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INTERNATIONAL INDOOR AND OUTDOOR EVALUATION OF *BACILLUS SPHAERICUS* PRODUCTS: COMPLEXITY OF STANDARDIZING OUTDOOR PROTOCOLS

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ABSTRACT. Only one *Bacillus sphaericus* strain, strain 2362, is currently used commercially to control *Culex* larval populations. A reliable methodology, easily used, was developed to identify new strains for field application. Larvicidal activities of 3 highly mosquitocidal strains, strains C3-41, Mal, and LB24, previously selected in the laboratory, were compared with that of strain 2362 in tropical and European countries. The following steps were performed: production and titration of acetonic powders from these 4 strains on local *Culex* species, survey of initial and residual activity under standardized indoor and outdoor conditions, and evaluation of the efficacy of liquid formulations of the 4 strains in natural breeding sites of *Culex.* In indoor conditions, strain C3-41 showed the highest activity on both *Culex pipiens* and *Culex quinquefasciatus*; strain Mal was the least active. The residual activity causing 80% mortality differed from 20 to 90 days according to the strains and the country. Outdoor experiments with powders (0.02–1.6 mg/liter) were performed and the initial toxicities were similar in all cases. Residual activities were very different, from 6 to 95 days posttreatment. Liquid formulations were applied to larval habitats (from 0.1 to 10 g/m^2). In tropical countries, larval recolonization in cesspits or ponds occurred after 10–35 days. In Europe, higher doses were needed in polluted water than in clear water (from 3 to 10 liter/ha) for the same control, and the time before 80% residual activity was reached was less than 9–12 days. However, in cesspits, residual activity could be observed for 12 days to 5 mo. A strain 3–5 times more active than the others in bioassays is not significantly detectable from those strains in field trials.

INTRODUCTION

Bacillus thuringiensis var. *israelensis* and *Bacillus sphaericus* are used for microbiological control of mosquito larvae. Since the discovery of the first highly larvicidal *B. sphaericus* strains (Singer 1977, 1980; Wickremesinghe and Mendis 1980; Weiser 1984), only one strain (2362) has been extensively used in the field. This strain was selected for development, after field experiments using various preparations of 1593, 2297, 2013-4, 2013-6, and 2362 (Lacey and Singer 1982, Obeta 1986, Lacey et al. 1988), not because it has a particularly high intrinsic toxicity but rather because the formulated product persists in the environment (Hougard and Nicolas 1986). Since then, much work has been done to find new bacterial isolates in many regions so as to obtain new toxins, higher pathogenicity, or new potentialities (host-range and persistence). Under laboratory conditions, most *B. sphaericus* isolates belonging to serotypes H3, H5, H6, H25, and H48 are as toxic against *Culex pipiens* Linnaeus as is the reference strain 2362 (Thiery and de Barjac 1989, Thiery et al. 1992). Some have higher toxicity than that of strain 2362 not only to *Culex* but also to *Anopheles stephensi* Liston larvae. Moreover, some of these strains present a toxicity towards *Aedes aegypti* Linnaeus larvae 100 times greater than that of 2362. The median lethal concentrations (LC_{50s}) of these strains to *Cx. pipiens* larvae are very low (*ca.* 30–100 spores/ml) as assessed by bioassays as described by de Barjac and Larget-Thiery (1984). From a preliminary selection of 12 strains (Thiery and de Barjac 1989; Thiery, unpublished), 3 strains were selected for this study to determine whether their toxicity was also higher than that of strain 2362 in natural conditions and to assess their value for mosquito control programs (Becker and Beck, 1992).

The goals of this study were to develop a common and reliable methodology for evaluating the efficacy of strains of *B. sphaericus* (prepared in the same standardized conditions), to determine the value of screening thousands of strains for years when the strains apparently promising in laboratory tests never pass field evaluations, and to strengthen links between laboratory and field experimentation, which is absolutely necessary to optimize evaluation.

MATERIALS AND METHODS

Bacterial strains: The laboratory-selected *B. sphaericus* (Neide) strains LB24 (isolated from the Czech Republic), Mal (isolated from Malaysia), and C3-41 (isolated from Wuhan, China) were compared with strain 2362 (isolated from Nigeria). All were from the IEBC Collection held by the Unit

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of Bactéries Entomopathogènes at Institut Pasteur, Paris, France, and belonged to the H5a5b serotype.

Production of acetonic powders: Bacillus sphaericus strains LB24, Mal, C3-41, and 2362 were grown in 16 liters of medium for *Bacillus sphaericus* (MBS) (Kalfon et al. 1983) in a draft-tube fermentor (Biolafitte) for 48 h at 30°C, then harvested with a Sharples (Stokes, Rueil-Malmaison, France) centrifuge. The pellets were treated by lactose–acetone precipitation as described in Dulmage et al. (1970). The acetonic powders were transported in 10-g aliquots in plastic vials and kept at 4°C until use. All experiments, except in field larval habitats, were performed using acetonic powders.

