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Larvicidal activity and influence of *Bacillus thuringiensis* (Vectobac G), on longevity and fecundity of mosquito species

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ABSTRACT

Mosquitoes are vectors of many human diseases and cause environmental nuisances. Due to their large geographical distribution and abundance, Culex pipiens and Culiseta longiareolata represent the most important mosquito species in Algeria. The management of these disease vectors using conventional pesticides has failed because of the high reproductive ability, development of insecticide resistance of mosquito species and environmental pollution. These reasons are leading to a search for novel molecules. As so the product Vectobac G that is based on the naturally occurring insecticidal toxin proteins of Bacillus thuringiensis israelensis (Bti) may provide economical control of mosquito larvae. Bioassay was conducted to test the larval toxicity, longevity and mosquito fecundity. The mosquitocidal activity of Bt. Vectobac G was tested at different concentrations, ranging between 5 and 25 µg/l, against the different larval stages (L1-L4) of Culex pipiens and Culesita longiareolata. Bioassays were done on newly moulted larvae under laboratory conditions. The technical material showed a high level of activity with mortality recorded for both treated and following stages and happened after incomplete development. For the same treated series a significant decrease was also recorded in the longevity of the 4th instar and the adult. From a dose-response curve, the LC_{50} and the LC_{90} with their confidence limits were determined. The $LC5_0$ of 16.21µg/l and 23.98 µg/l and the values of CL_{90} of 75.85µg/l and 73.31µg/l, were estimated for the both species Cx. pipiens and Cs. Longiareolata respectively. In other experiments the compound was applied at LC_{50} and LC_{90} against the fourth instars larvae and its effects was investigated on fecundity of female emerged from larval treated series. The results showed that Bt. vectobac G reduced significantly the laying egg number and the percentage of fecundity.

Key words: Mosquito, *Culex pipiens*, *Culiseta longiareolata*, toxicity, *Bacillus thuringiensis*.

INTRODUCTION

Culex pipiens and *Culiseta longiareolata* are considered among the most abundant mosquito species in Algeria. *Culex pipiens*, called the domestic mosquito, is present in the urban areas due to the higher number of breeding places in today's `throw away' society practically during all the year, with preference to *Culiseta longiareolata* to be more present particularly in semi-arid areas [1; 2]. Mosquitoes are vectors of several diseases like malaria, filariasis, dengue fever, yellow fever, etc., causing serious health problems to human beings. These insects are generally controlled by conventional pesticides [3; 4]. Further, the indiscriminate use of neurotoxic insecticides caused various environmental aspects [5; 6] toxic problems to non target organisms and insecticide resistance [7]. Globally, there have been conscientious efforts to overcome these problems and great emphasis has been placed recently on enviro-friendly and economically viable methodologies for pest control. An alternative approach for mosquito control is the use of natural products such as plant and microorganisms. The microbial pesticides have

undergone extensive testing prior to registration. They are essentially nontoxic to humans, so there are no concerns for human health effects with *Bacillus thuringiensis* [8] or *Bacillus sphaericus* [9]. Extensive testing shows that microbial larvicides do not pose risk to wildlife, non-target species or the environment and retain a good activity in polluted water [10].

The mosquitocidal activity of active strains of *B. sphaericus* and *B. thuringiensis* resulted in their development as commercial larvicides. It is being used currently in many pest and vector programs for many years ago in several countries. The first insecticidal component of the *B. sphaericus* strains used in mosquito control is acting by the production of a toxin during sporulation and vegetative stages of the *Bacillus* [11; 12]. Many reports have shown that mosquito larval midgut is the primary target of the toxin [13; 14; 15], by binding to the receptor expressed on the epithelial cells and it confers toxicity [16]. In recent years, although some toxic strains have been widely used, as biopesticides in the field in mosquito control programs [17], microbial pesticide, *Bacillus species* have received much attention as potent bioactive compounds against various species of mosquitoes [8; 18; 17]. In the present study bioassay was conducted to test the larval toxicity and longevity of *Bacillus thuringiensis* vectobac G on *Culex pipiens* and *Culesita longiareolata*. In addition, its effects at LC_{50} and LC_{90} were studied on the fecundity of the females emerged from the treated fourth instar larvae.

MATERIALS AND METHODS

Maintenance of larvae:
Bioassays based on standard methods for testing larval susceptibility [19], were conducted in the laboratory to determineLC_{50} and LC_{90} of *Bt.* Vectobac G against the instars larvae of *Cx. pipiens* and *Cs. longiareolata*. Different instars larvae of the two vector species were obtained from laboratory colonies reared as previously described [1]. Larvae were reared in storage jars containing 500 ml of stored tap water and maintained at temperature between 25-27°C, 85% RH and a photoperiod of 14:10 (L:D). Larvae were daily fed with fresh food consisting of a mixture of Biscuit-dried yeast (75:25 by weight), and water was changed every four days. The feeding was continued till the larvae were transformed into the pupa stage.

Maintenance of pupae and adult:
The pupae were collected from the culture trays and were transferred to glass jars containing 500 ml of water with the help of a dipper. The jars were kept in (50 × 50 × 50 cm) size mosquito cage for adult emergence. The adults were fed with 10% sugar solution for a period of three days before they were provided by an animal for blood feeding.

