FLOATING BAIT FORMULATIONS INCREASE EFFECTIVENESS OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AGAINST *ANOPHELES* LARVAE

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ABSTRACT. The development and screening of floating-type bait formulations designed to improve the activity of bacterial toxins against larval *Anopheles* is described. Floating and spreading abilities of carrier particles (wheat flour) were compared using corn oil, lecithin, and two products yielding surface films on water (Arosurf® and Liparol). Mixtures containing 1 or 5% Arosurf showed the best spreading abilities on a water surface, but strongly inhibited the ingestion of wheat flour by *Anopheles albimanus* larvae. Corn oil and lecithin improved spreading satisfactorily at a concentration of 5% and inhibited larval feeding by only 6–25%. To select a suitable concentration of active ingredient in formulations, *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) primary powder in concentrations ranging from 0.01 to 0.5% was mixed with wheat flour/corn oil mixtures and tested in quantities exceeding the gut volumes of treated larvae. Complete mortality was obtained with concentrations of 0.1% (*Anopheles stephensi*), 0.2% (*An. albimanus*), or 0.3% (*An. quadrimaculatus*) *B.t.i.* When in 175-liter containers the activity of formulations (5% corn oil, 0.2% *B.t.i.*) and of toxin suspensions was compared by conventional dosage-mortality regression, formulations were more active by a factor of 68 against *An. stephensi*, 39 against *An. albimanus* and 67 against *An. quadrimaculatus*.

INTRODUCTION

During the last decade, resistance of mosquitoes to chemical insecticides and environmental concerns have stimulated efforts to develop bacterial agents like *Bacillus thuringiensis* var. *israelensis* (de Barjac) (*B.t.i.*) as an alternative to chemical insecticides for the control of mosquitoes. The toxin produced by this bacterium has shown promise for mosquito control programs due to its high and selective activity against the larval instars. At present, a variety of formulations of primary powder (dried bacterial spores and toxin particles) of *B.t.i.* like liquid concentrates or granular formulations are used in operational control programs yielding effective control of *Aedes, Culex* and *Psorophora* larvae (Mulla 1985).

These formulations are less effective against *Anopheles* larvae due to the differential feeding behavior among mosquito species. Larvae of *Culex* and *Aedes* species are water filterers, whereas *Anopheles* larvae are adapted to collect particles from the air-water interface. Feeding individuals remain at the water surface in a horizontal position and filter only particulates present in the uppermost layers of water; particulates which sink deeper than 1-2 mm are not ingested. In addition, filtration rates of *Anopheles* larvae are approximately 10-20 times smaller than filtration rates of larval *Culex* or *Aedes* (Aly, in press). Thus, *Anopheles* larvae ingest water suspended *B.t.i.* toxin particles at low rates, which results in reduced effectiveness of the stomach toxin (Aly et al., in press). In contrast, *Anopheles* larvae are capable of rapid ingestion of surface-bound food particulates like yeast or wheat flour (Aly and Mulla 1986a). Therefore, formulation of *B.t.i.* as a bait confined to the water surface could increase activity of the toxin against *Anopheles* larvae.

In this study, the development of a surface-bound bait formulation of *B.t.i.* for the control of *Anopheles* larvae is described. Selection of a suitable spreading agent and the optimal toxin concentration in the bait powder, as well as the performance of a powder formulation in laboratory and simulated field tests are described. Although operational problems with *B.t.i.* formulations like UV-stability or pelletizing of baits for application have not yet been solved, results from this study should lead to further development of potent *B.t.i.* formulations for the control of *Anopheles* mosquitoes.

MATERIALS AND METHODS

Mosquito larvae and bacterial isolate. Mosquito larvae from laboratory colonies were reared in 25°C water on a diet of yeast and dog bisquits as described by Aly (in press). Early fourth instars were obtained 4-5 days [*Aedes aegypti* (Linn.)], or 8–11 days (*Anopheles*) after hatch, separated from food and debris and used within 2–3 hr for experiments.

