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Individual and combined effects of *Bacillus thuringiensis* var. *israelensis*, temephos and *Leptolegnia chapmanii* on the larval mortality of *Aedes aegypti*

Sebastian A. Pelizza · Ana C. Scorsetti · Vilma Bisaro · Claudia C. López Lastra · Juan J. García

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Abstract Larvicidal effects of interaction between *Bacillus thuringiensis* var. *israelensis* (*Bti*), temephos and *Leptolegnia chapmanii* zoospores on larvae of *Aedes aegypti* were determined under laboratory and seminatural conditions. In laboratory bioassays, two concentrations of *Bti* (0.012, 0.027 ppm), two of temephos (0.00035, 0.001 ppm), and a single concentration of *L. chapmanii* zoospores (6.1×10^4 zoospores ml^{-1}) were evaluated. Trials under field-like conditions were performed in a single container and then placed either in the shade or in direct exposure to sunlight. We evaluated concentrations of *Bti* and temephos at 3-fold those normally used in laboratory tests: 0.09 and 0.003 ppm, respectively, plus 1.8×10^5 zoospores ml^{-1} of *L. chapmanii*. The combined effect of sublethal concentrations of *Bti*, temephos, and *L. chapmanii* zoospores thus indicated that this fungus is not inhibited by the larvicides and also demonstrated the synergistic effect of the action of *L. chapmanii* when used together with *Bti* and temephos.

Keywords *Leptolegnia chapmanii* · *Aedes aegypti* · Zoospores · Temephos · *Bacillus thurigiensis* · Entomopathogenic fungi

Introduction

Culicidae are vectors for human pathogens causing serious illnesses, such as malaria, filariasis, yellow fever, and other arboviroses. Dengue and dengue hemorrhagic fever are considered the most dangerous and widespread viral diseases transmitted by mosquitoes (PAHO 1995). *Aedes aegypti* L. (Diptera: Culicidae) plays a crucial role in the transmission of these infections (Nogueira et al. 2001). The global use of insecticides for mosquito-vector control in recent decades has caused environmental pollution of aquatic ecosystems and has also resulted in insecticide resistance in many mosquito species (Scholte et al. 2004). It is therefore not surprising that the interest in alternative nonchemical strategies has increased in recent years. The use of biological control agents such as bacteria, protozoa, nematodes, and fungi has accordingly been developed with an attempt at gaining control over mosquito populations (Federici et al. 2007).

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S. A. Pelizza (✉) · C. C. López Lastra · J. J. García
CEPAVE (Centro de Estudios Parasitológicos y de Vectores) CCT La Plata-Conicet-UNLP, Calle 2 # 584, 1900 La Plata, Argentina
e-mail: pelizza@cepave.edu.ar

A. C. Scorsetti
Instituto de Botánica Carlos Spegazzini. Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Argentina, 53 # 477, 1900 La Plata, Argentina

V. Bisaro
Cátedra de Estadística, Facultad de Agronomía, Universidad Nacional de Rosario, Rosario, Argentina

Leptolegnia chapmanii Seymour (Straminipila: Peronosporomycetes) is an aquatic fungal pathogen[1] that has been isolated from several mosquito species (Mc Innis and Zattau 1982; Seymour 1984; Fukuda et al. 1997). In Argentina, this fungus has been cited as infecting *Ochlerotatus albifasciatus* Macquart (Diptera: Culicidae) by López Lastra et al. (1999). These investigators subsequently determined the susceptibility of ten species belonging to five genera of mosquitoes to this native isolate under laboratory conditions (López Lastra et al. 2004). Even though the natural distribution of *L. chapmanii* in Argentina is poorly known, the Argentinean isolate tolerates a wide range of environmental conditions indicating that this fungus could persist in several mosquito habitats (Pelizza et al. 2007a, b, 2009).

Another biological control agent, the bacterium *Bacillus thuringienis* var. *israelensis* (*Bti*) infects and kills several species of mosquito larvae. This microbe is highly selective for mosquitoes and black flies (Mulla 1990) and does not affect nontarget vertebrate or invertebrate species (Mardini et al. 2000). In general the duration of the efficacy of commercial *Bti* under field conditions is not greater than seven days, so that reapplications of *Bti* are required to obtain a prolonged efficacy (Mulla et al. 1993; Becnel et al. 1996; Batista Filho et al. 1998).

