensis* ssp. *israelensis* was greater than *B. thuringiensis* ssp. *israelensis* primary powder (fun 89C06D) or wettable powder (WP) (Bactimos products) when either mixed with silt or exposed to sunlight outdoors. Reduction of Bactimos primary powder toxicity was at least 10-fold higher than *Anabaena's* after mixing with silt. In outdoors experiments, Bactimos WP remained toxic (over 30% mortality of 3rd instar *Aedes aegypti* larvae) for 2–4 days only, while transgenic *Anabaena's* toxicity endured 8–21 days.

Introduction

Synthetic chemicals have failed to control mosquitoes due to indiscriminate use that caused the development of resistance by the vector (Georghiou and Lagunes-Tejeda, 1991). The most powerful environmentally friendly agent is *Bacillus thuringiensis* ssp. *israelensis* (Margalith and Ben-Dov, 2000). This Gram-positive spore-forming bacterium produces a proteinaceous crystal (δ-endotoxin) during sporulation. The crystal dissolves in the alkaline conditions prevailing in the mid-gut of ingesting mosquito larva and is cleaved into the toxic polypeptides by specific proteases that are active there. The toxic polypeptides bind to the gut epithelium (Margalith and Ben-Dov, 2000) and cause paralysis and death within a short time that depends on the concentration used.

The efficacy of *B. thuringiensis* ssp. *israelensis* is influenced by environmental factors such as sunlight and temperature, amounts and composition of available food in the developmental site, water quality, larval density and cohabitation with other filter feeding organisms competing for food (Ramoska and Pacey, 1979; Mulla, 1990; Mulla *et al*., 1990; Becker *et al*., 1992; Kondo *et al*., 1995; Ben-Dov *et al*., 2003). Application of *B. thuringiensis* ssp. *israelensis* for mosquito control is limited, in addition, by short residual activity of current preparations under field conditions (Eskils and Lovgren, 1997; Margalith and Ben-Dov, 2000). The major reasons for this short residual activity are: (a) sinking of the protoxin to the bottom of the water body (Rashed and Mulla, 1989); (b) adsorption onto silt particles and organic matter (Ohana *et al*., 1987); (c) consumption by other organisms to which it is non-toxic (Vaishnav and Anderson, 1995); and (d) inactivation by sunlight (Liu *et al*., 1993; Cucchi and Sanchez de Rivas, 1998). Retention of *B. thuringiensis* ssp. *israelensis* in the larval feeding zone and prolonging its activity are being facilitated by development of new formulations utilizing conventional methods and advanced tools of molecular biology. The strategy of transferring *B. thuringiensis* ssp. *israelensis's* mosquito toxin genes for expression in alternative hosts that are digested by mosquito larvae and multiply in their habitats is safe and economical.

A series of transgenic *Anabaena* PCC 7120 expressing combinations of *B. thuringiensis* ssp. *israelensis* genes has been constructed (Wu *et al*., 1997) and found effective in the laboratory (Lluisma *et al*., 2001; Manasherob *et al*., 2002). The conventional bioassay to determine product potency and longevity in the laboratory is, however, not sufficient to deduce residual activity in field conditions. Actual experiments must vary according to environmental conditions in each case (e.g. location, season).

Received 17 March, 2003; accepted 27 June, 2003. *For correspondence. E-mail ariehz@bgumail.bgu.ac.il; Tel. (+972) 8 6461 712; Fax (+972) 8 6278 951. †Dedicated to our colleague, Professor Yoel Margalith, the discoverer of *Bacillus thuringiensis* ssp. *israelensis*, on his 70th birthday and upon being awarded the Tyler Prize for environmental achievement.

Table 1. Toxicities of transgenic *Anabaena* PCC 7120 carrying three genes (*cry4Aa*, *cry11Aa*, *p20*) from *Bacillus thuringiensis* ssp. *israelensis* and Bactimos primary powder against late 3rd instar larvae of *Aedes aegypti*.

