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RAISING ACTIVITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST *ANOPHELES STEPHENSI* LARVAE BY ENCAPSULATION IN *TETRAHYMENA PYRIFORMIS* (HYMENOSTOMATIDA: TETRAHYMENIDAE)

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ABSTRACT. Toxicity of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) against surface-feeding mosquito larvae of *Anopheles stephensi* was enhanced by encapsulation in the protozoan *Tetrahymena pyriformis.* In the laboratory, larvae died about 8 times faster when exposed to protozoan cells filled with *B.t.i.* than when exposed to the same concentrations of *B.t.i.* alone. Best larvicidal activities were achieved with ratios of 1:200–1:500 *T. pyriformis* cells to *B.t.i.* spores. The concentration of *B.t.i.* needed to kill 50% of exposed populations was 4-fold lower with *T. pyriformis* than with *B.t.i.* alone in 100 ml-test cups. Toxicity enhancement is very likely a consequence of concentrating *B.t.i.* insecticidal crystal proteins in *T. pyriformis* cells and floating them to the water surface in the larval feeding zone. Reduction in the exposure time of *B.t.i.* to unfavorable field conditions, as a result of the decrease in larval mortality time, might improve the persistence of this biological control agent in nature.

INTRODUCTION

Use of conventional broad spectrum chemical insecticides to control the vectors of arthropod-borne diseases often has resulted in development of resistance by mosquitoes and environmental problems. These problems may be circumvented with the use of biological control agents (Kirschbaum 1985). The bacterium *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) (Goldberg and Margalit 1977, de Barjac 1978) is considered one of the best of such agents found so far (de Barjac and Sutherland 1990). Its larvicidal activity is caused by insecticidal crystal proteins (I.C.P.) that are produced during sporulation (Whiteley and Schnepf 1986). Following ingestion by mosquito larvae, the crystal dissolves in the alkaline pH prevailing in the larval midgut, releasing protoxin polypeptides which are then activated by proteolytic enzymes. The use of *B.t.i.* is, however, limited by the short persistence of current preparations under field conditions, because it does not reproduce efficiently (Mulla 1985, Becker et al. 1992).

Various mosquito species exhibit different levels of susceptibility to *B.t.i.* Surface-feeding anopheline larvae are 10-fold more tolerant to *B.t.i.* formulations than those of *Culex* and *Aedes* species (Mulla 1990). Sinking of the toxin below the air–water interface and slow feeding rates (10–20 times slower than those of *Culex* and *Aedes*) have been cited as the reasons for this reduced sensitivity (Aly et al. 1988). Floating food particles are rapidly ingested by *Anopheles* larvae (Aly and Mulla 1986), and floating type formulations of *B.t.i.* were therefore developed, with significantly increased activity against *Anopheles* larvae (Aly et al. 1987, Cheung and Hammock 1985).

We previously reported that *B.t.i.* toxins can be encapsulated in a motile protozoan, *Tetrahymena pyriformis,* in which I.C.P. are not inactivated (Zaritsky et al. 1991). Each cell is able to concentrate in its food vacuoles between 180–240 spores and their crystals (Ben-Dov et al. 1994), and to deliver them to and kill mosquito larvae upon ingestion. This active process of concentrating and delivering intact I.C.P. to the target organisms was termed "bioencapsulation" (Zaritsky et al. 1991). *Aedes aegypti* (Linn.) larvae died 3 times as fast when fed with bioencapsulated *B.t.i.* than when fed the same concentrations of *B.t.i.* alone (Manasherob et al. 1994).

The *T. pyriformis* bioencapsulation system concentrates *B.t.i.* spores and crystals and keeps them at the water surface; therefore, it is expected to particularly raise *B.t.i.* activity against *Anopheles* larvae. The degree of enhancement of toxicity against *Anopheles* larvae should be greater than that obtained against *Ae. aegypti* larvae (Manasherob et al. 1994). Here, we demonstrate in the laboratory that feeding larvae of *An. stephensi* Liston on *B.t.i.*-loaded *T. pyriformis* causes rapid mortality and decreases the minimal concentrations needed to kill these larvae.