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Bacillus thuringiensis var. *israelensis* was isolated from a commercial product (Abbott ABG-6145; Abbott Laboratories, Chicago, IL) and cultured in a liquid medium consisting of peptonized milk, dextrose and yeast extract at 30°C and with constant agitation (H. Dulmage, personal communication). After 3 days of fermentation, the fully sporulated cultures were centrifuged, repeatedly washed with distilled water and lyophilized. The resulting product, a so-called primary powder, contained spores, toxin particles and cell debris.

The bioactivity of the primary powder was determined by comparing its effectiveness against fourth instars of *Ae. aegypti* with the effectiveness of the standard IPS-82 (Institut Pasteur, Paris, France). Groups of 30 larvae were exposed in 100 ml deionized water (25°C) to serial dilutions of test or standard preparation. Each preparation was tested in 4 concentrations arranged in logarithmical sequence to induce between 10 and 90% mortality within 24 hr of exposure. At this time, actual mortality rates were determined. Dosage mortality regression was analyzed separately for each of 5 replicates using the computerized procedure of Ray (1982). In comparison with the standard, the primary powder revealed a potency of 22,700 IU/mg.

Selection of a spreading agent. The following materials were tested as adjuvants to improve the spreading and floating abilities of wheat flour: corn oil (commercial grade), soy bean lecithin (commercial grade), Arosurf® (active ingredient: ethoxylate of isostearyl alcohol), and Liparol (active ingredients: isoparaffines and lecithin; Schnetter and Engler 1978). Adjuvants were dissolved in carbon tetrachloride and mixed with wheat flour in concentrations of 1, 5 and 25% (w/w). The solvent was evaporated in an air stream under frequent stirring.

As a first parameter for suitability, spreading of the mixtures on a water surface was quantified. Tests were carried out in disposable waxed paper cups filled with 100 ml tap water at 25°C. With a ring tensiometer (Central Scientific Co., Chicago, IL) the surface tension of clean water in the test cups was first determined as a reference value. Subsequently, 10 mg test mixture were applied on the water surface of 43 cm^2, and the surface tension was again measured. Means of surface tensions before and after application of test mixtures were calculated from 9 measurements obtained from 3 replicates.

A second parameter for suitability of formulations was the ingestion of mixtures by larvae of *Anopheles albimanus* Wiedemann. To allow measurement of the ingestion of test mixtures by optical assessment, larvae were exposed before treatment for 1–2 hr to suspensions of Chinese ink, a suspension of carbon particles. During this pretreatment period, larvae filled 1-2 gut segments (marked by body segmentation) from a total of 6 with black carbon particles. Materials ingested by larvae during later treatments push the carbon particles posteriorly, and ingestion rates can be assessed optically by counting the substrate-filled gut segments through the transparent cuticle of the larva under a dissecting microscope (Dadd 1968, Aly 1985). Following the pretreatment with suspensions of Chinese ink, larvae were washed and introduced in groups of 20 in plastic containers holding 500 ml of tap water at 25°C. After an acclimatization period of 30 min, 10 mg test material were sprinkled on the water surface (185 cm^2). Larvae were allowed to feed for 15 min, then collected in a net and rapidly killed by brief submission in hot water. The number of gut segments filled with the white test materials, which was sharply separated from the previously ingested ink column in the gut, was counted under a dissecting microscope. Three tests were conducted on 3 different occasions, each including pure wheat flour as a reference material.

Concentration of active ingredient in bait powder. Primary powder of *B.t.i.* was suspended in ice cold carbon tetrachloride in a concentration of 10 mg/ml by low power ultrasound treatment. Appropriate amounts of this stock suspension were mixed with wheat flour and corn oil in pure carbon tetrachloride. After the evaporation of the solvent in an air-stream the mixtures contained 5% oil, and 0.01, 0.02, 0.05, 0.1, 0.2 or 0.5% (w/w) primary powder. For controls, mixtures of wheat flour and corn oil were similarly prepared with carbon tetrachloride only.

