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Fate of *Bacillus sphaericus* 1593 and 2362 Spores Used as Larvicides in the Aquatic Environment

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Dry powders produced from insecticidal *Bacillus sphaericus* strains 1593 and 2362 were applied against *Culex tarsalis* and *Anopheles franciscanus* mosquito larvae in small-plot field trials. Good control of *C. tarsalis*, but not of *A. franciscanus*, was produced at 0.1 and 0.2 lb/acre [ca. 0.122 and 0.244 kg/ha]. *B. sphaericus* spores settled rapidly from upper water layers and accumulated in bottom muds. Control of third/fourth-instar *C. tarsalis* larvae was maintained through day 4 after testing and was related to the presence of at least 100 spores per ml in the upper water layer 2 days earlier. *B. sphaericus* was shown to recycle in dead larvae both in the laboratory and in the field, producing an increase of 100- to 1,000-fold in spore numbers. There was no evidence of recycling in treated water nor of significant spore persistence upon reflooding of ponds after a very hot, dry period.

Bacillus sphaericus Neide strain 1593 has been shown to be highly insecticidal to larvae of *Culex* spp. and some *Anopheles* spp. mosquitoes in laboratory and small-plot field trials (5, 9, 12; reviewed in references 3 and 16). However, dry powder preparations of strain 1593 spores utilized in earlier field trials were unsatisfactory for various reasons, and none of these preparations is currently available from the manufacturer (5, 10, 17). Although insecticidal activity may be present for several weeks or months in the bottom of treated areas, most field trials of *B. sphaericus* have not detected persistence of insecticidal activity in the larval feeding zone (5, 7, 9, 10, 15). It has been suggested that this organism may recycle in the larval environment or in larval cadavers (7, 16).

Recently, a new strain of *B. sphaericus* has been isolated from a black fly breeding site in Nigeria by J. Weiser (J. Weiser, Zentralbl. Bakteriol., Parasitenkd. Infektionskr. Hyg. Abt. 2, in press). This strain, designated 2362, is of the same phage type and H serotype as strain 1593 but is reported to be somewhat more insecticidal than strain 1593 to larvae of susceptible mosquito species (18; A. A. Yousten, J. Invertebr. Pathol., in press; H. deBarjac, personal communication; J. Weiser and J. Vankova, personal communication).

The studies described here were undertaken with the following objectives: (i) to produce dry powder preparations of *B. sphaericus* 1593 and 2362 spores with good suspension properties in sufficient quantity for a series of small field trials; (ii) to test these powders against a variety of mosquito larvae in the laboratory and in the field; (iii) to follow the fate of the spores in treated field plots; and (iv) to investigate possible recycling of these organisms in the laboratory and in the field. Entomological aspects of this study are discussed in detail elsewhere (M. S. Mulla, submitted for publication).

MATERIALS AND METHODS

Preparation of *B. sphaericus* powders. Nutrient agar slants of *B. sphaericus* 1593 and 2362 were incubated for about 3 days at 28 to 30°C and then stored at 5°C until used. A loopful of surface growth from a slant was used to inoculate the 2,000-ml Erlenmeyer "seed" flask containing 750 ml of 1% Hysoy medium (Humko-Sheffield, Memphis, Tenn.). This flask was incubated on a rotary shaker at 28 to 30°C and 340 rpm for 8 to 12 h. A 250-liter fermentor containing 150 liters of peptonized milk medium (containing [g/liter] Humko-Sheffield peptonized milk nutrient, 10; series 1003 autolysed yeast extract [Amber Laboratories, Milwaukee, Wis.], 2; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.02) (J. A. N. Obeta, N. Okafor, and H. T. Dulmage, unpublished data) was inoculated from the seed flask at 0.5% by volume and incubated for 20 to 22 h at 28 to 30°C at 350 rpm, and at an aeration rate of 0.66 volumes per volume of liquid per min. Nearly complete sporulation was achieved. The cultures were centrifuged at 15,000 rpm at a flow rate of 3.3 liters/min in a continuous flow centrifuge. Dry powders were prepared from the creamy residues of the centrifugations by using the lactose coprecipitation method of Dulmage et al. (6). The final dry powder preparation of strain 1593 (lot no. IF 94–95) contained ca. 8.2×10^7 CFU per mg; the strain 2362 preparation (lot no. IF-97) contained ca. 8.5×10^7 CFU/mg.