MATERIALS AND METHODS

Anopheles stephensi: Adults and larvae were grown at 28°C and a photoperiod regime of 14 h light : 10 h dark. Adult mosquitoes were fed glucose solution (10%). Blood was supplied by laboratory mice. Eggs were collected and submerged in tap water supplemented with tropical fish food. Fourth-instar larvae were selected and washed before every experiment.

Bacillus thuringiensis *var.* israelensis: A powder (Roger Bellon Laboratories, Belgium, R-153-78),

which contained *B.t.i.* spores (10^8 mg^{-1}), I.C.P. (1,000 International Toxic Units mg^{-1} [Dulmage et al. 1990]), diatom algae, and debris of unknown origin was used. Spore concentration and larvicidal activity of the powder were both determined and found to stay unchanged and to maintain the original levels throughout the present set of experiments. The *B.t.i.* powder (stored at 4°C) was suspended in sterile distilled water (1 mg ml^{-1}) and pretreated by heat shock (10 min, 70°C) and sonication (MSE Sonifier, 4 times 30 sec each with 30 sec intervals, 0°C) before the experiments to disperse spore aggregates and minimize contamination.

Tetrahymena pyriformis: The protozoan was maintained and grown axenically as previously reported (Manasherob et al. 1994). Experiments were performed with 1×10^5–3×10^5 cells ml^{-1} cells of exponentially growing cultures shaken in a waterbath (28°C, 60 strokes min^{-1}). Cells were microscopically counted in a Sedgwick chamber after fixation in 1% formaldehyde. The cells were washed twice with sterile distilled water by centrifugation (90 sec at 3,000 rpm).

Bioencapsulation: Washed *T. pyriformis* cells (20,000 ml^{-1}) were preincubated for 90 min (28°C, 60 strokes min^{-1}) with *B.t.i.* powder (100 μg ml^{-1}) in 5 ml of sterile distilled water in a 20-ml vial. Bioencapsulation was tested microscopically (Ben-Dov et al. 1994) prior to each experiment to assure that *T. pyriformis* cells were fully loaded with *B.t.i.*, i.e., contained between 25 and 35 *B.t.i.*-loaded (average of 8 spores per vacuole) food vacuoles per cell.

Bioassays—LT_{50} (time taken to kill 50% of exposed larvae): Twenty fourth-instar *An. stephensi* larvae, in duplicates, were incubated with 100 ml of appropriately diluted *B.t.i.* alone or encapsulated in *T. pyriformis.* Incubation was carried out in sterile tap water inside 150-ml disposable plastic cups. To allow determination of larval mortality every 20–60 min, incubation at 28°C was performed in an open waterbath.

LC_{50} (spore concentration that kills 50% of exposed larvae): Larvae were treated in the same manner as for the LT_{50} test, but incubated in a closed incubator at 28°C (both water and air) and scored for larval mortality after 24 h. Mortality data were subjected to probit analysis (Finney 1971).

RESULTS

Bioencapsulation at concentrations of 2×10^5 *B.t.i.* spores (2 μg) and 500 *T. pyriformis* cells per ml improved the larvicidal activity against *An. stephensi* (Fig. 1): Death started earlier (lag time was shorter) and 50 and 80% mortalities (LT_{50} and LT_{80}, respectively) were reached faster. Toxicity amplification factor—T.A.F. [the ratio of larval death time caused by *T. pyriformis*-encapsulated *B.t.i.* to that caused by *B.t.i.* alone (Manasherob et al. 1994)]—was around 7.4 for the different percentages of mortality (Fig. 1, inset). Mortality in the absence of *B.t.i.* with and without *T. pyriformis* cells under the conditions of this experiment as well as in all the following experiments was negligible (and see Zaritsky et al. 1992).