Treatment	LC_{50} at 24 h[a]	Toxicity ratio[b]	LC_{50} at 48 h[a]	Toxicity ratio[b]
Anabaena without silt	19.14 (14.44–25.46)	1	15.20 (10.14–22.82)	1
Anabaena not mixed	134.64 (92.03–199.65)	0.142	72.79 (37.31–122.25)	0.208
Anabaena mixed once	201.43 (146.18–268.62)	0.095	130.77 (77.34–202.37)	0.116
Anabaena mixed five times	112.50 (81.38–149.22)	0.170	80.95 (50.68–117.96)	0.187
Bactimos without silt	21.39 (17.06–26.87)	1	11.83 (8.94–14.89)	1
Bactimos not mixed	275.86 (207.40–358.05)	0.077	241.68 (156.85–390.75)	0.048
Bactimos mixed once	1212.57 (914.43–1846.26)	0.017	507.50 (445.25–575.44)	0.023
Bactimos mixed five times	899.05 (685.68–1192.21)	0.023	489.34 (338.68–717.96)	0.024

a. LC_{50} values represent the average numbers (ng ml^{-1} of dry Bactimos powder and chlorophyll in *Anabaena*) in at least three bioassays performed. Numbers in parentheses are 95% confidence limits (lower and upper), as determined by probit analysis.
b. Ratio between LC_{50} value obtained in tap water without silt to that with silt.

Here, the efficacy and residual activities of the transgenic *Anabaena* are compared with those of *B. thuringiensis* ssp. *israelensis*-based Bactimos formulations under semifield conditions as well as under mixing with silt in the laboratory. We conclude that the transgenic cyanobacterium is better than *B. thuringiensis* ssp. *israelensis* itself in the conditions tested.

Results and discussion

Mosquito larvicidal activity of the recombinant *Anabaena* PCC 7120 expressing *cry4Aa*, *cry11Aa* and *p20* from *B. thuringiensis* ssp. *israelensis*, which displayed high potency in the laboratory by standard bioassays (Wu *et al*., 1997; Lluisma *et al*., 2001), demonstrated here better persistence than *B. thuringiensis* ssp. *israelensis* (as Bactimos products) when either mixed with silt (Table 1) or exposed to sunlight outdoors (Tables 2–4).

Adverse effect of silt on toxicity

The adverse effect of silt on toxicity of Bactimos primary powder was higher than on the transgenic *Anabaena*, more so when mixing was more extensive (Table 1): without mixing, toxicity of Bactimos decreased to 0.077 and 0.048 of its control value (without silt) after 24 and 48 h respectively. The decrease in *Anabaena's* toxicity by the same treatment was smaller, to 0.142 and 0.208. Following mixing (once or five times), toxicity of Bactimos decreased dramatically, to 0.017–0.024 (about 2% of its original values) and to 0.095–0.187 (between 10% and 20% of original toxicity) for *Anabaena*. Thus, silt quenches toxicity of Bactimos 10-fold more than that of transgenic *Anabaena*. Moreover, the real residual toxicity of Bactimos after 48 h is likely to be even lower, because larvae of *A. aegypti* have most likely scavenged carcasses of *B. thuringiensis* ssp. *israelensis*-killed larvae, in which spores and their toxicity recycle (Khawaled *et al*., 1988). This interpretation is supported by the rise in toxicity (reduction in LC_{50} from 21 to 12 ng ml^{-1}) in the control experiment between 24 and 48 h of incubation. The small (about 20% – hardly significant) increase in toxicity of the original *Anabaena* preparation (without silt) may be caused by some multiplication during the 24 h difference, anticipated to be very slow under these conditions.

Longevity of toxicity in outdoor experiments

Toxicity of the transgenic *Anabaena* in semifield trials (outdoor tests) performed in the summer (June, 2002) lasted

Table 2. Toxicity comparisons between transgenic *Anabaena* and Bactimos against *A. aegypti* larvae in tap water with 40% shade (17.6–27.6, 2002).

Day	Max temp (°C)	Light intensity (μmol m^{-2} s^{-1})[a]	Larval mortality (%)			Chlorophyll (μg ml^{-1})
			Bactimos	*Anabaena*	Control	
1	26	1100	100	82	1	1.0
2	25	1100	94	64	2	0.7
3	26	1200	27	60	5	0.4
4	27	1200	10	67	1	<0.1
6[b]	28	1150	3	70	7	<0.1
8[b]	27	1100	2	85	5	<0.1
10[b]	27	1200	1	47	2	<0.1

a. Range of light intensity outdoors was 1900–2100 μmol photon m^{-2} s^{-1}.
b. Results were recorded after 2 days.