To select mixtures with a sufficient content of active ingredient, fourth instars of *Anopheles albimanus, An. quadrimaculatus* Say or *An. stephensi* Liston were exposed to a surplus of material. In dishpans holding 8 liters of tap water (25°C) 50 larvae were treated with 30 mg of mixture sprinkled on the water surface (640 cm^2). Mortality was determined 24 hr posttreatment. Mean mortality rates were calculated from 3 replicates performed with different groups of larvae. From mixtures inducing 100% mortality, the one with the lowest content of active ingredient was selected for further testing.

Bioassays in a 100 ml system. Although the full advantage of floating- versus water-suspended formulations can be assessed best in deep water, tests in a 100 ml system were performed to check if small water volumes can be used for initial screenings.

Efficacy of formulations containing 0.1 or 0.2% primary powder was compared with the efficacy of suspensions of the primary powder

used in the preparation of the formulations. Formulations were weighed on an electronic balance with an accuracy of ±10 μg. Stock suspensions of the primary powder were prepared at a concentration of 1 mg/100 ml in a buffered detergent solution (2.5 mM phosphate buffer pH 7.2, 0.01% Tween 80). Microscopical checks showed that bacterial spores and toxin particles suspend well in such a solution. Larvae were introduced in groups of 30 in disposable styrofoam cups filled with 100 ml deionized water. Formulations were sprinkled on the water surface, whereas stock suspensions of primary powder were applied in appropriate amounts with a pipet. During each replicate, 4 doses of each formulation and 4 concentrations of primary powder suspension were tested. Mortality rates were determined 24 hr posttreatment. Results from 3 independent replicates were pooled and subjected to dosage mortality regression analysis (Ray 1982).

Bioassays in a 175 liter system. Plastic containers located in a greenhouse were used to compare the effectiveness of formulations and toxin suspensions in a system with deep water. For each replicate, 9 containers with diameters of 63 cm were filled with 175 liters of tap water. After 20 hr of temperature adjustment and dissipation of gases, larvae of one test species were introduced in groups of 100 individuals/container. During acclimatization of larvae (1–2 hr), stock suspensions of the primary powder (1 mg/10 ml) were freshly prepared, and formulations were weighed as described above. In each test (replicated 3 times at different occasions), four amounts of formulation containing 0.1% (or 0.2%) primary powder, and 4 concentrations of primary powder suspensions were tested simultaneously. One container remained untreated as a control. Tested amounts of formulations ranged within the following limits: *An. stephensi*, 1–6 (0.1% formulation) and 1–4 mg/container (0.2% formulation); *An. albimanus*, 2–18 (0.1% formulation) and 2–9 mg/container (0.2% formulation); and *An. quadrimaculatus*, 1–9 mg/container (0.2% formulation). Formulations were applied at the center of containers regardless of the distribution of larvae. Stock suspensions were tested in amounts of 0.5–10 ml/container (*An. stephensi*), 2.5–10 ml/container (*An. albimanus*), or 1–16 ml/container (*An. quadrimaculatus*). For each replicate, four amounts of stock suspension within these limits were mixed with 100 ml of tap water in a squeeze bottle. Suspensions were distributed evenly over the water surface of the containers, and the water was not stirred after application to allow a higher toxin concentration in the upper water layers. Twenty-four hr after treatment, the number of surviving larvae was recorded. Mortality was corrected for mortality in the control container (<5%), and dosage mortality regression analysis was performed as described above.

RESULTS

Selection of a spreading agent. The largest reduction of surface tension (36%) was caused by Arosurf, when mixtures containing 5% adjuvant were compared (Table 1). At this concentration, lecithin and corn oil caused a moderate reduction (13–15%), while use of Liparol resulted in only a small reduction of surface tension. Based on these findings, Arosurf should be a promising candidate for enhancing the spreading abilities of a wheat flour formulation.