Temephos is a pesticide applied to drinking water in households to control the proliferation of insect disease vectors. In Brazil, this organophosphate has been used to control *Ae. aegypti* larvae since 1967 through its application to mosquito breeding sites. Lima et al. (2003), however, reported resistance to temephos in *Ae. aegypti* larvae in temephos-treated areas.

The goal of the work reported here was to evaluate the interactions among *Bti*, temephos, and the fungus *L. chapmanii* against larvae of *Ae. aegypti* under both laboratory and seminatural conditions in order to assess the utility of the combined utilization of these larvicidal agents against *Aedes*-mosquito disease vectors.

[1] We acknowledge that all taxa of oomycete water molds are now classified among the Kingdom Chromista (=Straminipila) and formally excluded from the true fungi. For the sake of convenience, however, we continue to refer to oomycetes in this paper in the historically sense as fungi.

Materials and methods

Mosquito larvae

The larvae of *Ae. aegypti* used in this study were obtained from a colony maintained at Centro de Estudios Parasitológicos y de Vectores (CEPAVE), La Plata, Argentina, maintained using standard mosquito-rearing techniques (Gerberg et al. 1994).

Larvicidal agents

The sample of *L. chapmanii* (CEP 010, ARSEF 5499), isolated from *Oc. albifasciatus*, was maintained on Emerson-YpSs–agar medium (Difco, Detroit, MI) in 60 × 15 mm Petri dishes at 25°C. The fungal inoculum was obtained by cutting cubes of hypha-containing agar (0.5 cm^2) and placing one of them into a 90 × 15-mm Petri dish with 70 ml distilled water. Third-instar larvae of *Ae. aegypti* were then placed into the dish. *Leptolegnia chapmanii*-infected *Ae. aegypti* larvae (48 h postinfection) were used as the *Aedes* inoculum. Each infected larva (one larval equivalent) was estimated to contain $6.1 \pm 0.2 \times 10^4$ zoospores (Pelizza et al. 2007a). The zoospores for inoculation were obtained following the techniques cited by Pelizza et al. (2008).

The other larvicides included the two commercial products *Bti*, as a liquid-concentrate formulation (*Bti* Liquid®, Laboratorios BIAGRO, Las Heras, Buenos Aires Province, Argentina) containing 1,200 international toxicity units (ITU) per mg, and the organophosphate temephos (Abate®, BASF). The concentrations of *Bti* and temephos evaluated here (Table 1), when used alone, killed fewer and more than 50% of larvae treated, respectively. The *L. chapmanii* doses evaluated killed fewer than 50% (44.6 ± 2.5%).

Laboratory bioassays

Twenty-five third-instar *Ae. aegypti* larvae were placed in 200-ml plastic containers with 150 ml of distilled water. Two doses of *Bti* (0.012 and 0.027 ppm), two doses of temephos (0.00035 and 0.001 ppm), and a single dose of *L. chapmanii* zoospores (6.1×10^4 ml^{-1}) were evaluated both individually and in combination (Table 1). Five replicates were processed under each condition, and a

Table 1 Different concentrations and combinations of *Bacillus thurigiensis* var. *israelensis*, temephos, and *Leptolegnia chapmanii* zoospores used in laboratory bioassays

Treatment	Concentrations		
	Zoospores (ml)	Temephos (ppm)	*Bti* (ppm)
A	6.1×10^4		
B		0.00035	
C		0.001	
D			0.012
E			0.027
F	6.1×10^4	0.00035	
G	6.1×10^4	0.001	
H	6.1×10^4		0.012
I	6.1×10^4		0.027
J		0.00035	0.012
K		0.001	0.027
L	6.1×10^4	0.00035	0.012
M	6.1×10^4	0.001	0.027

control group was included containing the larvae alone.

Bioassays were performed at 25 ± 1°C and a 12:12 LD photoperiod. The cumulative larval mortality was recorded at 48 h after the beginning of the assay. Dead larvae were examined for fungal infection in wet-mount preparations under phase-contrast microscopy (×400). The experiments described above were repeated three times under comparable laboratory conditions.