Production of liquid formulations: Two liters of liquid formulation per strain were produced by Ecogen Corporation (Langhorne, USA) in the same conditions as used for the production of Spherimos® from Novo Nordisk company (Copenhagen, Denmark). Each group received 400 ml of formulation, which were kept at 4°C until use. Liquid formulations were applied in field habitats.

Mosquito strains: Culex pipiens (Linnaeus) and *Cx. pipiens* (molestus form) were reared at 25°C and 80% relative humidity in France (Bordeaux and Montpellier) and in Germany, respectively. Larvae of *Culex quinquefasciatus* (Say) were sampled from urban breeding sites in Yaoundé, Cameroon, and in the Urabà area, Colombia.

Laboratory bioassays: Larvicidal activity of each of the 4 acetonic powders and the liquid formulations was checked against *Cx. pipiens* and titrated against the reference lyophilized powder SPH88 before distribution to the field groups. Bioassays were performed on 2 groups of 25 larvae in plastic cups filled with 150 ml of bacterial suspension as described by de Barjac and Larget-Thiery (1984). Each product was assayed at 6 dilutions. Two cups containing 150 ml of deionized water were used as the controls. Bioassays were run in triplicate. Larval mortality was recorded at 48 h. Lethal concentrations (median [LC_{50}] and 90% [LC_{90}]) were estimated with a log-probit program (created by E. Frachon, Institut Pasteur, Paris, France) on a Macintosh computer. Lethal concentration LC_{50} values are expressed as means ± standard errors (SE). Each product was titrated against SPH88 powder, the titer of which is 1700 international toxic units (ITU)/mg (Thiery, unpublished) as previously assessed by titration against the first *B. sphaericus* standard powder, RB80 (Bourgouin et al. 1984).

Indoor experiments: The indoor experiments, as standardized as possible, were performed in 50-liter containers or 100-liter aquariums containing a 1 cm depth of soil substrate from a natural breeding site and 50 liters or 100 liters of 10% water from a natural breeding site, diluted 1:9. In Germany, no substrate was added. According to the volume, 50–100 3rd-instar *Culex* sp. larvae were added to each container every 3 days. The concentration of each powder used corresponded to 2, 4, and 8 times the LC_{90} of the most toxic powder determined in laboratory bioassays (Becker and Rettich 1994). Mortality was recorded every 3 days, living larvae were removed, and the cadavers were left in the containers. New batches of larvae were added to establish the residual activity of each concentration. The experiments, run at 25 ± 2°C, were stopped when less than 50% mortality was recorded. These experiments allow determination of the operational doses appropriate for the subsequent outdoor experiments.

Outdoor experiments: Outdoor experiments were conducted in containers (Germany; Bordeaux and Montpellier, France) filled with 50 liters of rain water or in cement cylinders (1-m × 0.5-m diam) filled with 30 liters of rain water and 10 liters of filtered cesspool water (Cameroon). Except for experiments in Germany, substrate was used as in the indoor experiments, and the water was approximately 50 cm deep. For each powder concentration, 2 containers were placed in sunny conditions and 2 were placed in the shade or were covered. Two control containers were exposed to the same conditions. The covers allowed mosquito colonization. Each powder was added to containers when mosquito colonization was observed or as follows. In Cameroon, apart from natural oviposition, 200 larvae were also added to each container every 3 days. In Montpellier, treatment started when 300–500 3rd- to 4th-instar larvae were recorded per container. Every 3 days, samples of water were collected by dipping and the percent reduction of the natural larval population was calculated according to the formula of Mulla et al. (1971). Dead larvae were left in the water. If the larval population was greater than 50% of the control population, a new treatment was applied at the same dose. After 3 treatments, experiments were stopped.

In the experiments in Germany and Bordeaux, mosquito colonization was limited, so 50 *Cx. pipiens* larvae were added to each container on the first day, every 3 days, and when 100% mortality was reached. Nets were used to avoid any further mosquito colonization. The persistence of each powder at each different concentration was determined as described above. Initial efficacy and residual activity of the 4 acetonic powders were recorded every 3 days.