To measure the dependence of larval mortality on *T. pyriformis* concentration, a series of similar experiments were carried out with 50–1,200 cells ml^{-1} and a constant concentration of *B.t.i.* spores (2×10^5 ml^{-1}) and I.C.P. (2 ITU ml^{-1}). Highest larvicidal activities (reflected in all 3 time parameters—lag, LT_{50}, and LT_{80}) were obtained by 200–500 *T. pyriformis* cells ml^{-1}.

The effect of variable *B.t.i.* concentrations on mortality rate of *An. stephensi* was tested with constant *T. pyriformis* concentration (200 cells ml^{-1}). The LT_{80} values obtained with 1, 2, and 3.5 μg powder ml^{-1} were 265, 118, and 80 min, respectively, compared to 16, 12.5, and 10 h obtained with *B.t.i.* alone. Toxicity amplification factors (T.A.F.) calculated from these data were thus 4, 6, and 7, respectively.

All the previous quantitative measurements of *B.t.i.* toxicity in this study were based on the time of larval mortality and required, therefore, complete death of the tested population. This could not be achieved with *An. stephensi* when *B.t.i.* concentration was lower than 0.33 μg ml^{-1} (0.33 ITU ml^{-1}), as mortality reached only 35% with the bioencapsulation system and 10% with *B.t.i.* alone. Thus, to allow quantitative comparison of larvicidity between the bioencapsulated and the free *B.t.i.* even at low concentrations, we had to use the LC_{50} bioassay system that measures percentage of larval mortality after an excess time of incubation (24 h).

Bioassays were carried out with variable *B.t.i.* concentrations with and without a constant *T. pyriformis* concentration (200 cells ml^{-1}) (Fig. 2). At *B.t.i.* concentrations of 0.4, 0.6, and 0.8 μg ml^{-1} encapsulated in *T. pyriformis,* mortalities were approximately 50, 80, and 95%, respectively, while they were negligible with *B.t.i.* alone. The results of a set of independent experiments were plotted on a probit graph (Fig. 3). The average LC_{50} obtained for fourth-instar *An. stephensi* larvae was 0.43 μg ml^{-1} with the bioencapsulation system and 1.66 μg ml^{-1} with *B.t.i.* alone. Thus, the system decreases 4-fold the *B.t.i.* concentration needed to kill 50% of exposed larvae.

DISCUSSION

Effectiveness of the *B.t.i.* I.C.P. against mosquitoes depends on efficient application and long retention in the larval feeding zone until lethal doses are ingested. *Anopheles* larvae collect particles from the air–water interface and rapidly ingest them (Aly and Mulla 1986); suspended particles are filtered at low rates (Aly 1988). Surface-bound formulations of *B.t.i.* are needed to achieve high ac-

