SMALL SCALE FIELD TRIALS OF *BACILLUS SPHAERICUS* (STRAIN 2362) AGAINST ANOPHELINE AND CULICINE MOSQUITO LARVAE IN SOUTHERN MEXICO

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ABSTRACT. Experimental breeding sites simulating natural conditions were used to evaluate the efficacy of 2 formulations of *Bacillus sphaericus* (strain 2362) against *Anopheles albimanus* and culicine (mostly *Culex coronator* and *Cx. quinquefasciatus*) mosquito larvae of southern Mexico. Three doses of each formulation were used in a first field trial: 2, 3 and 4 g/m^2 (granular) or 2, 3 and 4 ml/m^2 (liquid); and in a second field trial: 0.125, 0.24 and 0.5 g/m^2 (granular) or 0.125, 0.25 and 0.5 ml/m^2 (liquid). The optimum concentrations of each formulation for effective control of larval populations over periods of 3–4 months were 0.125 ml/m^2 of liquid product for *Culex* spp. and 2 g/m^2 of granular product for *An. albimanus* (ca. 70% mean reduction).

INTRODUCTION

Interest in the possibility of using *Bacillus sphaericus* Neide as an alternative insecticide in malaria control programs is based on the increasing spread of resistance to synthetic chemical insecticides and the increasing need to integrate other nonchemical insecticides into the current control programs. *Bacillus sphaericus* elicits a residual effect as a result of spore recycling in mosquito cadavers, which are released to their aqueous environment while the cadavers disintegrate (Davidson et al. 1984, Des Rochers and García 1984, Charles and Nicolas 1986). This form of control has the advantage of being noncontaminant and relatively innocuous to nontarget organisms (Shadduck et al. 1980, Mulla et al. 1984).

The efficacy of *B. sphaericus* strain 2362 (Weiser 1984) in controlling mosquito larvae has been tested on several occasions (Vanková 1984, Majori et al. 1987, Mulla et al. 1987, 1988a, 1988b). Long-term larvicide action of this pathogen has been observed, and evidence for persistence of *B. sphaericus* 4 years after its introduction in breeding sites for *Culex pipiens* Linn. larval control has been documented (Karch et al. 1988).

The objectives of the present study were 3-fold: to determine under simulated natural conditions the susceptibility of *Anopheles* spp. and *Culex* spp. mosquito larvae from southern Mexico to 2 formulations of *B. sphaericus* (strain 2362), to determine the most effective concentration of these products, and to assess their residual activity.

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MATERIALS AND METHODS

Laboratory assays: Two formulations of *B. sphaericus* were evaluated: one granular (ABG-6185) from Abbott Laboratories (North Chicago, IL), and one liquid (Flowable Concentrate FC, BSP-2) from Solvay & Co. (Brussels, Belgium). Counts of viable spores of each formulation were carried out using a spread plate technique. At the time of the bioassays (August 1986), the number of viable spores of the granular formulation (3.9×10^9 spores/mg) was in the same order of magnitude as that of the liquid product (2.15×10^9 spores/ml). Both granular and liquid formulations were initially tested in triplicate laboratory bioassays to determine lethal dose concentrations. To obtain the desired concentrations, serial dilutions were made by shaking the starting materials (granules or liquid) in a stirrer with crystal pearls for a 2-h period using 10 g of either formulation (density of the liquid formulation was almost equal to 1). This was then diluted in a liter of water and used as the stock suspension. Samples of 25 field-collected *Anopheles albimanus* Wied. larvae (2nd and 3rd instars) were used for each concentration. Larvae were placed in plastic cups with 100 ml of distilled water which contained either 2, 4 or 6 mg/liter of granular product or 0.5, 1, 2 or 4 µl/liter of liquid product. Controls were run in triplicate in each trial. In a similar way, *Culex quinquefasciatus* Say larvae were exposed to 0.05, 0.1, 0.5 and 1 µl/liter of liquid formulation. Larval mortality was recorded after 24-, 48- and 72-h exposure.