However, mixtures of Arosurf and flour were poorly ingested by *An. albimanus* larvae (Table 2). In comparison to pure wheat flour, ingestion rates were reduced by 49% and 89% in the presence of 1% and 5% Arosurf. Since *B.t.i.* is only active upon ingestion as a stomach toxin, Arosurf appeared to be an unsuitable adjuvant for a bait formulation.

Feeding rates were reduced by 6–25% in the presence of corn oil, Liparol or lecithin at concentrations of 1 or 5% (Table 2). Since corn oil had induced a satisfactory reduction of surface tension at concentrations of 5%, this cheap and generally available material was chosen as the adjuvant for further studies.

Concentration of toxin in bait powder. Formulations with a concentration of primary powder at or below 0.05% failed to induce complete mortality, even when offered in amounts far exceeding the total gut volumes of test groups (Table 3). Larvae surviving treatment with these low concentrated formulations were found actively feeding 24 hr post-treatment and had

Table 1. Surface tension of distilled water after application of wheat flour mixed with various adjuvants.[a]

Adjuvant	Surface tension (units) at % adjuvant in wheat flour	
	5	25
Arosurf	30.7 ± 0.2a[b]	30.7 ± 0.2a
Corn oil	40.5 ± 0.3b	40.2 ± 0.2b
Lecithin	41.6 ± 0.2c	35.1 ± 0.2d
Liparol	46.8 ± 0.1e	31.6 ± 0.4f

[a] Ten mg of mixtures applied to 43 cm^2 water surface; surface tension was determined with an adhesion balance (ring method), and is presented in units (direct reading from the instrument).

[b] Mean and standard deviation of 3 replicates; means followed by different letters are significantly different at the 0.05 level (Duncan's Multiple Range Test, Ray 1982); all means were significantly different from the surface tension after application of pure wheat flour (47.9 ± 0.2 units).

Table 2. Ingestion rates of fourth-instar *Anopheles albimanus* in the presence of wheat flour treated with various adjuvants.[a]

Wheat flour + adjuvant	Relative ingestion rate at % adjuvant[b]		
	1	5	25
Corn oil	92.9a[c]	73.6ab	40.2cd
Lecithin	78.3ab	82.9ab	35.1de
Liparol	84.0ab	91.7a	34.7de
Arosurf	60.5bc	11.4ef	8.4f

[a] Groups of 20 larvae exposed to 10 mg material sprinkled on 185 cm^2 water surface; number of filled gut segments (marked by body segmentation) determined optically.

[b] Ingestion of mixtures relative to ingestion of pure wheat flour during the same replicate.

[c] Means of 3 replicates; means followed by the same letter (rows and columns) are not significantly different at the 0.05% level; means followed by "a" not significant from ingestion of pure wheat flour.

Table 3. Mortality of fourth-instar larvae of *Anopheles* following the application of surplus amounts of formulations with variable concentrations of *B.t.i.* primary powder.[a]

Concentration of *B.t.i.* in formulation (%)	Mortality 24 hr posttreatment (%)		
	An. stephensi	*An. albimanus*	*An. quadrimaculatus*
0.01	38 ± 10[b]	2 ± 2	2 ± 4
0.02	84 ± 10	18 ± 14	18 ± 18
0.05	98 ± 3	82 ± 18	68 ± 8
0.1	100	98 ± 2	90 ± 10
0.2	100	100	96 ± 4
0.5	100	100	100

[a] Fifty-fourth instars exposed in 8 liters of water; 30 mg formulation sprinkled on the water surface (640 cm^2).

[b] Mean and standard deviation of 3 replicates.

removed the applied material completely from the water surface. In contrast, formulations containing 0.1% or more primary powder induced 90–100% mortality within 24 hr after application (Table 3). The few surviving individuals were not feeding any more at this time point, and the applied amounts of formulations were not completely removed from the water surface. Complete mortality was obtained by formulations with 0.1% (*An. stephensi*), 0.2% (*An. albimanus*) or 0.3% (*An. quadrimaculatus*) primary powder. For further evaluation, formulations containing 0.1 and 0.2% primary powder were selected.