Treatment of natural breeding sites: In this study, the naturally occurring larval habitats in urban or rural areas were called natural breeding sites and were different from the artificial containers that we created for the outdoor experiments. Small breeding sites were selected where possible, with areas less than 10 m^2. In Yaoundé, cesspits and septic tanks (area less than 1 m^2) were treated first with 0.1 and 0.5 g/m^2 then with 1 and 10 g/m^2. Four cesspits, 2 open and 2 covered, were treated per concentration and per liquid formulation. Samples

Table 1. Titration of *Bacillus sphaericus* powders against SPH88 standard.[1]

Location	Larvae[2]	Strain LB24			Strain MAL		
		LC_{50}	LC_{90}	Titer**	LC_{50}	LC_{90}	Titer
Paris, France	*Culex pipiens*	0.044 ± 0.031	0.079	547	0.06 ± 0.02	0.11	342
Bordeaux, France	*Cx. pipiens*	0.017 ± 0.002	0.063	655	0.031 ± 0.004	0.15	358
Montpellier, France	*Cx. pipiens*	0.016 ± 0.003	0.062	634	0.022 ± 0.003	0.088	471
Germany	*Cx. pipiens* (molestus form)	0.125 ± 0.05	0.289	131	0.071 ± 0.006	0.118	230
Colombia	*Culex quinquefasciatus*	0.011 ± 0.004	0.089	1,701	0.012 ± 0.003	0.062	1,589
Cameroon	*Cx. quinquefasciatus*	0.0086 ± 0.0008	0.063	277	0.00089 ± 0.0003	0.020	2,674

[1] LC_{50} = median lethal concentration, LC_{50}s (mg/liter) are means of at least 3 experiments; LC_{90} = 90% lethal concentration.
[2] Old 3rd- or young 4th-instar larvae.
[3] Titers are expressed in international toxic units/mg (ITU/mg) or powder using SPH88 (1,700 ITU/mg) as the reference.

were collected by dipping 2 days after treatment then every 5 days.

In Colombia, near Urabà, 20 ponds (*ca.* 2 m^2, 50 cm depth) were treated with 3 liters/ha (*ca.* 0.3 g/m^2) of the 4 formulations. Ten ponds were shaded and 10 were exposed constantly to the sun. Once a week, larvae were scored by taking 8 dip samples per pond. Mosquito populations were recorded for 2 wk before and for 7 wk after treatment. Other insects observed, particularly Odonata and Hemiptera, were also recorded. In southern France, cement containers like those used for the outdoor experiment were filled with manure compost and 3.6 m^3 water to a depth of 50 cm (surface: 7.3 m^2) and exposed to the sun. Two concentrations, 0.1 g/m^2 (1 liter/ha) and 1 g/m^2 (10 liters/ha) of each formulation were applied. Living 3rd- and 4th-instar larvae were counted by dipping twice a week. Four water treatment plants free of any vegetation and in sunny conditions were also selected. Each breeding site was treated with 1–3 and 10 liter/ha after each mosquito recolonization (3 treatments). Sanitary voids were also treated at 3 liters/ha once per formulation. Samples were collected by dipping before and 72 h after treatment and every week.

Marshlands in the Isle of Ré, on the Atlantic coast of France, were divided into lots and each 30-m^2 area (depth 20–80 cm) was treated, under sunny or shade exposures. Water treatment plants (20 m^2 treated) and cesspits (0.2 m^2 and 60–80 cm deep) were treated in an urban area of Bordeaux. All breeding sites were treated with 1 g/m^2 and after recolonization (total of 2–3 applications). Samples were collected by dipping every 3 days posttreatment.

RESULTS

Titration: Each of the 4 strains (LB24, Mal, C3-41, and 2362) were successfully produced in a fermentor. Each final whole culture (FWC) was tested on *Cx. pipiens* 4th-instar larvae before being harvested. The LC_{90}, *ca.* 1×10^{-6} dilution of the FWC, was identical to that for FWCs grown in flasks (data not shown). Each pellet from the 16-liter culture yielded, after lactose–acetone precipitation, *ca.* 70–80 g of acetonic powder. The larvicidal activity of each of the 4 acetonic powders was evaluated on *Cx. pipiens* larvae in France, *Cx. pipiens* (molestus form) in Germany, and *Cx. quinquefasciatus* in Colombia and Cameroon (Table 1). Lethal concentrations were much lower for *Cx. quinquefasciatus* strains than for *Cx. pipiens* strains. European *Cx. pipiens* was at least twice as susceptible to C3-41 powder as to the other powders, whereas the Colombian *Cx. quinquefasciatus* strain was more susceptible to 2362, and 4 times less susceptible to strains LB24 and Mal. Strain Mal was the most toxic towards the Cameroonian *Cx. quinquefasciatus* strain, nearly 10 times more toxic than the strain LB24.