Trials under field-like conditions

Trials were carried out under field-like conditions. The experiments were performed in 1-l transparent plastic containers placed either in the shade or in direct exposure to sunlight. A layer of 2 cm of dry sand, simulating natural conditions, was added at the bottom of the containers along with 500 ml of dechlorinated water (simulated flooding). The concentrations of *Bti*, temephos, and *L. chapmanii* added to the appropriate containers were three times higher than used for laboratory bioassays: 0.09 ppm, 0.003 ppm, and 1.8×10^5 zoospores ml^{-1}, respectively. Five replicates and a control (containing the larvae alone) were performed for each treatment. Every 24 h, 20 third-instar *Ae. aegypti* larvae were added to each container. Dechlorinated water was also added to maintain a final volume of 500 ml when necessary. The mortality was recorded daily for 15 days.

After 15 days, the contents of the containers were emptied and left to dry for one week, keeping them in either the sunlight or the shade as occurred during the incubation. The same volume (500 ml) of dechlorinated water was then added. The containers were thereafter treated the same way, but without the addition of a second round of antilarval agents. This step and its final repetition were carried out to test the extent to which the chemical agent and the bacteria retained larvicidal activity in the dry state and whether the fungus could also persist under such conditions in a dormant form as encysted zoospores. The flooding procedure was performed twice at which of which no larval mortality was detected in any containers.

Statistical analyses

The larval mortality data were expressed as percentages, then arcsine–square-root transformed and evaluated by means of a bifactorial model of analysis of variance (ANOVA). A comparison of the means by the Duncan test ($P = 0.05$) was utilized to compare the larval mortality obtained in laboratory bioassays.

To analyze the data set for the field-like conditions, the mortality data were expressed as percentages, then arcsine–square-root transformed to achieve homoscedasticity and normality, and analyzed by an ANOVA model for repeated measurements over time (Andersen et al. 1981; Johnson and Wichern 1982; Littell et al. 1996).

We detected a lack of independence among the observations because larval-mortality values at consecutive times were better correlated with each other than with the data obtained at widely separated times. We thus fitted the data to a model that took into account the covariance structure over time along with testing the following covariance structures according to the hierarchical criteria of Akaike and Schwarz Bayesian: compound-symmetry covariance, unstructured covariance, and autoregressive–order-1 covariance. As the last possibility gave the best fit with the covariance of the data from both the flooding and the sunlight-shade results, we adopted this form of covariance for further analyses. The model followed a second-degree-polynomial function with respect to the time variable.

Results

Evaluation of the results of the laboratory bioassays

The laboratory bioassays performed obtained mortality rates at 48 h ranging from 33.3% to 100% (Fig. 1). In bioassays using different individual concentrations of *L. chapmanii*, temephos, and *Bti*, the higher dose of temephos (treatment C) gave the highest larval-mortality rate, whereas the lower dose of *Bti* (treatment D) caused the lowest mortality. When the two conventional larvicides (*Bti* and temephos) were used in combination (treatments J and K), the overall mortality was increased under all conditions to 100% (Fig. 1). Furthermore, in tests with the three control agents used together (treatments L and M), the rates of mortality reached 100% throughout. No mortality was recorded in the control group. Significant differences in the percent mortality of the *Ae. aegypti* larvae were found between the assays performed with the larvicides used individually and those with the agents present in combinations (ANOVA, $F = 13.02$, df $= 12, 64$, $P < 0.0001$).

Evaluation of the results under field-like conditions

Significant differences in larval-mortality rates were found on different treatment days, during the three floods, and between containers placed in the sunlight and the shade (Tables 2, 3).

During the first flooding cycle, the *Ae. aegypti* third-instar larvae incubated in containers with only a suspension of 1.8×10^5 zoospores ml^{-1} of *L. chapmanii* and exposed to sunlight had a mortality rate of $95 \pm 0.8\%$, but attained a 100% mortality rate in containers placed in the shade by the 4th day of treatment (Fig. 2). By contrast, the larvae subjected to temephos alone registered a mortality level of 100% in containers either exposed to sunlight or placed in the shade up to either the 3rd or the 4th day of treatment (Fig. 2). Moreover, larvae subjected to *Bti* alone exhibited a mortality rate of $71.6 \pm 3.2\%$ in the sunlight and $75 \pm 2.4\%$ in the shade on the 1st day of treatment (Fig. 2).