Field tests: The study area is situated in a pasture area within a cattle ranch called Venecia (14° 49′ 44″ N, 92° 21′ 45″ W), located 20 km south of Tapachula, Chiapas. The climate is hot-subhumid (García 1973), with 2 well-defined seasons: the rainy season, which extends from May to October, and the intervening dry season. Mean annual rainfall between 1986 and 1987

was 2,010 mm, while the RH ranged from 68 to 88% with an average annual temperature of 26.7°C.

Four sets of 12 plots (each plot, $1m^2$) of variable depths were prepared with an average distance of 200–300 m between each set. Plots within one set were separated by no more than 2 m. One set was constructed in a shaded area while the other 3 sets were located under direct sunlight (exposed areas 1, 2 and 3). The plots were dug deep enough to maintain a minimum water level of 15–25 cm. Vegetation around plots consisted primarily of *Panicum* and *Paspalum* grasses. Water temperature in experimental plots during all experiments ranged from 26.1 to 31.7°C. Once the plots were prepared they were allowed to stabilize for 3 weeks. During this period mosquitoes naturally oviposited in each site.

Larvae were sampled from each plot 3 times per week. Each sample (10 dips of 500 ml each) was taken along each side of the plot, in each corner and 2 in the center. Mosquitoes from each sample were classified to genus and larval stage (I—IV instars), quantified and returned into their respective plots. Larval densities per sample were calculated on the basis of III—IV instar larval count and divided by the number of dips. First and second instars were omitted from the calculation because it was considered likely that their densities would depend more on oviposition rates than on the effectiveness of the treatment. Water temperature and level of each plot were measured during each sampling. Potential larval predators were numerous (fish, frogs, other insects), and care was taken to reduce their numbers every day. Fast growing algae (*Spirogyra* sp.) which tended to cover the surface of some plots were also continuously removed.

After a preliminary field trial to standardize the methodology, a first trial was initiated on December 18, 1986, and included the application of both granular and liquid formulations in both shaded and sun exposed areas. Prior to larvicide applications, plots were filled to a depth of 25 cm with water, and the total volume was calculated. Concentrations of *B. sphaericus* for field applications were at least double the necessary dosage to kill 100% of *An. albimanus* larvae in laboratory bioassays. Three doses of each formulation were used: 2, 3 and 4 g/m^2 (granular Abbott) or 2, 3 and 4 ml/m^2 (liquid Solvay). Application of each concentration was made by hand using a polyethylene squeeze bottle (Mulla et al. 1984) to completely cover the water's surface of each plot. Three sets of plots were used in this test: the shaded area, exposed area 1 and exposed area 2. While plots in the shaded area and exposed area 1 were filled with water from an irrigation canal, plots in exposed area 2 were filled with water from a small creek containing sugar cane residues or "vinaza" from a sugar cane liquor distillery located nearby (i.e., the unused liquid remnants from a sugar cane mill after sugar production are the prime materials for the sugar cane liquor production; these materials are first fermented, then distilled, and the unfermented portion, the "vinaza," is discharged to the creek). Two plots each were selected using concentrations of 3 and 4 g/m^2 (granular Abbott) or 3 and 4 ml/m^2 (liquid Solvay). Only one plot was selected to evaluate the concentration 2 g/m^2 (granular Abbott) or 2 ml/m^2 (liquid Solvay). Two plots in each set were used as controls. The plots were evaluated for a period of 18 weeks posttreatment.

A second field trial was carried out to determine whether lower concentrations of bacteria than those used in the first field trial were sufficient to reduce anopheline larval populations. This trial began on June 3, 1987, and was carried out in only one set of plots; exposed area 3. The concentrations tested in this experiment were 0.125, 0.25 and 0.5 g/m^2 (granular Abbott) or 0.125, 0.25 and 0.5 ml/m^2 (liquid Solvay). Samples from plots were taken 3 days per week, covering a period of 12 weeks posttreatment.

Data analysis: The effect of the treatments in both trials on larval populations was calculated at percent reduction using the formula developed by Bown et al.[3] and Mulla et al. (1971). Statistical analysis to compare the differences in larval densities and mean reduction was performed using a Fisher's Protected Least Significant Difference (PLSD) test (Milliken and Johnson 1984) in one way factorial ANOVA (Winer 1971).