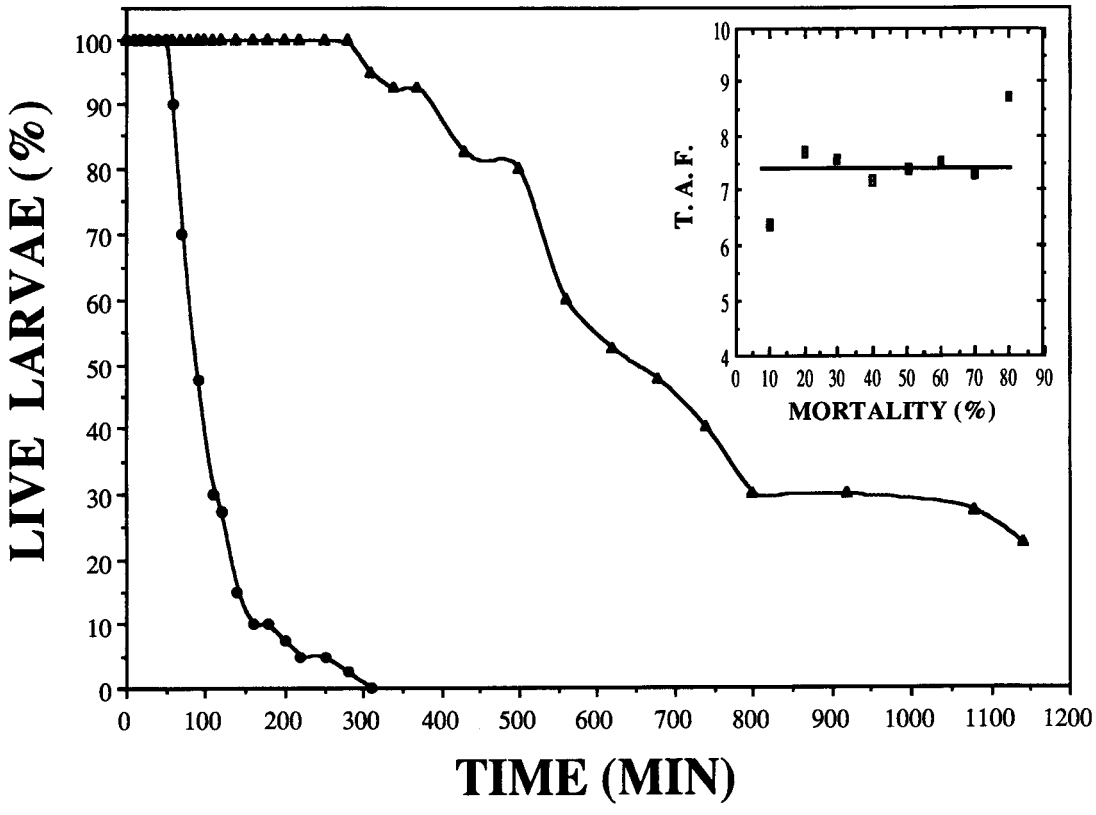

Fig. 1. Raising mortality rate of *Anopheles stephensi* larvae by encapsulating *B.t.i.* in *Tetrahymena pyriformis*. Young fourth-instar larvae were exposed to 2 μg ml^{-1} of a *B.t.i.* powder, either alone (▲) or encapsulated in 500 *T. pyriformis* cells ml^{-1} (●). Inset (■), Toxicity amplification factor (T.A.F.) in different lethal times.

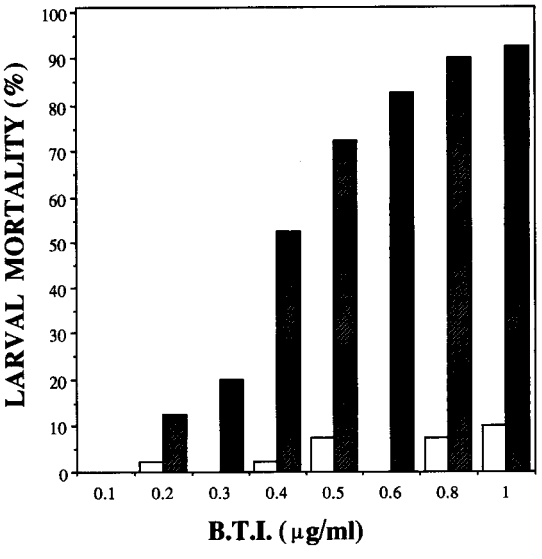

Fig. 2. Percent mortality of old fourth-instar *Anopheles stephensi* larvae after 24-h exposure to variable concentrations of *B.t.i.* (0.1–1.0 μg ml^{-1}), alone (□) and encapsulated in 200 *Tetrahymena pyriformis* cells ml^{-1} (▨).

tivity against these less susceptibile larvae. Toxicity of *B.t.i.* against larvae of *An. stephensi* was improved by loading the I.C.P. in the protozoan *T. pyriformis* (Fig. 1), which concentrates I.C.P. and floats them to the water surface. The calculated toxicity amplification factor (T.A.F.) was significantly higher than that previously obtained with *Ae. aegypti* larvae (Manasherob et al. 1994).