The larvae exposed to *L. chapmanii* zoospores plus temephos showed a mortality rate of 100% both on the 9th day of treatment, in the presence of sunlight and on the 10th day in the shade (Fig. 2). In the presence of both *L. chapmanii* zoospores and *Bti*, a higher mortality was observed on the 1st day of treatment, $83.3 \pm 0.4\%$ in the sunlight and $88.3 \pm 08\%$ in the shade.

When concentrations of temephos and *Bti* were combined, 100% mortality rates were observed until the 9th and 10th day of treatment in the sunlight and the shade, respectively. The larvae exposed to the three agents in combination exhibited 100% mortality rates until 12 or 13 days of treatment in containers placed in the sunlight or in the shade, respectively (Fig. 2).

During the second flooding cycle, the containers incubated in the sunlight in the presence of only *Bti*

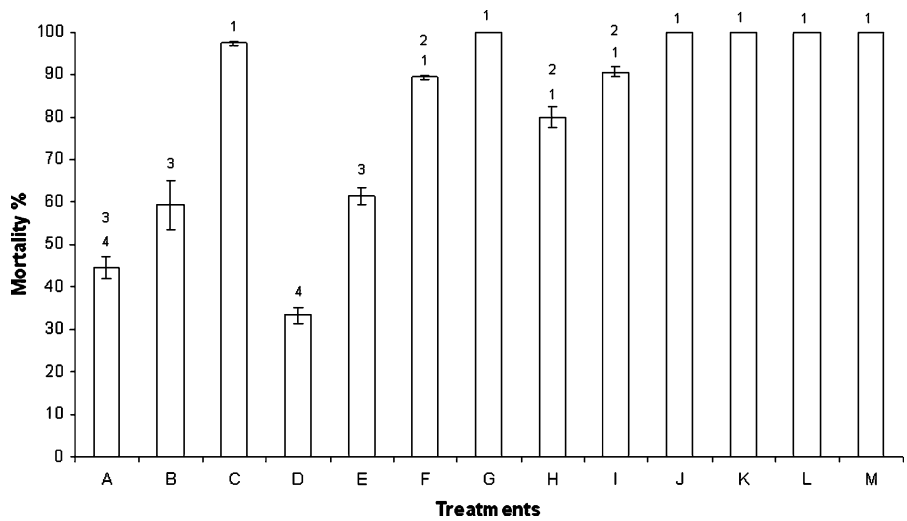

Fig. 1 Percent mortality (means ± SD) of *Aedes aegypti* immature stages exposed to different concentrations and combinations of *Bacillus thurigiensis* var. *israelensis*, temephos, and *Leptolegnia chapmanii* zoospores. The percent mortalities followed by the same number are not significantly different according to the Duncan test ($P = 0.01$). No mortality was recorded in controls in the absence of larvicidal agents. Treatment codes are given in Table 1

Table 2 Results of analysis of variance (ANOVA) for the effects of the treatments used, the number of days of exposure (linear and quadratic), and the interaction between these variables from the first to the third flooding in the sunlight

Effect	First flood			Second flood			Third flood		
	df	*F* value	*P*	df	*F* value	*P*	df	*F* value	*P*
Treatment	6,14	193.28	<.0001	6,14	226.25	<.0001	6,14	8.25	0.0006
Days	1,280	669.59	<.0001	1,280	1563.95	<.0001	1,280	1.82	0.1785
Days × treatment	6,280	23.21	<.0001	6,280	32.12	<.0001	6,280	0.27	0.9516
$Days^2$	1,280	127.92	<.0001	1,280	1.24	0.2666	1,280	68.98	<.0001
$Days^2$ × treatment	6,280	14.37	<.0001	6,280	9.82	<.0001	6,280	9.32	<.0001

Table 3 Results of analysis of variance (ANOVA) for the effects of the treatments used, the number of days of exposure (linear and quadratic), and the interaction between these variables from the first to the third flooding in the shade