Indoor experiments: The bioassays were used to determine the concentrations used in containers for *Cx. quinquefasciatus* and *Cx. pipiens* (Fig. 1). In Germany and Cameroon powders were added to containers at final concentrations of 2–4 and 8 times the LC_{90} of the most toxic strain, C3-41 (Germany, LC_{90} = 0.035 mg/liter; Cameroon, LC_{90} = 0.01 mg/liter). In Montpellier, 0.25–0.5 and 1 times the LC_{90} of the most toxic strain, C3-41 (LC_{90} = 0.01 mg/liter), were used. In Colombia, 2–4 and 8 times the LC_{90} of each strain were used, that is, Mal (0.06 mg/liter), LB24 (0.09 mg/liter), C3-41 (0.004 mg/liter), and 2362 (0.0026 mg/liter), (Fig. 1A, 1B, 1C, 1D). In all cases, the initial toxicity of all powders was 100%. The days on which 80% and 50% larval mortality were observed were recorded. In Montpellier and in Cameroon, 50% mortality was observed after 27 and 45 days, respectively, and residual activity increased with the applied concentrations (Fig. 1B, 1D). In Germany and in Colombia, residual activity of each of the 4 powders was detected after at least 90 days (Fig. 1A, 1C). The efficacy of the powders on *Cx. pipiens* (molestus

Table 1. Extended.

Strain C3-41			Strain 2362		
LC_{50}	LC_{90}	Titer	LC_{50}	LC_{90}	Titer
0.016 ± 0.008	0.029	1,328	0.049 ± 0.025	0.097	393
0.007 ± 0.003	0.040	2,222	0.023 ± 0.012	0.085	574
0.011 ± 0.004	0.037	914	0.013 ± 0.001	0.068	792
0.016 ± 0.005	0.035	1,026	0.057 ± 0.004	0.094	286
0.035 ± 0.0002	0.0040	5,488	0.0023 ± 0.0001	0.0026	8,147
0.0015 ± 0.0003	0.0098	1,587	0.0037 ± 0.0008	0.0198	643

form) in barrels with no addition of substrate was tested in Germany. The larvicidal activity was followed for 120 days for strains 2362 and Mal and for 200 days for strains C3-41 and LB24. Strain C3-41 retained activity longer than did the other strains. Residual activities differed between countries but not clearly between bacterial strains.

Outdoor experiments: Initial efficacy, residual activity, and the mean percentage of larval mortality were determined in Germany and Bordeaux (Fig. 2A, 2B). Initial larval mortality was over 92% in Germany; mortality decreased progressively to reach 50% for all powders on day 43. The rate of loss of activity was slightly higher in the sun than

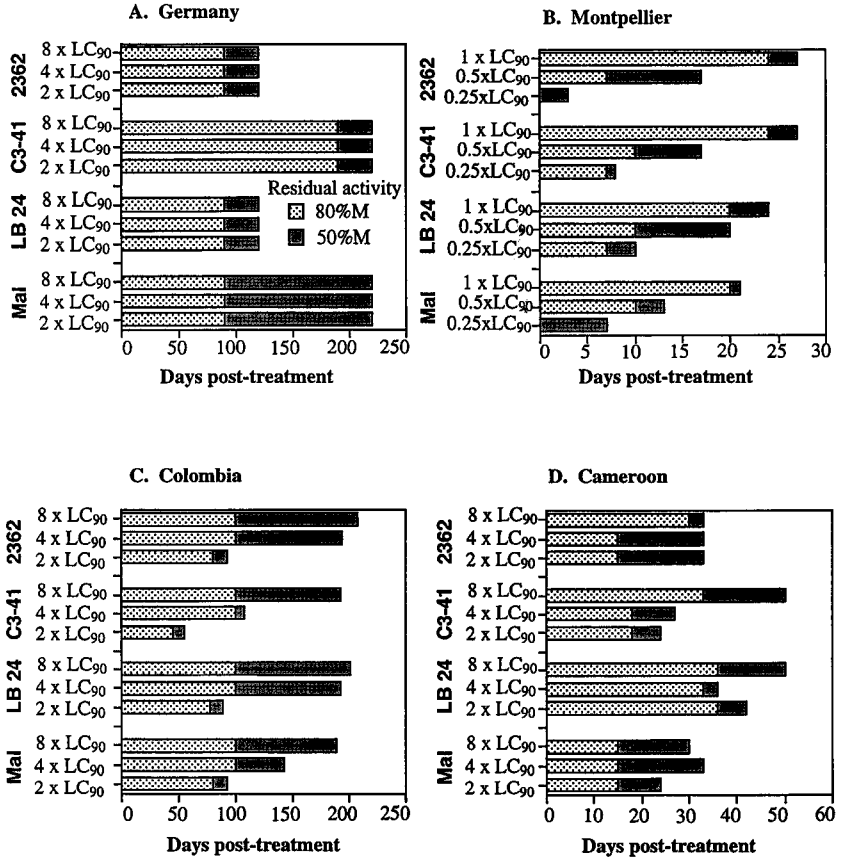

Fig. 1. Indoor experiments to determine the residual activity of four *Bacillus sphaericus* strains applied at 3 concentrations. A: Germany; B: Montpellier, France; C: Colombia; D: Cameroon. The spotted bars represent the days where at least 80% mortality was recorded and the gray bars correspond to the last days where greater than 50% mortality was observed.