Larvicidal activity of the *B.t.i.* powder (2 μg ml^{-1}, with 2×10^5 spores and 2 ITU ml^{-1}) was best enhanced when encapsulated in 200–500 *T. pyriformis* cells ml^{-1} namely at 1,000–400 *B.t.i.* spores, which corresponds to 10×10^{-3}–4×10^{-3} ITU per *T. pyriformis* cell, respectively. These results are similar to those with *Ae. aegypti* larvae. At these ratios, cells of *T. pyriformis* are filled to their maximum capacity—an average of 8 spores in each of 30 food vacuoles (Ben-Dov et al. [1994] and see Materials and Methods). However, one should bear in mind that spore counts are only an indirect measure of I.C.P. ingestion. Electron microscopy studies (unpublished data) of the content of the protozoan food vacuoles revealed that the ratio I.C.P.: spores was indeed approximately 1.

When varying *B.t.i.* concentrations, T.A.F. values

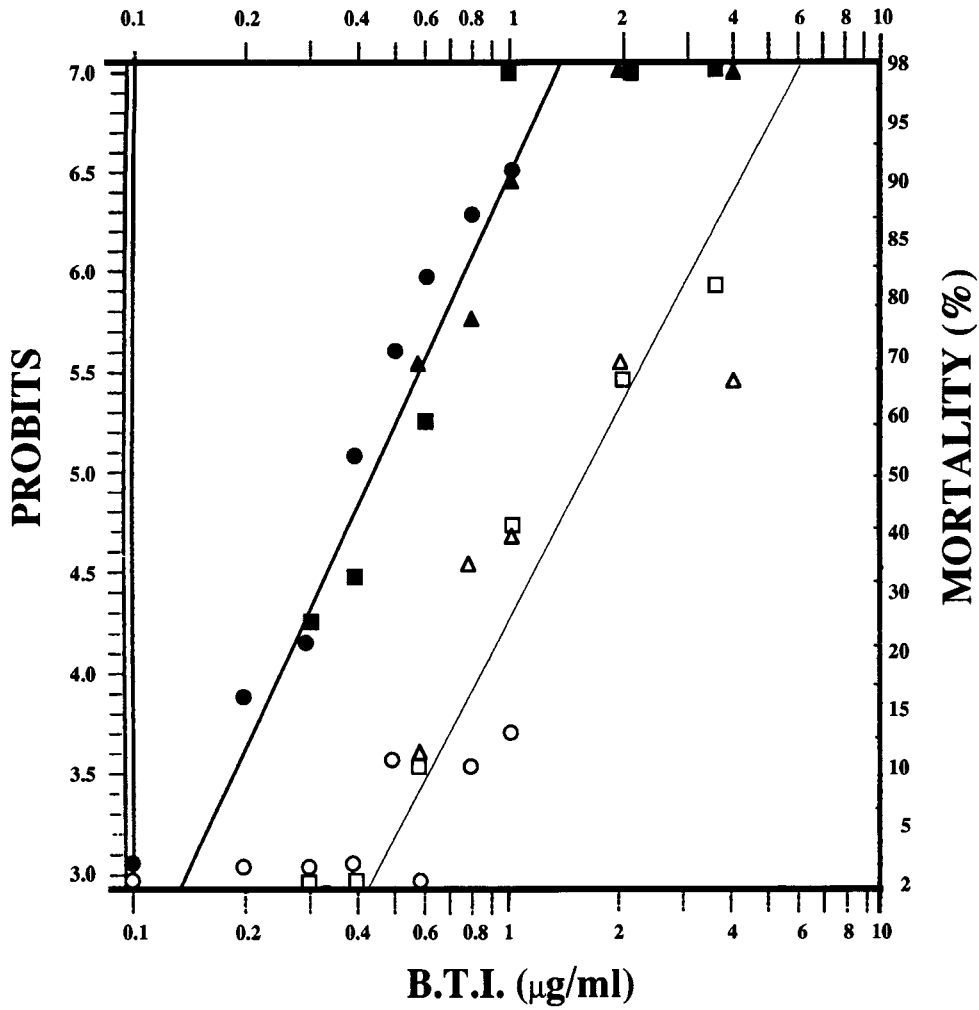

Fig. 3. Mortality of *Anopheles stephensi* larvae (fourth-instar) as a function of spore concentration. Data are drawn on a probit scale for LC_{50} determinations of *B.t.i.* alone (open symbols) or encapsulated in *Tetrahymena pyriformis* cells (200 ml^{-1}; closed symbols). Different symbols represent separate experiments.

for LT_{80} increased from 4 in 1 μg ml^{-1} to 7 in 3.5 μg ml^{-1} with *An. stephensi* larvae. A constant T.A.F. of around 2 was found in this range with *Ae. aegypti* larvae, but it increased with decreasing *B.t.i.* concentrations (from 1.0 to 0.2 μg ml^{-1}; Manasherob et al. 1994). The lower susceptibility of *An. stephensi* larvae to suspended I.C.P. precludes the possibility to compare the two tests at a low concentration range.

To allow such comparisons even at low *B.t.i.* concentrations, the LC_{50} bioassay system was used (Figs. 2 and 3). These data clearly reveal another advantage of the bioencapsulation system over the free *B.t.i.*, when high percentages of mortality were obtained even at *B.t.i.* concentrations of 0.4–1.0 μg ml^{-1}, a range at which no significant mortality was obtained with *B.t.i.* alone (Fig. 2). The concentration needed to kill 50% of exposed larvae was 4-fold lower with *T. pyriformis* than with *B.t.i.