Effect	First flood			Second flood			Third flood		
	df	*F* value	*P*	df	*F* value	*P*	df	*F* value	*P*
Treatment	6,14	46.75	<.0001	6,14	294.47	<.0001	6,14	13.86	<.0001
Days	1,280	233.04	<.0001	1,280	2056.25	<.0001	1,280	4.22	0.0410
Days × treatment	6,280	8.59	<.0001	6,280	34.92	<.0001	6,280	0.54	0.7803
$Days^2$	1,280	79.13	<.0001	1,280	8.55	0.0037	1,280	108.05	<.0001
$Days^2$ × treatment	6,280	8.15	<.0001	6,280	8.22	<.0001	6,280	13.88	<.0001

evinced a maximum mortality of 10 ± 1.4% during the first four days, and this degree of larval killing decreased until day 8, after which no further mortalities were recorded (Fig. 3). However, when the same concentration of *Bti* was combined with *L. chapmanii* zoospores, the larval mortality increased to 28 ± 0.8%, and the residual toxicity was extended up to day 15 with retention of larval mortality at 10 ± 1.2% at that time. With temephos alone, the mortality rates were 30 ± 2.3% during the 1st days but declined to 0% by day 15 (Fig. 3). In combination with *L. chapmanii* zoospores, however, this concentration of temephos produced a larval mortality of 45 ± 1.8% on day 2, though this toxicity decreased to a level of 15 ± 0.9% larval mortality by day 15. When the three antilarval agents were combined, the larval mortality was 75 ± 2.6% on day 2, but declined to 18 ± 0.7% by day 15. By contrast, containers incubated in the shade evinced a higher larval mortality for *Bti* 18 ± 1.2% and less by temephos on the 1st day 26.6 ± 1.5%, but then caused no larval mortality from days 8 and 15, respectively. When, however, each of these agents was combined with the *L. chapmanii* zoospores, the mortality increased to 40 ± 0.6% and to 60 ± 2.7%, respectively, and then decreased to values of 11 ± 0.5 and 18 ± 1.6%, respectively, by day 15. Finally, the two agents together in combination with the *L. chapmanii* zoospores resulted in the highest rates of mortality, at 80 ± 1.2%, on day 2 but then declining to 20 ± 2.2% on day 15 (Fig. 3).

During the third flooding cycle, containers containing only *Bti* or temephos produced no larval mortality in either the sunlight or the shade. Nevertheless, in the presence of the *L. chapmanii* zoospores, larval mortality occurred with both agents at levels of 20 ± 0.6% and 18 ± 1.5%, respectively, in the sunlight, and at a value of 23.3 ± 1.2% by *Bti* and 26.08 ± 0.8% by temephos in the shade. After 12 days in this third cycle no mortality was observed in any of the containers in either the sunlight or the shade (Fig. 4).

Discussion

The native isolate of the fungus *L. chapmanii* (ARSEF 5499-CEP 010), the main object of this study, was obtained from a puddle with infected larvae of *Oc. albifasciatus* in the city of Melchor