Treatment of field plots. Ten sod-lined ponds, 35 m^2 and 30 to 40 cm deep, located in the Coachella Valley near Indio, Calif., were flooded 8 days in advance of testing (April 1982). Water level was maintained by means of a float valve. These ponds developed natural populations of *Culex tarsalis* Coquillett and *Anopheles franciscanus* McCracken. Water temperatures were 25.5 to 29°C maximum, 18.5 to 19°C minimum.

Each *B. sphaericus* preparation was applied at a dosage of either 0.1 or 0.2 lb/acre (ca. 0.122 or 0.244 kg/ha) with two replicates per treatment and two untreated controls. An appropriate amount of powder was suspended in tap water and delivered uniformly over the surface of the pond by using a squeeze bottle.

Three weeks after testing, ponds were permitted to dry and remained dry for 5 months. In October 1982, ponds were reflooded.

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Microbiological sampling. Samples for microbiological studies were taken immediately before treatment, 1 to 2 h posttreatment (day 0), and on days 1, 2, 3, 4, 14, and 21 posttreatment. Samples were also taken immediately after reflooding ponds and 10 days later. At each sampling time, one set of samples was taken near the edge of each pond and another set was taken from the middle of the pond. Plastic vials (20 ml) were used to sample the undisturbed water surface, the water just above the bottom, and the mud just at the surface of the bottom. During the first 5 days of sampling, water and mud samples were held on ice and plated within 3 to 4 h of sampling. Samples taken at 14 and 21 days were transported on ice and then refrigerated overnight before plating. After reflooding, surface and bottom water and mud samples were taken from the center of each treated pond and were refrigerated before plating. All water and mud samples were pasteurized at 80°C for 12 min before dilution and plating to kill vegetative and non-spore-forming bacteria. Pasteurized samples were plated on a selective medium designed for the recovery of *B. sphaericus*, consisting of nutrient agar with 0.05% yeast extract and 0.01% streptomycin (19). Colonies were identified at 24 to 48 h by distinctive colony morphology confirmed by microscopic observation. In a few cases where the identity of colonies was uncertain, bacteria from colonies in question were bioassayed against field-collected *C. tarsalis* larvae or against laboratory-reared *Culex quinquefasciatus* Say larvae.

For experiments on the development of pathology in the larvae, *C. tarsalis* larvae were collected from each treated pond at 2 h after treatment and confined to floating screened cages in the same pond. Four hours after treatment (day 0) and on days 1, 2, and 3, 25 of these larvae from each pond were rinsed with 95% ethanol followed by distilled water and homogenized in 5-ml sterile water with a glass tissue grinder. This homogenate was plated on selective medium before and after pasteurization. Colonies were identified as described above. In laboratory experiments, a similar technique was used to trace the fate of *B. sphaericus* 1593 spores from liquid cultures ingested by *C. quinquefasciatus* larvae during 15 min of feeding in a suspension of ca. 10^5 to 10^6 spores per ml at 25°C. In the laboratory, samples were taken immediately and at 1, 2, 3, 4, 5, 6, 9, 12, 24, 36, 48, 72, and 96 h after feeding and were plated on brain heart infusion agar plates before and after pasteurization.