* alone (Fig. 3) in the 100-ml test system. This factor of enhancing toxicity is twice that obtained with floating bait formulations against *An. stephensi* larvae in the same 100-ml test system (Aly et al. 1987). Attaining the full advantage of this bioencapsulation system may need larger and deeper test systems, as is the case with the floating formulations of Aly et al. (1987).

Cells of *T. pyriformis* at concentrations used here may serve as a gustatory phagostimulant, enhancing larval ingestion rates (Aly 1985, Aly and Mulla 1986).

The increased mortality rate of *An. stephensi* larvae achieved by encapsulating I.C.P. in *T. pyriformis* cells is very likely the consequence of the concentration of large quantities of crystals and their efficient delivery to the target organism. Shortening larval mortality time by this bioencapsulation should reduce the exposure time of *B.t.i.* to harmful

field conditions that inactivate its larvicidal activity and thus should raise its larvicidal efficacy. However, this prediction must still be tested in nature.

Other species of ciliated protozoa should be used as vehicles for *B.t.i.*'s toxin, depending on the constraints imposed by the conditions prevailing in any particular mosquito-infested area. For example, the heat-resistant species *T. thermophila* is expected to be most useful in tropical countries, where higher ambient temperatures prevail.

Utilization of *B.t.i.* encapsulated in *T. pyriformis* for controlling *Anopheles* larvae would be preferable to treatment with the commercial formulations because the protozoa prevent fast sinking and adsorption of the toxin, concentrate it, retain it in the top layer of the water body, and may serve as a phagostimulant. Recycling of *B.t.i.* spores in the excreted food vacuoles of *Tetrahymena* would further enhance the efficacy of the system. Furthermore, the use of *Tetrahymena* for biotechnological purposes (Munro 1985) has become promising since the recent development of an efficient method to raise them in large amounts (Kiy and Tiedtke. 1992).

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