Bioassays. Effectiveness of formulations and suspensions of primary powder was compared first in a 100 ml test system to evaluate if improvement of activity of surface bound formulations can be assessed in small water volumes. It appeared that formulations were 3–4 times (*An. albimanus* and *An. quadrimaculatus*), or 2 times (*An. stephensi*) more active than suspensions (Table 4). Larvae of test groups were observed ingesting the offered amounts of formulation within short time spans (maximum 20 min). Thus, small water volumes can be used to economically demonstrate the activity of surface-bound formulations, although the full potential of these formulations may be seen best in large test systems.

In greenhouse tests, effectiveness of bait formulations and water suspended primary powder was compared in 175-liter containers. The formulation containing 0.2% primary powder was 39 times (*An. albimanus*), 67 times (*An. quadrimaculatus*) or 68 times (*An. stephensi*) more active than suspensions of primary powder (Fig. 1). These improvements in the effectiveness of the floating formulation were observed although suspensions were applied to the water surface without subsequent stirring; stirring would have distributed the water-suspended material in a much larger water volume, and would have resulted in an even larger advantage of the floating materials over the suspensions. With the 0.1% bait formulation, improvements of the effectiveness over suspended material by a factor of 37 (*An. albimanus*) or a factor of 72 (*An. stephensi*) were found. For *An. quadrimaculatus,* even large amounts of this formulation did not result in more than 70% mortality. In all other cases, mortality regression lines could be established; slopes were similar for formulations and toxin suspensions (Fig. 1).

DISCUSSION

To achieve high effectiveness of stomach toxins, these materials should be applied at the feeding zone of the target and retained there until ingestion is completed. Phagostimulation will further increase the effectiveness, since smaller amounts of toxins are sufficient to result

Table 4. Effectiveness of formulated and water suspended *B.t.i.* primary powder in a 100 ml test system.[a]

Mosquito species	LC_{50} (mg primary powder/liter)		Increase of effectiveness (factor)
	Formulation[b]	Suspension	
An. albimanus	1.58 (1.8)[c]	6.15 (1.7)	3.89
An. quadrimaculatus	1.88 (1.3)	6.43 (1.3)	3.42
An. stephensi	1.41 (2.4)	2.67 (2.0)	1.89

[a] Groups of 30 fourth instars exposed to 4 concentrations/replicate, 3 replicates.

[b] Content of primary powder 0.2%.

[c] In parenthesis: slopes of dosage-mortality regression lines.

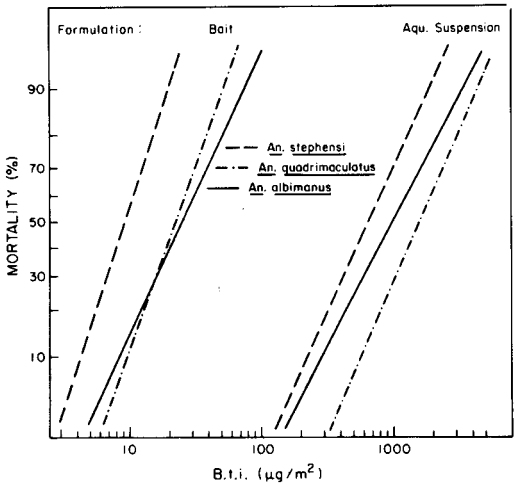

Fig. 1. Mortality of mosquito larvae induced by bait-formulated and water-suspended *B.t.i.* primary powder in containers holding 175 liters of water. Formulations consisted of wheat flour with a content of 5% corn oil and 0.2% primary powder. Formulations and suspensions were applied to the water surface without subsequent stirring, mortality was recorded 24 hr post-treatment. Four concentrations were tested per replicate, data from 3 replicates were pooled. Chi-square values of replicates <8.

in certain levels of mortality if ingested at increased velocity (Aly et al., in press); phagosuppressants of deterrents will reduce the effectiveness of the toxin.