RESULTS

Entomological data. When numbers of third- and fourth-instar *C. tarsalis* larvae in treated ponds were compared to controls, it was found that these larvae were reduced by 83 to 100% at 2 days and 94 to 100% at 4 days posttreatment. The strain 1593 and 2362 powders were equally effective, and 0.1 lb/acre provided nearly as good control as 0.2 lb/acre. The small numbers of *A. franciscanus* present were not reduced in treated ponds. Residual insecticidal activity was not seen beyond day 4 in most treated ponds (Fig. 1). After reflooding, treated ponds developed large populations of mosquito larvae, and there was no evidence of residual control (M. S. Mulla et al., submitted for publication).

Microbiological data. Pretreatment samples contained some bacteria which were capable of growing on the selective medium after pasteurization. A few colonies resembled *B. sphaericus*; however, bacteria from these colonies did not kill mosquito larvae in bioassays. These noninsecticidal colonies, which never exceeded 100 per ml, could be distinguished on the basis of colony morphology from the colonies produced by the insecticidal strains during counting of colonies from samples taken after treatment.

Figure 1 shows that *B. sphaericus* spores rapidly settled from the upper water layers into the lower water and mud. In 14- and 21-day samples, spore numbers in mud also declined, probably due to percolation of the spores into lower mud strata. The rapid appearance of large numbers of spores in the mud within 2 h of treatment was due to the settling of some large clumps of spores. However, the bulk of the powders was composed of fine particles which remained in suspension for much longer periods. It is apparent that a large inoculum of viable spores was present in the bottom muds of treated ponds for at least 21 days after treatment.

In samples taken after reflooding, insecticidal *B. sphaericus* spores were recovered only from three treated and one control pond. The number of spores never exceeded 10 per ml. Bacteria may have been moved from a treated pond to the control pond by equipment, personnel, or wading birds.

Data from homogenized larvae showed that the course of the pathology in field-treated larvae closely followed that found in the laboratory (Fig. 2 and 3). The drop in numbers of heat-resistant forms which occurred at 4 to 6 h in the laboratory (Fig. 2) agrees with electron microscopic observations of germination of strain 1593 spores in the larval gut at 4 to 6 h after ingestion (2). Field-treated larvae sampled on day 0 were homogenized at ca. 4 h after treatment; the large difference between heat-resistant and total unpasteurized counts (Fig. 3) indicates that spore germination in the larvae had already taken place. In the laboratory, sporulation of bacteria in cadavers was observed to begin between 36 and 48 h, culminating at 96 h with an increase in spore numbers of ca. 10-fold over the initial number of *B. sphaericus* ingested (Fig. 2). In the field, the increase in *B. sphaericus* numbers in cadavers was greater than that found in the laboratory (up to 100-fold), and sporulation began by 24 h in most cases (Fig. 3).

DISCUSSION

This study confirmed the efficacy of *B. sphaericus* 1593 and 2362 dry powder preparations against *C. tarsalis* under field conditions. At the temperatures experienced during this field trial, *C. tarsalis* larvae should require ca. 2 to 3 days to pass from the first/second-instar cohort to the third/fourth-instar cohort. The bacteria in the upper water layer (the larval feeding zone) during the 2 to 3 days preceeding the third/fourth instar, therefore, would have the greatest influence on the survival of larvae to this cohort. In laboratory assays, ca. 100 to 1,000 *B. sphaericus* strain 1593 or 2362 spores per ml are required to kill second-instar larvae of a similarly susceptible species, *C. quinquefasciatus* (11; Davidson et al., unpublished data). Larval populations were reduced 83 to 100% whenever *B. sphaericus* numbers exceeded ca. 100 spores per ml in the surface water 2 days earlier (Fig. 1). Whenever bacterial numbers dropped below ca. 100 spores per ml in the upper water layer (i.e., day 3 to 4), third/fourth-instar *C. tarsalis* increased within 3 days (i.e., day 7). Hornby et al. (8) similarly reported control of *C. quinquefasciatus* and *Culex nigripalpus* Theobald larvae in sewage tanks and freshwater sites whenever *B. sphaericus* 1593 spores exceeded ca. 100 per ml, but failure of control whenever spore concentration fell below this number.