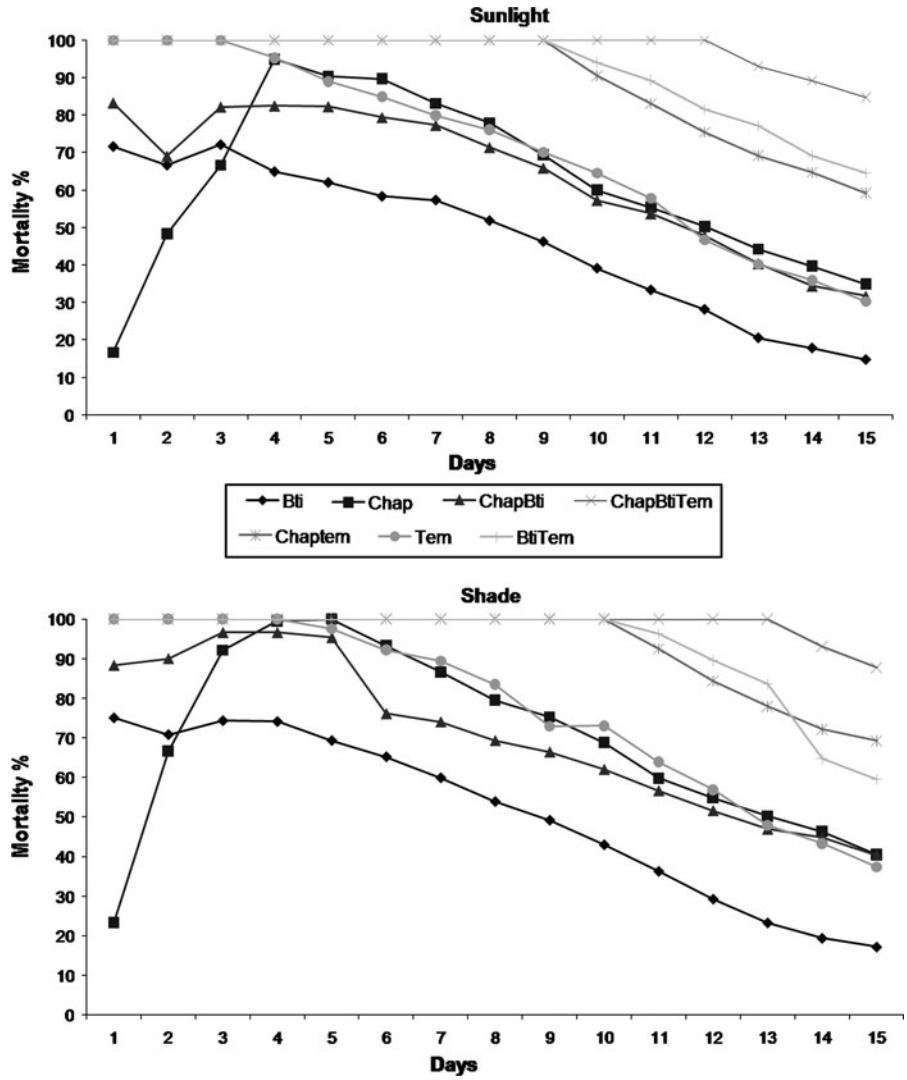

Fig. 2 Percent mortality of *Aedes aegypti* immature stages exposed to different doses and combinations of *Bacillus thurigiensis* var. *israelensis*, temephos and *Leptolegnia chapmanii* zoospores under seminatural conditions. First flooding under sunlight and shade

Romero, La Plata, Buenos Aires province, Argentina. Up to now, the known world distribution of *L. chapmanii* has been restricted to three states of the USA, California, Florida, and Ohio (Mc Innis and Zattau 1982; Seymour 1984; Lord and Fukuda 1988; Fukuda et al. 1997), with those specimens being the only native isolates within the Neotropical and temperate zones.

Historically, the control of *Ae. aegypti* throughout the world has been achieved through natural, non-chemical methods involving the elimination of breeding sources and by the use of standard insecticides, typically organophosphates. Since the 1980s several commercial products with active substances such as the *Bti* endotoxins were utilized for the control of *Ae. aegypti* and other culicids of relevance to public health, since these bacteria have a high specificity for aquatic diptera and are safe for vertebrates along with most of the aquatic invertebrates (Lacey 2007).

Castellanos (1997) described how the joint action of entomopathogenic fungi in conjunction with a chemical agent caused an enhanced larval-mortality rate with target insects relative to the use of the chemical alone. This observation constituted a possible breakthrough for the management of problematic insects and would have offered the advantage of reducing the occurrence of resistance to new insecticides. In this regard, resistance of *Ae. aegypti* to temephos has been reported (Fumasa 2000; Lima et al. 2003; Braga et al. 2004). Moreover, at times