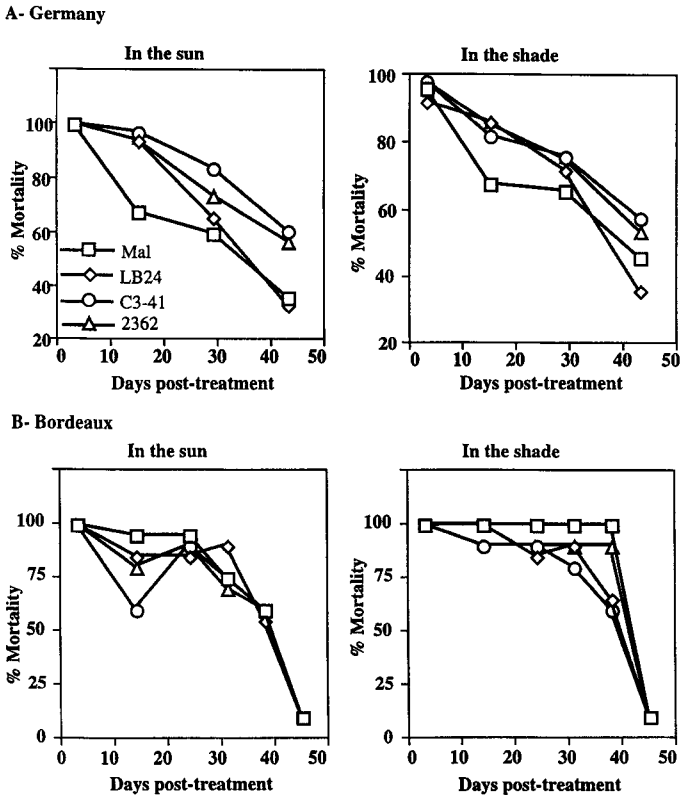

Fig. 2. Residual activity of acetonic powders of 4 *Bacillus sphaericus* strains in containers either exposed to the sun or covered. A. A concentration of 2 times the 90% lethal concentration (LC_{90}) of each powder was applied in Germany (see Table 1). B. A concentration of 0.4 mg/liter of each powder was applied per container in Bordeaux, France (corresponding to 2 times the LC_{90} of the less toxic strain).

in the shade although the difference was not significant (Fig. 2A). No significant difference was found between treatment at 2 times the LC_{90} and 1 times the LC_{90} (data not shown). Because of its lowest applied concentration (see LC_{90} in Table 1), C3-41 was considered the most permanent active strain, followed by strains 2362 and LB24; the less lasting active strain was Mal powder.

Three different concentrations (0.4, 0.8, 1.6 mg/liter) were applied in the Bordeaux experiment, corresponding to 2, 4, and 8 times the LC_{90} of the least toxic strain (Table 1). The residual activity of the lowest concentration (0.4 mg/liter) of each powder was determined in Fig. 2B. All powders caused 100% initial mortality and residual activity after 30 days was high (Fig. 2B). The use of higher concentrations did not increase the residual activity (data not shown). None of the strains was more persistent than the others. The powders retained activity slightly longer in the covered than open containers. During this experiment, populations of *Chironomus plumosus* Linnaeus, *Eristalis* sp., *Culiseta litorea* (Shute), and *Culex hortensis* (Ficalbi) larvae were found in some containers: only *Cx. hortensis* was susceptible to *B. sphaericus* powders.

Two treatments at 0.02 mg/liter of each powder

Table 2. Residual activity of the 4 acetonic powders in containers exposed to the sun or covered in Cameroon.

Treatment (0.02 mg/liter)	Days posttreatment when residual activity of each powder was still observed							
	Mal		LB24		C3-41		2362	
	Sun	Covered	Sun	Covered	Sun	Covered	Sun	Covered
1st treatment	9–9[1]	2–2	0–12	6–18	2–2	2–6	6–2	2–18
	15–21[2]	18–27	18–27	27–27	6–24	18–18	6–9	14–21
2nd treatment	0–2[1]	0–2	0–11	0–2	0–8	0–0	2–5	0–0

[1] Days with 100% mortality in the 2 containers (1–2).
[2] Presence of larvae in 3 consecutive samples.