Very few viable *B. sphaericus* spores were found in ponds after reflooding. Air temperatures in this region reached 49°C during the period when the ponds were dry. The high soil temperatures, and in particular UV exposure (1), probably led to the inactivation of spores in bottom muds. In

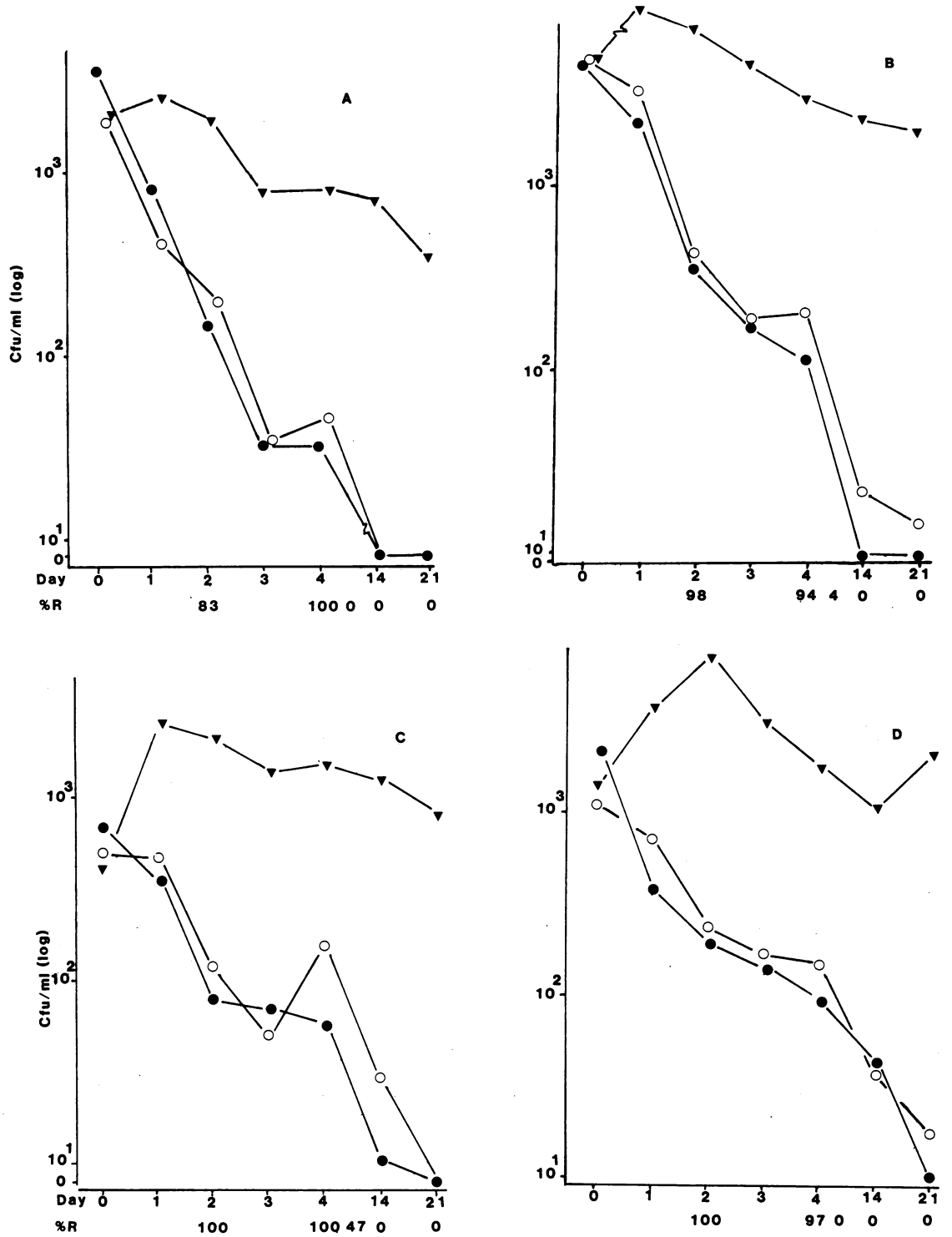

FIG. 1. Heat-resistant *B. sphaericus* spores present in upper water layers (●), lower water layers (○), and mud (▲) of experimental ponds after treatment with dry powder preparations. (A) Strain 1593, 0.1 lb/acre. (B) Strain 1593, 0.2 lb/acre. (C) Strain 2362, 0.1 lb/acre. (D) Strain 2362, 0.2 lb/acre. Abbreviations: Cfu/ml (log), CFU of *B. sphaericus* per ml (log scale); %R, percent reduction of third/fourth-instar *C. tarsalis* mosquito larval populations.

contrast, viable, insecticidal *B. sphaericus* 1593 spores were found in the lower strata of bottom muds of roadside ditches in Florida subjected to alternate drying and flooding for up to 9 months after treatment (7).

Recycling and sporulation of *B. sphaericus* 1593 and 2362 in the larval cadaver, with overall gain in bacterial numbers, occurred both in the laboratory and in the field. At 3 days after treatment, 10^5 to 10^6 *B. sphaericus* per larva were

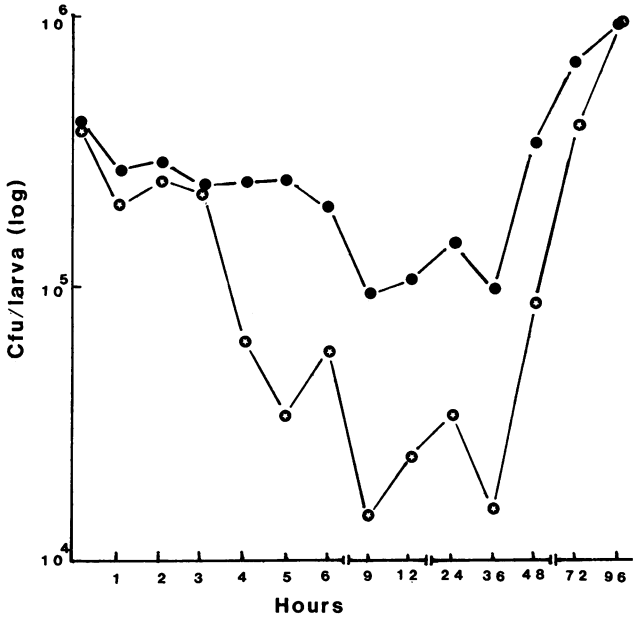

FIG. 2. Total (●) and heat-resistant (○) *B. sphaericus* present in second-instar *C. quinquefasciatus* larvae fed ca. 10^5 spores per ml for 15 min. Laboratory study.

consistently found, regardless of the initial numbers consumed. This figure was also found by Davidson et al. (4) for *B. sphaericus* SSII-1 and by Ramoska and Hopkins (13) for strain 1593 cells, and seems to be the maximum number of *B. sphaericus* cells which can be contained by one *Culex* larva. This number of cells will probably be produced by each larva fatally infected by this bacterium, regardless of the initial dose ingested, and may provide significant recycling of this bacterium in the field when large numbers of susceptible larvae are present at the time of treatment. In the study described here, it was impossible to determine whether some of the spores which were found in bottom muds in 14- and 21-day samples had been released from larval cadavers. There was no evidence of recycling of the bacterium in the water; however, this was not expected in water of low organic content (A. A. Yousten, personal communication). In laboratory studies (data not shown), cadavers 3 days postmortem were found to be insecticidal when placed in the same container with healthy *C. quinquefasciatus* larvae. Recycling of *B. sphaericus* in the cadaver leading to mortality of residual larvae may occur in laboratory bioassays as well; for this reason, mortality in *B. sphaericus* bioassays should be assessed no later than 2 to 3 days after initiation of the assay.