RESULTS

Laboratory tests of granular and liquid formulations showed 4 mg/liter (1 g/m^2) to be the minimum lethal dose for *An. albimanus* larvae after 48 h of exposure. *Anopheles albimanus* was 20–30 times more tolerant to *B. sphaericus* than *Cx. quinquefasciatus* larvae (LD_{100} = 0.1 mg/liter). Bacterial concentrations for field trials were calculated on the basis of *Anopheles* larval susceptibility, except for the second experiment which was made to determine if concentrations

[3] Bown, D. N., Y. H. Bang and N. Rishikesh. 1973. WHO three month annual report: bio-control methodology. *Anopheles* Control Research Unit 1. Kaduna, Nigeria.

lower than the minimum lethal dose were sufficient to control *An. albimanus*.

First field trial: This experiment was carried out during the dry season (December 1986 to mid-April 1987). The pretreatment period for this trial consisted of 4 weeks of sampling. Because there were no differences between the replicate plots, the larval densities were pooled together as an average. Mean larval densities in the shaded area were 1.1 larvae/dip for *An. albimanus* (range 0.03–5.2) and 9.2 larvae/dip for *Culex* spp. (range 0.1–45.8). In the exposed area 1, *An. albimanus* mean larval density was 2.5 larvae/dip (range 0–11.6) and for *Culex* spp. 0.63 larvae/dip (range 0–2.9). In exposed area 2, *An. albimanus* mean larval population was 2.1 larvae/dip (range 0–16.3) and for *Culex* spp. 57.5 larvae/dip (range 0–1,166.7). No significant differences were found in larval densities of *An. albimanus* among the 3 areas following an ANOVA on the numbers of larvae per dip. *Culex* spp. larval densities in exposed area 2 were 6- to 90-fold higher than those of shaded and exposed 1 areas ($P < 0.01$).

In the exposed area 1, *An. albimanus* larvae were effectively reduced during the entire experiment by most concentrations of both products (Table 1). Data from larval densities in control plots were generally consistently higher than those in treated plots ($P < 0.01$)(Table 2). The highest effect was observed with 3 g/m^2 of the granular product (96% mean reduction) and with 2 ml/m^2 of the liquid product (95% mean reduction). In this area no significant differences were observed when using either of the 2 formulations against *An. albimanus*.

In the exposed area 2, larvicide effect was evaluated for 10 weeks. During this period, notable changes in the water employed to fill the plots occurred. This happened because the sugar cane liquor distillery, located about 100 m upstream, began to disacharge wastewater containing "vinaza" into the river at an unconstant rate. Maximum discharges of vinaza occurred between weeks 0 and 1 and 6 and 10, resulting in an inverse relation being generated between *An. albimanus* larval populations and the volume of vinaza (Table 2). The mean reduction value for the concentration of 2 g/m^2 of the granular product was 74%, and that of the liquid product was 67% at the application rate of 3 ml/m^2 (Table 1). No significant differences were found between the most effective concentrations.

In the shaded area, larval densities of both genera fluctuated sharply, while control plots continued to demonstrate higher larval densities during most observations (Table 2). The lowest concentrations of granular (2 g/m^2) and liquid (2 ml/m^2) formulations had the best sustained effect in reducing *An. albimanus* larval populations through 12 weeks of treatment (Table 1). Reduction of anopheline larvae fluctuated during the period, but had an overall mean reduction of 75% with the granular formulation and

Table 1. Mean overall reduction (%) of *Anopheles albimanus* and *Culex* larvae in experimental areas using different concentrations of both granular and liquid products of *Bacillus* sp. over variable periods of posttreatment in the first and second field trials (Rancho Venecia, Chiapas, Mexico, Dec. 1986–Apr. 1987; Jun.–Sep. 1987).†