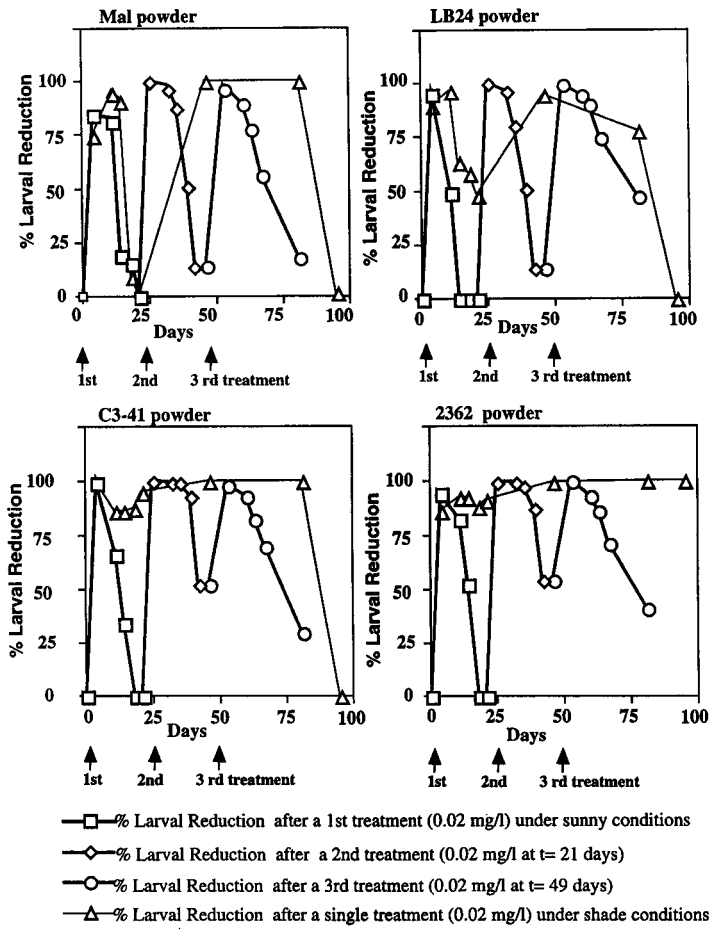

Fig. 3. Residual activity in Montpellier, France, of 0.02 mg/liter of each *Bacillus sphaericus* powder in containers either exposed to the sun or shaded. Three treatments were applied to the containers exposed to the sun and only one treatment to containers in the shade.

(2 times the LC_{90} of the most toxic strain) were applied in Cameroon (Table 2). Larval reduction as a percentage of the controls could not usefully be calculated because of the presence of *Culex decens* (Theobald) (nonsusceptible to *B. sphaericus*) and *Culex tigripes* (De Grandpre and De Charmoy) (predator of other mosquito larvae). The period of maximum efficacy varied from 12 days (sun) to 18 days (shade) with no strain performing significantly better than the others. The loss of activity of strains LB24 and Mal was slower than the other strains. Sunlight caused a more rapid loss of activity, particularly of C3-41 and 2362 powders. The second treatment was poorly toxic, possibly because of the large population of *Cx. decens,* which is not susceptible to the toxin, but which ingests the bacteria, thus decreasing its concentration in the larval feeding zone.

In Montpellier, 3 treatments at 0.02 mg/liter of each powder under the sun were applied on days 0, 21, and 49 to containers containing natural populations of *Cx. pipiens.* Larval reduction as a percentage of the controls was expressed after each treatment (Fig. 3). Initial efficacy of all powders under the sun was 94% or higher, except for the first treatment with Mal strain. Fifty percent residual activity was reached in 11 days or less with the first treatment, and 18 days with the 2nd and 3rd treatments, thus showing a slight additive effect. Only one treatment, at a concentration of 0.02 mg/liter was applied in covered containers. Mal and LB24 powders caused fluctuating reductions of the populations, suggesting recycling in the cadavers. C3-41 and 2362 powders maintained a good larval control for 81 and 95 days, respectively.

Evaluation of formulated products in natural breeding sites: Liquid formulations of the 4 *B. sphaericus* strains were produced. The larvicidal activity of each of the 4 liquid concentrates was first titrated on *Cx. pipiens* at the Institute Pasteur.

Table 3. Residual activity of *Bacillus sphaericus* powders in indoor and outdoor experiments (number 1, the best control of mosquito larvae; number 4, the poorest control).