Anopheles larvae mainly collect particles prevailing at the air-water interface. Whereas food particles connected to the water surface are rapidly ingested (Aly and Mulla 1986a), larvae filter water-suspended particles at low rates (Aly, in press). Therefore, optimal ingestion of a stomach toxin can only be expected when surface-bound formulations are used. Similar considerations had previously resulted in surface applications of chemical stomach toxins in *Anopheles* control programs (Metcalf and Hess 1944). Recently it was demonstrated that the formulation of *B.t.i.* in buoyant droplets concentrated this toxin in the upper water zones, and consequently resulted in a large increase of its effectiveness against *Anopheles freeborni* Aitken larvae (Cheung and Hammock 1985).

Inhibition of feeding by formulation ingredients counteracts the effective use of a stomach toxin. In our experiments Arosurf was found to strongly deter larval feeding and therefore could not be used as an adjuvant, although this material is a potent spreading agent. Earlier attempts have been made to distribute and retain bacterial toxins on the water surface by the addition of Arosurf; applied at high rates, mixtures of *B.t.i.* and Arosurf were found useful to control larvae and pupae of mosquitoes simultaneously (Levy et al. 1984). However, in field tests the mixture failed to control larvae of *Culex tarsalis* Coquillett and *Culex quinquefasciatus* Say at rates containing amounts of *B.t.i.* effective when applied without Arosurf (M. S. Mulla, unpublished data). Most likely, feeding inhibition by Arosurf as it was demonstrated in the present study prohibited the efficient ingestion of the bacterial agent by the mosquito larvae.

Gustatory stimulation of target insects has been used to enhance ingestion rates and effectiveness of pathogens in a number of insect species. In the bollworms *Heliothis virescens* (Fabr.), *H. zea* (Boddie) and *Pectinophora gossypiella* (Saunders), ingestion of viruses was stimulated and infection rates were increased when formulations contained phagostimulant components like cottonseed oil, aqueous plant extracts or sucrose (Montaya et al. 1966, Bell and Kanavel 1975, Smith et al. 1982). Similarly, molasses or wheat bran was used as an adjuvant in formulations of *B. thuringiensis* Berliner or *Nosema locustae* to control the gypsy moth *Lymantria dispar* (Linn.) or several species of *Melanoplus* grasshoppers (Yendol et al. 1975, Henry et al. 1978).

For a variety of insects, bait formulations have been developed and successfully tested under field conditions (reviewed by Couch and Ignoffo 1981). For mosquito larvae, phagostimulation by components associated with food has been reported for *Culex pipiens* Linn. (Dadd 1970), *Aedes vexans* Meigen (Aly 1985) and *An. albimanus* (Aly and Mulla 1986a), the last species ingesting food particulates up to 8 times faster than inert materials. Therefore, formulation of *B.t.i.* in a bait seemed suitable to increase its effectiveness against mosquito larvae. Such an increase of effectiveness was observed, when primary powder was formulated as bottom pellets containing fishmeal, a phagostimulant for larval *Ae. vexans* (Aly 1985), and tested against the browsing larvae of this species (Aly 1983).

In the present study, the combination of 2 effects—retaining of toxin at the primary feeding zone of larvae and addition of a gustatory carrier particle—resulted in a substantial increase in the effectiveness of *B.t.i.* against larvae of 3 *Anopheles* species. However, a number of technical problems such as shelf life, sensitivity to UV light, and difficulties associated with the application of the proposed formulation remain to be investigated and resolved. Nevertheless, it is important to note that the information gathered here could lead to further development of environmentally compatible bacterial agents (Aly and Mulla 1986b). More effective formulations for the control of disease transmitting anopheline mosquitoes, which are less suscepti-

ble to currently-used formulations of bacterial pathogens, are needed.

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