	Formulation				
	Granular Abbott			Liquid Solvay	
	20 kg/ha (2 g/m^2)	30 kg/ha (3 g/m^2)	40 kg/ha (4 g/m^2)	20 liters/ha (2 ml/m^2)	30 liters/ha (3 ml/m^2)
An. albimanus					
Exposed area 1*	81b	96a	90a	95a	83a
Exposed area 2Ψ	74a	36b	54a	32b	67a
Shaded areaΩ	75a	74a	52b	62a	47b
All areas	68a	66a	57b	59b	57b
	1.25 kg/ha (0.125 g/m^2)	2.5 kg/ha (0.25 g/m^2)	5 kg/ha (0.5 g/m^2)	1.25 liters/ha (0.125 ml/m^2)	2.5 liters/ha (0.25 ml/m^2)
Culex spp.					
Exposed area 3Ω	63b	65b	67b	87a	57b

a, b = Comparisons within concentrations and species only. Letter b means a significantly lower figure at the 95% level than the best concentration (a).

† 6 weeks posttreament.

Ψ 9 weeks posttreatment.

Ω 12 weeks posttreatment.

* 18 weeks posttreatment.

Table 2. Effect of granular (in kg/ha) and liquid (in liters/ha) formulations of *Bacillus sphaericus* (strain 2362) against *Anopheles albimanus* and *Culex* spp. III–IV instar larvae in experimental plots (Rancho Venecia, Chiapas, México, Dec. 1986–Apr. 1987; Jun.–Sep. 1987).

	Anopheles albimanus									*Culex*		
	Exposed area 1			Exposed area 2			Shaded area			Exposed area 3		
Weeks	Control	30 kg/ha† (3 g/m^2)	20 liters/ha (2 ml/m^2)	Control	20 kg/ha (2 g/m^2)	30 liters/ha (3 ml/m^2)	Control	20 kg/ha (2 g/m^2)	20 liters/ha (2 ml/m^2)	Control	1.25 kg/ha (0.125 g/m^2)	1.25 liters/ha (0.125 ml/m^2)
0**	0.5*	8.5	5	0.5	1	1	2.5	3	4	11.1	2	36
2	3.3	1.6 (97)Ψ	0.7 (98)	3.5	3.2 (55)	1.3 (82)	2.6	1.3 (59)	1.6 (62)	45.7	0 (100)	6.1 (96)
4	3.8	4.2 (94)	1.2 (97)	3.4	1.4 (80)	0.5 (93)	3.4	1.5 (63)	0.6 (39)	10 (10)	0.3 (83)	2.7 (92)
6	3.4	1.2 (98)	2.1 (94)	1.8	0.9 (75)	1.2 (66)	3.2	0.4 (90)	2.1 (59)	3.9 (65)	0.1 (83)	2.1 (83)
8	2	1.7 (95)	1.8 (91)	0.6	0.2 (85)	0.2 (85)	2.6	0 (100)	1.6 (62)	2.6 (77)	0.3 (45)	0 (100)
10	2.3	2.3 (94)	1.7 (93)	0	0	0	2.8	0.6 (82)	0 (100)	3.1 (72)	0.3 (45)	0.3 (97)
12	2.3	0.5 (99)	1 (96)				1.8 (28)	0.6 (72)	1.1 (62)	1 (91)	3.1 (0)	1.1 (63)
14	5.9	3.8 (96)	2 (97)									
16	7.5	1.1 (99)	0.3 (100)									
18	7.8	8.5 (94)	0.8 (99)									

† Data of best effect concentrations is shown.
* Average no. of III-IV instar larvae per 10 dips.
Ψ Percent reduction in parentheses.
** Larval densities in day 0.

62% with the liquid product. No differences were found in the effect of both formulations upon anopheline larvae.

After integrating results from all areas, the most effective concentrations for reducing *An. albimanus* populations were 2 g/m^2 of the granular product (68% mean reduction) and 2 ml/m^2 of liquid formulation (57% mean reduction) over an 18-week period (Table 1). Statistical comparison of these results showed a significant difference ($P < 0.05$). *Culex* spp. larval populations were also effectively reduced in all areas by all concentrations of the granular and liquid formulations, but these results are not included since lower concentrations of bacteria were sufficient to control *Culex* spp. (see results from the second field trial).