The dry powder preparations tested in these studies were greatly improved in suspension qualities over the *B. sphaericus* 1593 powder used in several earlier studies (Stauffer MV-716), which suspends poorly and settles rapidly (10, 17). The IF 94–95 (strain 1593) powder used in this study is at least as insecticidal to *C. quinquefasciatus* larvae in laboratory assay (50% lethal concentration = 40 μg/liter; Mulla, submitted for publication) as is the Stauffer powder (50% lethal concentration = 80 to 90 μg/liter) (14). The IF 97 (strain 2362) powder is significantly more insecticidal to *C. quinquefasciatus* than is the Stauffer powder (50% lethal concentration = 5 μg/liter; Mulla, submitted for publication). Future formulation research should be directed to

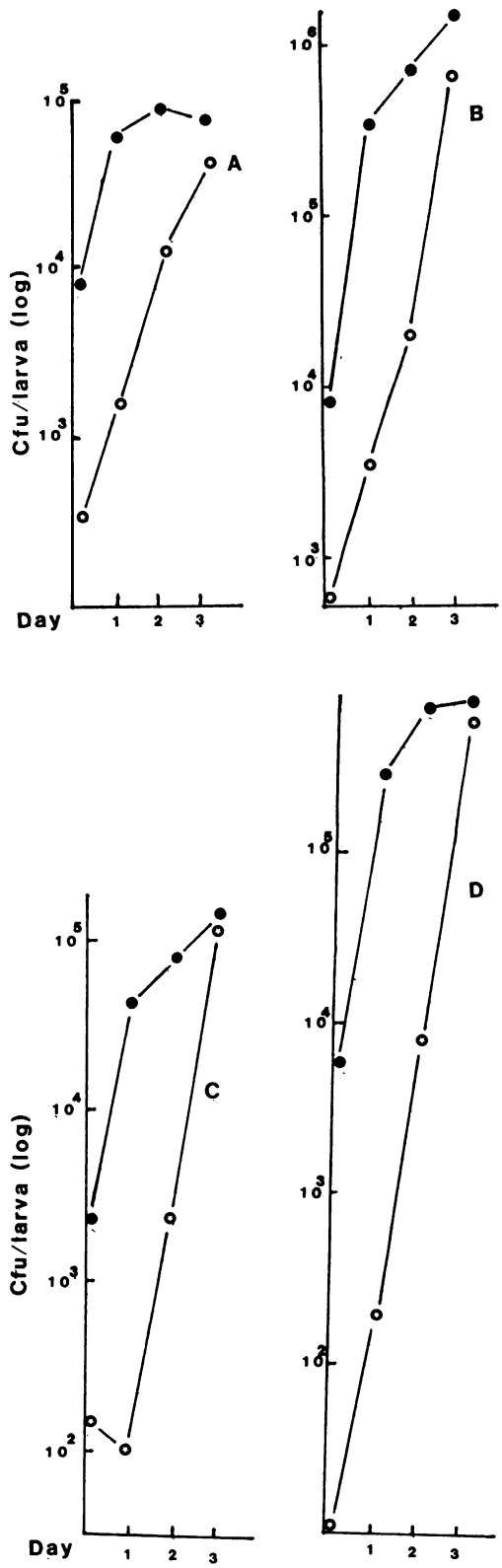

FIG. 3. Total (●) and heat-resistant (○) *B. sphaericus* present in *C. tarsalis* larvae treated in the field. (A) Strain 1593, 0.1 lb/acre. (B) Strain 1593, 0.2 lb/acre. (C) Strain 2362, 0.1 lb/acre. (D) Strain 2362, 0.2 lb/acre.

prevention of clumping and maintenance of spores in the upper water layers, thereby increasing the activity and persistence of these materials.

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