Table 3. Toxicity comparisons between transgenic *Anabaena* and Bactimos against A. aegypti larvae in tap water with 70% shade (10.7–23.7; 30.7–12.8, 2002).[a]

Day	Max temp (°C)	Light intensity (μmol m^{-2} s^{-1})[b]	Larval mortality (%)			Chlorophyll in *Anabaena* (μg ml^{-1})
			Bactimos	*Anabaena*	Control	
1	25.5	525	100	82	3	0.7
2	24.5	510	100	83	0	0.58
3	25.5	550	75	84	0	0.48
4	26.5	550	55	77	0	0.35
5	27.5	600	22	70	4	0.22
6	27	590	7	65	2	0.1
7	27	575	5	64	2	<0.1
8	26	550	3	44	5	<0.1
9	26	550	8	28	2	<0.1
10	25	560	5	25	5	<0.1
11	25.5	550	5	24	4	<0.1
12	25	575	8	21	6	<0.1
13	25	550	5	5	2	<0.1

a. Average values in two experiments.
b. Range of light intensity outdoors was 1900–2100 μmol photon m^{-2} s^{-1}.

for the same period (2 days) as that of Bactimos WP (data not shown). While this activity (causing over 30% mortality) remained the same for Bactimos when 40% shading was applied, *Anabaena's* toxicity endured for 8 days (Table 2), even though chlorophyll was bleached after 3 days. The conclusion that toxin inactivation by sunlight or water temperature is protected in the transgenic *Anabaena* was confirmed by additional experiments, in which the light was dimmed by 70% shading (Table 3) and in the winter with 40% shading (Table 4). With 30% light transmitted through the net (Table 3), Bactimos activity remained twice longer than with 60% light transmitted (Table 2), 4 rather than 2 days. Endurance of the transgenic *Anabaena's* toxicity remained for 8 days, but considering the lower initial concentration of chlorophyll (0.7 rather than 1 μg ml^{-1}; compare Table 3 with 2), it lasted for 11 days. Note that only in the experiments of Table 3 parameters were determined each and every day without skipping weekends.

The experiment conducted in the winter (Table 4) indicates that sunlight is not the only parameter that inactivates toxicity. Light intensity penetrating through the net with 40% shade in sunny winter days (about 480 μmol photon m^{-2} s^{-1}) was only about 15% less than that shaded 70% in the summer (about 560 μmol photon m^{-2} s^{-1}; Table 3). In this case, Bactimos toxicity (killing daily at least 30% of exposed larvae) was retained for 3 days only [cumulative mortality of 3 additional days (4–6) was 39% (data not shown)] while that of *Anabaena* was extended to at least 21 days (Table 4), twice as long as in the summer, most likely because cyanobacterial cells survived at the low light intensity (chlorophyll is only 30% bleached, to 0.5 μg ml^{-1}). The low temperature, on the other hand, may result in low activity of the promoter (P_{psbA}) or degradation of the toxins, parameters that should be measured in future tests.

Larval mortality in control tubs is revealing: when both

Table 4. Toxicity comparisons between transgenic *Anabaena* and Bactimos against *A. aegypti* larvae in tap water with 40% shade (17.11–8.12, 2002).

Day	Max temp (°C)	Light intensity (μmol m^{-2} s^{-1})[a]	Larval mortality (%)			Chlorophyll in *Anabaena* (μg ml^{-1})
			Bactimos	*Anabaena*	Control	
1	15	500	100	85	0	0.7
2	14	500	82	72	3	0.7
3	14	550	45	74	6	0.7
7	12	180	12	75	5	0.7
8	13	400	6	78	8	0.7
9	14	500	10	60	6	0.6
10	14	500	8	59	10	0.6
14	12	180	14	57	13	0.5
15	12	180	25	59	20	0.5
16	14	450	15	61	25	0.5
21	14	450	9	46	15	0.5

a. Range of light intensity outdoors was 900–1200 μmol photon m^{-2} s^{-1} (in the sunny days) and around 400 μmol photon m^{-2} s^{-1} (in the cloudy days).

light intensity and maximum daily temperature were high, such as in the summer (Tables 2 and 3), it remained at 2–5%, while it rose to 15% in the winter (Table 4). Apparently the cold temperature caused death (average temperatures in the summer and winter were 26°C and 14°C respectively). Moreover, winter low temperatures after cloudy days dropped at night to below 10°C (not systematically determined).

To the best of our knowledge, the only outdoor test for endurance of mosquito larvicidal activity by transgenic cyanobacteria was performed by Xu *et al*. (2000). These authors could not find larvae of *Culex pipiens* for 2 months in 500 ml dips from the surface of 800 l vats after a single application of *Anabaena* PCC 7120 (7×10^4 cells ml^{-1}) expressing the binary toxin of *Bacillus sphaericus*. These results cannot be compared with ours because no specific environmental and experimental conditions for them were detailed. For example, temperatures and light intensities that tremendously affect duration of residual activity (Tables 2–4) may have been much more favourable than those in our tests. Larger containers would mitigate such parameters increasing the life span and effectivity of our transgenic *Anabaena*. To be able to compare results from different laboratories, a standard method must be approved by international bodies such as the WHO. The convention would determine, for example, the dimensions of the treated water body and whether additional larvae are to be introduced and in what rate. We followed the conditions set by Ali *et al*. (1994), who demonstrated different efficacies and residual activities of various granular formulations of *B. thuringiensis* ssp. *israelensis*.