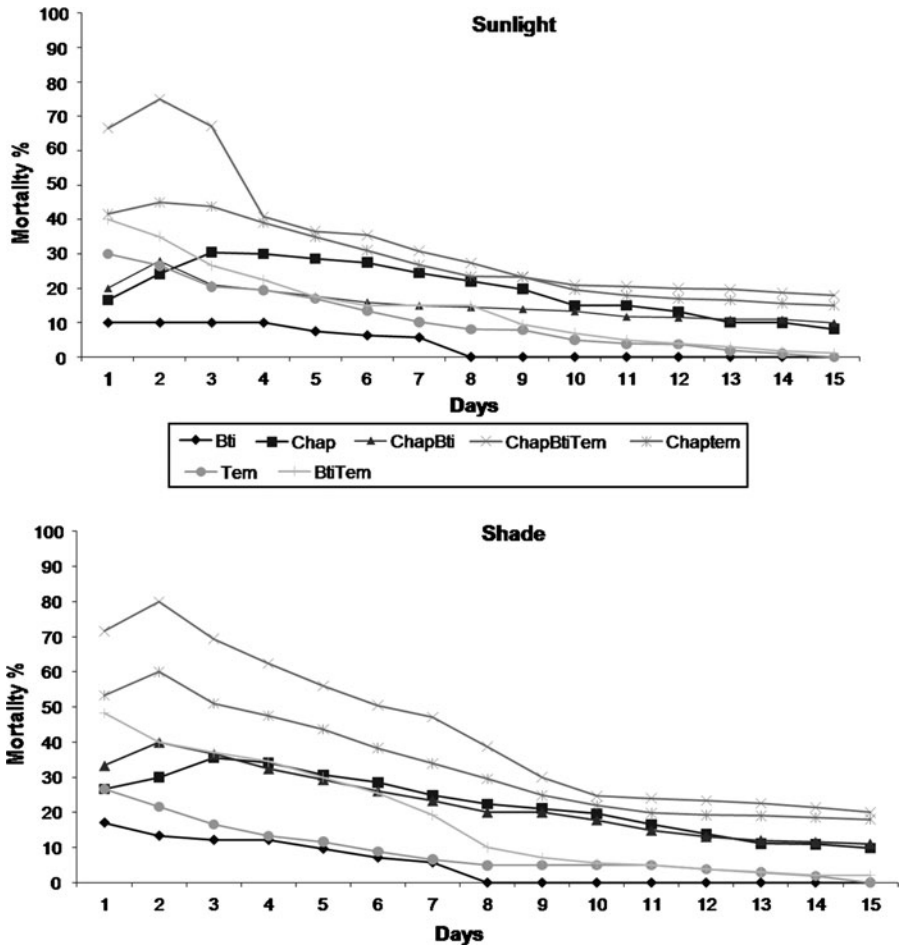

Fig. 3 Percent mortality of *Aedes aegypti* immature stages exposed to different doses and combinations of *Bacillus thurigiensis* var. *israelensis*, temephos and *Leptolegnia chapmanii* zoospores under seminatural conditions. Second flooding under sunlight and shade

chemical insecticides may have a greater detrimental impact on other natural enemies of mosquitoes than on the target insect.

The combined effect of sublethal concentrations of *Bti*, temephos, and the *L. chapmanii* zoospores thus indicated not only that this fungus is not inhibited by those two agents but also that the zoospores, when used together with these other agents, exert a synergistic larvicidic effect on *Ae. aegypti*. We have also observed an enhancement of larvicidal activity when the zoospores were used along with either of the two compounds alone, both in laboratory assays and under seminatural conditions. From small-scale treatments we determined that *L. chapmanii* zoospores were infective for up to 56 days. These data are consistent with the previous observation that zoospores or their cysts can survive for 51 days under field-like conditions (Pelizza et al. 2008).

Several authors have demonstrated the advantages of the synergistic interaction between fungi and chemical insecticides when applied simultaneously (Ferron 1985; Barjan et al. 1995; Pristavko 1966). Orduz and Axtell (1991) reported that the mosquito-pathogenic oomycete *Lagenidium giganteum* Couch was compatible with *Bti* (Vectobac-12 AS) as well as with *Bacillus sphaericus*. At lower concentrations of *Bti* (0.057 ITU/mg) and *B. sphaericus* (0.6×10^4 spores ml^{-1}) the larval mortality was lower than 50%. When, however, these agents at those same concentrations were combined with *L. giganteum* zoospores, the mortality increased dramatically to 97.5% and 100%, respectively.

Likewise, evaluations of the combined larvacidal action of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill (Hypocreales: Sordariomycetes) when used along with chemical insecticides such