Experiment[1]	Treated larvae	Titration[2] (best strain)	Residual activity							
			Powders applied indoors				Powders applied outdoors			
			Mal	LB24	C3-41	2362	Mal	LB24	C3-41	2362
1	*Culex pipiens* (molestus form)	C3-41	4	2	1	3	3	3	1	2
2	*Cx. pipiens*	C3-41	4	3	1	2	3	3	2	1
3	*Culex quinquefasciatus*	2362	3	3	2	1	—	—	—	—
4	*Cx. quinquefasciatus*	Mal	4	1	2	3	2	1	3	4
5	*Cx. pipiens*	C3-41	—	—	—	—	1	1	1	1
Total of efficacy			15	9	6	9	9	8	7	8

[1] Experiments: 1, Germany; 2, Montpellier, France; 3, Colombia; 4, Cameroon; 5, Bordeaux, France.
[2] The most toxic strain in titration experiments (see table 1).

The means of the titers of the liquid formulations of 2362, Mal, LB24, and C3-41 were 90 ± 17, 74 ± 13, 71 ± 8, and 73 ± 13 ITU/mg, respectively. The titers of Mal, LB24, and 2362 formulations on the *Cx. quinquefasciatus* strain from Cameroon were between 200 and 300 ITU/mg whereas that of C3-41 was 800 ITU/mg.

The larval populations in the cesspits and septic tanks in Cameroon were between 5 and 495 larvae per dip sample before treatment. Larvae were found 7 days after treatment with the lowest doses and after 12 days at higher dosages in most cases. At low concentrations, the longest period before 50% residual activity was reached was 20 days whereas 10 g/m^2 of C3-41 controlled 50% of the populations for 62 days in two cesspits. Doses 100-fold higher only increased the duration of residual activity by 2- or 3-fold. Sunlight did not increase the rate of loss of residual activity. Indeed, activity was lost by all formulations more rapidly in the shade than in the sun at 10 g/m^2.

In Colombia, complete control was obtained by application of 3 liters/ha of all formulations. The larval populations returned to the initial levels between 3.5 and 5 wk after treatment exposed to the sun, except for that with Mal, which provided 90–100% control after more than 7 wk. In the shaded ponds, mosquito populations returned to the pretreatment density 4–4.5 wk after treatment with Mal and LB24, and more than 6–7 wk after that with 2362 and C3-41. 2362 and C3-41 formulations retained activity longer in shaded than in sunny ponds, whereas LB24 and Mal were more effective in sunny than in shaded ponds.

In Montpellier, formulated products were applied at 0.1 g/m^2 (1 liter/ha) and 1 g/m^2 (10 liters/ha) in containers in which the larval populations were between 657 and 2,035 larvae per container. The initial control was complete and 6–9 days posttreatment residual activity was above 78%. After 12 and 19 days, mosquito colonization was observed. No formulation was substantially more active than the others. Residual activity was not higher at 10 liters/ha than at 1 liter/ha. In the 4 sunny water treatment plants, free of any vegetation, the 1- and 3-liter/ha doses were insufficient for complete larval control whatever the formulation used. A dose of 10 liters/ha led to more than 99% mortality and residual activity fell below 50% in less than 8 days. No significant differences were found between the 4 formulations. In the urban cesspits of buildings treated with 3 liters/ha of formulations LB24, C3-41, and 2362, mortality was 92, 95, and 100%, respectively, 3 days posttreatment. Formulation 2362 assured complete control for 5 months. The persistence of larvicidal activity of the 2 other formulations could not be determined as other pesticide treatments were applied, which undoubtedly affected larval populations. The Mal formulation was applied to cesspits that had been treated with Spherimos for more than 7 years. No mortality of the larval population was observed. In fact, the *Culex* larval population in this cesspit was found to be resistant to *B. sphaericus*. This was the first case of resistance in France to *B. sphaericus* (Sinègre et al. 1994).

All breeding sites near Bordeaux were treated 2–3 times with 1 g/m^2 of one of the formulations. Larval populations before treatment were between 40 and >1,000 larvae per site. This dose did not completely eliminate larvae from the sunny breeding sites, whatever the formulation used. In closed urban and polluted sites, initial efficacy and residual activity were 100% until 10, 12, or 15 days posttreatment depending on the larval density in the breeding sites. Residual activity of all the formulations in natural breeding sites were compiled and expressed according to a classification from the most (number 1) to the least lasting active formulation (number 4) in each country as reported in Tables 3 and 4.

DISCUSSION

The goal of this study was to define a simple methodology to select a strain obtained by laboratory screening with good larvicidal potential and persistence in breeding sites. This methodology was

Table 4. Residual activity of *Bacillus sphaericus* formulated products in natural breeding sites.