Second field trial: Low concentrations of either granular or liquid formulations were not effective in reducing anopheline larval populations, even 1 and 2 days following the first application. Mean reduction levels of 28% with the liquid product (0.5 ml/m^2) and 22% with the granular product (0.5 g/m^2) were observed 3 weeks posttreatment.

Culex larval populations were reduced best by concentrations of 0.125 ml/m^2 of liquid product (87% mean reduction) and 0.5 g/m^2 of granular product (67% mean reduction) through 12 weeks posttreatment (Tables 1 and 2). The liquid formulation was significantly more effective than the granular ($P < 0.01$).

DISCUSSION

The first field trial demonstrated that when spores of *B. sphaericus* 2362 are applied under conditions where a minimum water source is maintained, its effect in reducing larval populations of *Anopheles* mosquitoes can extend for a considerable period. Both formulations tested demonstrated capabilities to reduce *An. albimanus* at all concentrations, even in heavily organically polluted environments (exposed area 2). Mulla et al. (1988a), found also a good larvicidal effect of *B. sphaericus* 2362 in polluted waters. The lowest concentrations of bacteria appeared to maintain reduction levels for longer periods and demonstrated the highest overall mean percent reduction. These results are not well understood; however, due the host specificity of *B. sphaericus* (Davidson 1985), residual larvae could be necessary to maintain bacterial cultures. Although some attempts to recover *B. sphaericus* from plot water or dead larvae were unsuccessful, positive reduction levels were observed in treated plots throughout the 18 weeks after larvicide application. Fluctuations in reduction levels over time were probably a result of different rates of natural oviposition in experimental plots.

A clear difference between the effect of both larvicides on *An. albimanus* was found to exist with the location of the experimental breeding sites. Granular and liquid formulations reduced densities of anopheline larvae more effectively in areas exposed to direct sunlight (Tables 1 and 2). Habitats of this type are abundant in the highly modified agricultural areas found along the coastal plain of Chiapas and are known to be a preferred oviposition site for *An. albimanus*.[4] Results from the second field trial indicated that sub-LD_{100} concentrations for *Anopheles* larvae (1 g/m^2 in 48 h) were not an effective control measure in the field, even though highly reduced levels of *Culex* spp. larvae were produced.

Higher reductions of *Anopheles* larval populations were attained by the granular formulation as compared with the same concentrations of the liquid formulation. This could be explained by the feeding habits of these species (Dahl 1988). The granular formulation would tend to remain longer at the surface than the liquid formulation; and, since *Anopheles* species are generally surface feeders, they are more likely to have more contact with the granular product. Conversely, the liquid product proved to be better for control of *Culex* spp. larvae, which are mainly suspension feeders.

High concentration levels of either formulation used for 3 and 4 months against *Culex* spp. and *An. albimanus* showed that in planning an effective strategy for larval control, 4 rounds of treatment per year at doses of 2.0 g/m^2 would be sufficient to maintain overall reduction levels of ca. 70% for *Anopheles* larvae. Considerably higher doses (10 g/m^2) with a residual activity of only 6–10 weeks were reported necessary to control *Cx. quinquefasciatus* in Africa (World Health Organization 1985).

ACKNOWLEDGMENTS

The authors wish to thank Amílcar Zúñiga, Luis Domínguez, Francisco Maldonado, Enrique Pérez and Octaviano Girón for technical assistance. We also thank Dr. E. Loyola, Dr. J. M. Ramsey and Dr. J. Lines for their critical review of the manuscript and suggestions. This research was supported in part by grant T16/181/V2/104ID:860196 from the UNDP/World Bank/WHO Special Programme for Research

[4] Arredondo-Jimènez, J. I., M. H. Rodríguez, T. López, A. Orozco, G. Vázquez, E. G. Loyola and D. N. Bown. Larval ecology of *Anopheles albimanus* in southern Chiapas, México (unpublished report).

and Training in Tropical Diseases and by the Mexican Secretariat of Health.

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