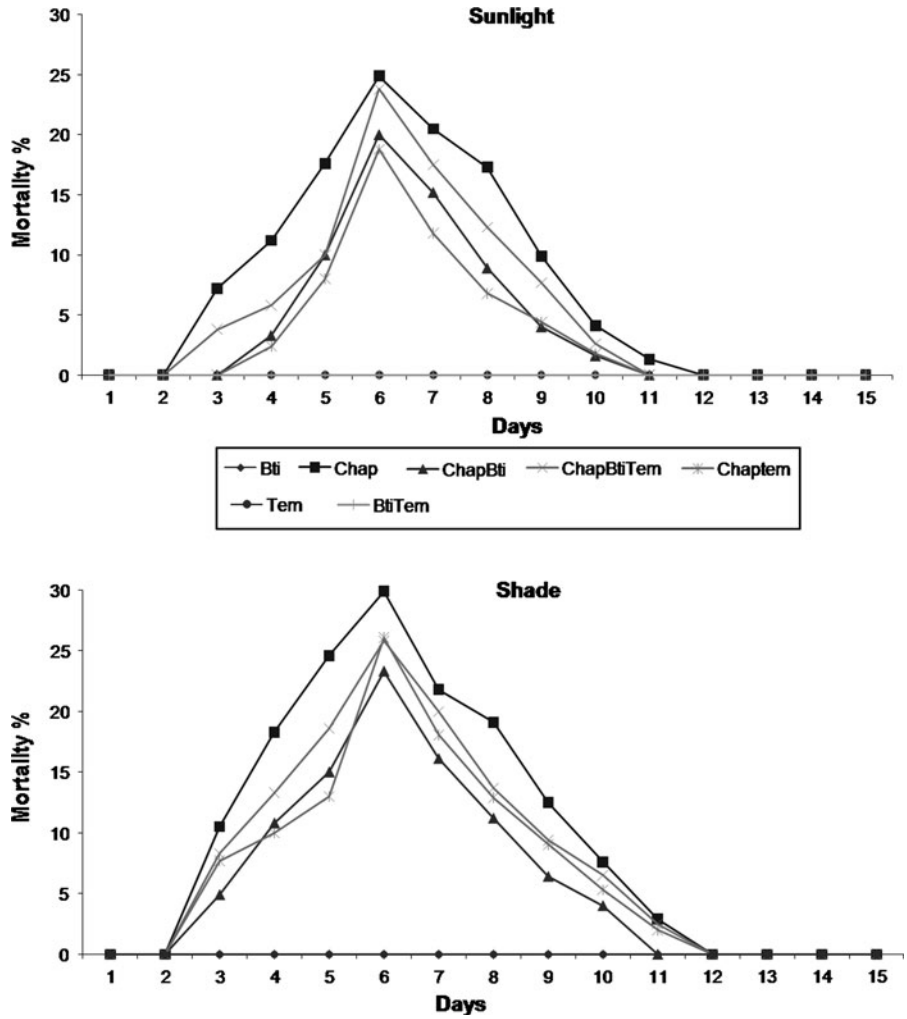

Fig. 4 Percent mortality of *Aedes aegypti* immature stages exposed to different doses and combinations of *Bacillus thurigiensis* var. *israelensis*, temephos and *Leptolegnia chapmanii* zoospores under seminatural conditions. Third flooding under sunlight and shade

as carbaril, fenvalerate, abamectin, and triflumuron plus *Bti* have indicated a compatibility between the conidiospores and both the latter categories of larvacides (Anderson et al. 1989; Vazquez et al. 2004, 2006).

Nevertheless, Rivera et al. (1994), when testing the effect of several insecticides (endosulfan, clorpirifos, fenitrothion, miazinon, malathion, isazofos, and pirimifos) along with *B. bassiana* against *Hyphothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), found that those particular chemical agents were fungistatic with this entomopathogen.

In this work we have demonstrated not only the absence of any inhibitory interactions between *Bti*, temephos, and *L chapmanii* zoospores but also the joint synergistic action of these agents in maximizing *Ae. aegypti* larval mortality. These results could point to the use of these three larvicides in combination for the development of new commercial products containing several larvicides in a single formulation. Such admixtures of active ingredients would have several advantages over the present products containing only one larvicide; the amounts of chemical insecticides such as temephos released into the environment would be reduced. Since the quantity of *Bti* present would likewise be decreased, such a formulation would also provide an economical benefit. A lower extant concentration of *Bti* in the environment would also minimize the possibility of resistance development within natural population of

the target pest, a drawback that has been cited for populations of *Culex pipiens* L. exposed to *Bacillus sphericus* (Tabashnik et al. 1990; Tabashnik 1994).

Finally, the major advantage of this multivalent-larvicide formulation would be the effective and efficient short-, medium-, and long-term control of *Ae. aegypti* larval development. This prolonged management of *Ae. aegypti* larval development would result through a combination of the short-term effects of temephos and *Bti* along with the medium- to long-term control evoked by the *L. chapmanii* zoospores. This fungus not only would produce a high larval mortality but would also remain in the environment for longer periods of time as encysted zoospores and/or resistant oospores until the environmental conditions were appropriate for the presence of a new generation of mosquitoes.

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