Experiment[1]	Conditions and dose of formulations	Formulation[2]			
		Mal	LB24	C3-41	2362
1	Not tested				
2	Sun, 0.1 g/m²	4	1	1	3
	1 g/m²	4	1	1	1
	Clear water, 10 liter/ha	1	1	1	1
	Cesspits, 3 liters/ha	?	1	1	1
3	Ponds, sun, 3 liters/ha	1	2	3	4
	Ponds, shade, 3 liters/ha	3	4	1	2
4	0.1 g/m²	1	4	2	3
	0.5 g/m²	1	4	2	4
	1 g/m²	3	2	1	3
	10 g/m²	1	2	4	3
5	Sun, 1 g/m²	1	1	1	1
	Shade 1 g/m²	1	4	2	2
Total of efficacy		21	27	20	28

[1] Experiments: 1, Germany; 2, Montpellier, France; 3, Colombia; 4, Cameroon; 5, Bordeaux, France.
[2] Efficacy of formulations was classified although their differences were not significant.

easy to handle, based on controlled indoor and outdoor conditions. This method could allow a rapid low-cost selection and eliminate the requirement for large-scale field testing.

Results from titration and indoor experiments agreed that powder C3-41 was best for control of European *Cx. pipiens* mosquito larvae and powder 2362 was best for control of the Colombian *Cx. quinquefasciatus* strain (Table 3). This confirmed the selection of strain C3-41 for control of *Cx. pipiens* by Thiery and de Barjac (1989). In Cameroon, the Mal strain, the most active in titration experiments, was the least residual in indoor containers (Table 3). In indoor experiments, mosquito larvae were most susceptible to strain C3-41, less to 2362 and LB24, and least to Mal. The long-term effects (200 days) in Germany were not correlated with the concentrations used. The persistence of the larvicidal activity may have been due to recycling in the cadavers left in the containers where no substrate was added (Nicolas et al. 1987). Under semi controlled outdoor conditions, differences between strain activities were not clear, and it is not possible to conclude which strain provided the longest residual activity in any particular conditions (Table 3). Persistence of larvicidal activity of the powders varied between experiments in each country and did not appear to depend on the susceptibility of the mosquitoes or on the bacterial strain. The outdoor experiment did not allow selection of one strain as the best from the group.

Rather than comparing one strain to the 2362 formulation, we therefore compared the larvicidal activities in natural breeding sites of the 4 strains formulated in the same conditions. In Europe, none of the formulations was identified as performing better than the others in clear water (Table 4). In both tropical countries, the Mal strain seemed to control *Cx. quinquefasciatus* populations satisfactorily, whereas the Mal strain has been the least effective in indoor and outdoor experiments. The *Cx. quinquefasciatus* natural breeding sites tested were ponds in Colombia, and cesspits in Africa with highly polluted water. In Colombia, sunlight appeared to increase the rate of loss of larvicidal activity, whereas no such effect was observed in Africa, possibly due to the density of the organic matter in the sites tested (Lacey and Smittle 1985). Increasing the doses in Cameroon increased the period for which residual activity remained high, whereas this effect was not observed for *Cx. pipiens* in clear water near Bordeaux.

Strains C3-41 and 2362 performed better than the 2 others in the indoor and outdoor controlled experiments, whereas the Mal strain was the most effective in the field. Overall, the data do not show any of the strains to be clearly superior. Indoor evaluations of acetonic powders identify strain C3-41 as the best, but this strain was no better than the others in outdoor evaluations and in natural breeding sites (Tables 3 and 4).

This study shows that standardized semicontrolled laboratory conditions and field conditions give conflicting results. It therefore appears impossible to select the optimal strain for field use from among strains for which laboratory bioassays differ by less than 5- to 10-fold. However, new strains with toxicities comparable to those of strains currently used could be valuable, in cases of larval resistance to commercialized strains (Nielsen-LeRoux et al. 1997). Furthermore, mosquitoes from different geographical regions had different susceptibilities to *B. sphaericus*. The higher susceptibility of *Cx. quinquefasciatus* larvae than that of *Cx. pipiens* to *B. sphaericus* was previously observed by Thiery (unpublished) and was confirmed herein.

Temperature, pH, and water pollution all affected the activity of the powders and the effects might be different between tropical and temperate climates. Consequently, it is important to evaluate strains in different countries to identify which is most appropriate for the local conditions and mosquito populations.

We were unable to standardize the evaluation conditions, as each participant chose different concentrations for outdoor experiments according to the results of the indoor trials. Nevertheless, we show that collaborations between laboratory and field workers and between temperate and tropical countries are essential for optimizing the efficacy of bacterial application. Our study also reveals that each breeding site is a unique biotope and that consequently no single protocol for field applications is optimal in all situations.

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