Experimental procedures

Bacterial strains and preparations

Two products (Bactimos) containing *B. thuringiensis* ssp. *israelensis* (Berliner) were used: (a) primary powder 1990, fun 89C06D, Duphar B.V., [12 000 International toxic units (ITU) mg^{-1}] for bioassay with silt; (b) commercial powder WP; Summit Chemical Co. (3500 *Aedes aegypti* ITU mg^{-1}) for semifield trials.

Anabaena PCC 7120 and its toxic recombinant clone (with pSBJ2 no. 11; 6 AA ITU mg^{-1}) expressing *cry4Aa*, *cry11Aa* and *p20* from *B. thuringiensis* ssp. *israelensis* (Wu *et al*., 1997) were used in this study. Each strain was maintained by shaking (160 r.p.m) in 250 ml flasks (100 ml, 30°C) with continuous illumination (50 μmol photon m^{-2} s^{-1}). For use in bioassays, 2.5 l glass-columns were employed (2 l, 30°C) under continuous illumination (85 μmol photon m^{-2} s^{-1}) and bubbling (air containing 1.5% CO_2). The medium used was BG11 (Lluisma *et al*., 2001) and paste was prepared from stationary phase (2 weeks) cultures by centrifugation.

Rearing mosquito larvae

Dry strips of paper bearing eggs of *A. aegypti* were submerged in 1 l of sterile tap water supplemented with 1.5 g of Pharmamedia (Traders Protein) and incubated at 28 ± 2°C for hatching and larval growth (Khawaled *et al*., 1988). Third instar larvae were selected for bioassays after 3–4 days of incubation.

Comparative laboratory bioassays with silt

Survival of 20 3rd instar *A. aegypti* larvae in 100 ml of sterile tap water (in 170 ml disposable plastic cups) was determined after 24 and 48 h at 28°C with appropriate dilutions of Bactimos or recombinant *Anabaena*. Silt from an open-air fish pond (0.6 g dry weight in 5 ml water) was added to the bioassay cups, maintaining the final volume at 100 ml.

Three different treatments were employed, as follows:

(i) silt was added to the cups with larvae before either *Anabaena* or Bactimos, and a single, gentle mixing was performed by sucking and releasing the suspension using a 5 ml pipette;
(ii) recombinant *Anabaena* or Bactimos with silt was mixed as in (a) five times in 2 h intervals;
(iii) recombinant *Anabaena* or Bactimos was added gently to separate cups 2 h after addition of silt (left to settle) and incubated 5 h before adding *A. aegypti* larvae.

Larval mortality was determined without removing the carcasses of the dead larvae (Khawaled *et al*., 1988). Each bioassay was performed at least thrice. Values of LC_{50} [concentration (in ng ml^{-1} of Bactimos powder; in ng of chlorophyll ml^{-1} of *Anabaena*)] were determined with six concentrations in duplicate. LC_{50} and 95% fiducial limits were obtained using probit analysis (Daum, 1970). Mortality of control larvae (without toxic material) never exceeded 5%.

Comparative bioassays in semifield conditions

Plastic containers (0.35 cm diameter, 54 cm depth) were buried 20 cm in the soil, filled with 50 l of tap water and left to stand for 24 h for the water to age before introducing mosquito larvae. When needed, the tubs were covered outdoors with a vinyl black net providing 40% or 70% shade, as specified in each experiment.

One hundred larvae (late 3rd–early 4th instars) of *A. aegypti* were placed in each tub and either *Anabaena* (3.85 g) or Bactimos (6.6 mg) was added (23 100 AA ITU each). (Application rates recommended by Bactimos' manufacturer are 0.125–0.750 kg $hectare^{-1}$ or 0.025–0.150 mg l^{-1}.) Larval mortality was monitored for at least 10 days. All larvae (dead and living) were removed into a shallow tub (either daily or at specified intervals) from each tub with the aid of a 2 l beaker for scoring larval mortality. Dead larvae were removed to avoid recycling of cells and their toxicity in the tubs with Bactimos (Khawaled *et al*., 1988), living larvae (with all water) were returned to their respective tub afterwards and untreated fresh larvae were introduced to top up to one hundred. To assess residual activity, all remaining larvae in each tub were removed and discarded in 4 day interval and a new batch of 100 larvae was introduced. Daily maximum

temperature and sunlight intensity were recorded. The tests were performed between June and December. Plain tap water served as control in all experiments.

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