Toxicological assays:
Toxin preparation used in this study was lyophilized powder of spore and crystal mixture of lysed cultures of *Bacillus thuringiensis* variety *israelensis.* Laboratory bioassays were conducted on the efficacy of a granule (G) formulation of *Bacillus thuringiensis* variety *israelensis* (Vectobac G; active ingredient [AI]: 2000 *Bti* international toxic units [ITU]/mg) at different concentrations, against newly ecdysed larvae for all different larval stages (L1 to L4) of *Culex pipiens* and *Culesita longiareolata*. In the assays, each 25 larvae were placed in a jar containing 500 ml of stored tap water. For testing, serial dilutions from the product were made from the stock to obtain the appropriate range of concentrations. For each vector species, the formulation was tested at 4 different concentrations (5, 7.5, 12.5, 25 µg/l) against the different larval developmental stage. The serial concentrations were freshly prepared on each occasion. Each concentration was applied to three jars (replicates) while three other jars were left untreated as controls. The rate of growth and development was examined and mortality was registered daily until adult emergence. The longevity of the developmental stages was carried out on the fourth larval stage of both mosquito species, after treatment with the lethal concentrations of *Cs. pipiens* and *Cs. longiareolata* (CL50= 16.21 µg/l, CL90=75.85 µg/l; CL50 = 23.98 µg/l, CL90= 7331 µg/l respectively). The fourth, pupae and adult longevity of mosquitoes was also recorded. This was calculated by the number of days lived by the developmental stage. The emergence day and mortal days of the adults were recorded and the means were calculated to give the mean longevity in days.

Statistical Analysis:
The mortality percentage observed for each stage and concentration was corrected [20] and subjected to the probit analysis [21]. LC_{50}, LC_{90}, confidential limits and the slope were calculated [22]. Data from insecticidal tests were subjected to analysis of variance after angular transformation of observed mortality percentages.

Fecundity tests:
The fecundity experiments were conducted on the eggs of *Cx. pipiens* and *Cs. longeareolata* collected from the breeding jars of the females emerged from treated fourth larval stage with the lethal concentrations (CL_{50} = 16.21

µg/l, CL_{90} = 75.85 µg/l; CL_{50} = 23.98 µg/l, CL_{90} = 73.31 µg/l respectively) of the *Bt.* Vectobac G of both mosquito species. For each concentration 20 females and 20 males were kept in separate breeding cage. The laying eggs for each series were collected, counted and transferred to a new jar containing 500 ml of water and kept for larval hatching. Different parameters of reproduction; the number of egg laying, hatching rate, the fecundity, were studied. The fecundity was calculated by the number of eggs laid in ovitrap divided by number of females let to mate (20 nos.) (The death of adults in the experiments was also considered). The obtained results were subjected to a statistical analysis using the *t* test of student. Hatching Rate (HR) was calculated according to the following formula:

$$HR = \frac{\text{number of hatching eggs}}{\text{total number of eggs}} \text{ X100}$$

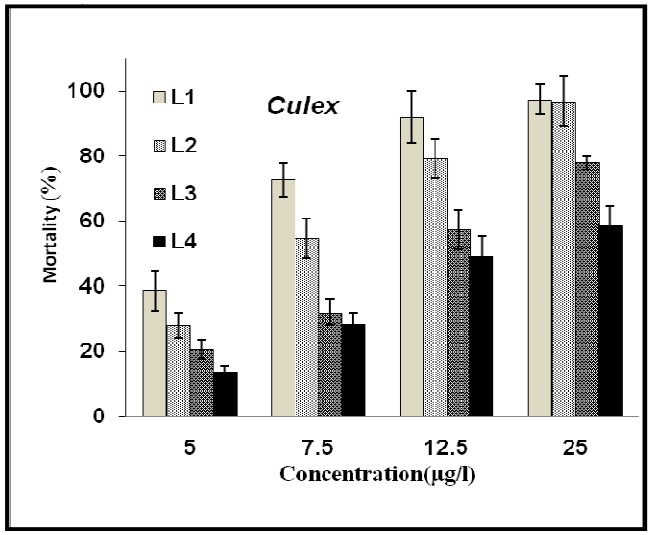
RESULTS

Insecticidal activity of *Bacillus thuringiensis* (Vectobac G):

Bioassays showed that *Bacillus thuringiensis* (Vectobac G) have a high toxicity against the two species, *Culex pipiens* and *Culiseta longiareolata*. The percentage mortality of the different instar larvae (L1, L2, L3, L4) were represented in figure 1. The mortality varied with concentration for the different larval stages and the both mosquito species. Mortality was observed during the treated larval stage and the following ones. The lower concentration (5µg/l) of *Bt.* vectobac G shows the mortality varies between 30% and 40%, further the mortality rate was found to be higher with the increased concentration and the mortality range was about 50-100% within adult emergence. The analysis of variance of the data showed a significant ($p<0.001$) insecticidal effect with a dose response relationship. The highest concentration of *Bt.* Vectobac G (25µg/l) tested against *Cs. longiareolata*, caused 100% mortality for first and second instar larvae and the mortality decreased within the older stages, whereas 58.66% mortality was registered for the fourth-instar larvae of *Cx. pipiens* and 52% of *Cs. longiareolata*. Furthermore it was noticed that the mortality was much higher for the first-instar larvae for all the concentrations used (Figure 1). The target mosquito species tested were extremely sensitive to the *Bt.* Vectobac G formulation, with the most sensitive species *Cs. longiareolata*. With probit, the LC_{50} and LC_{90} were calculated and the confidence limits (LCL & UCL) for all stages were estimated (Table 1).

Table 1: Toxicity of *Bacillus thuringiensis* Vectobac G) against *Culex pipiens* and *Culiseta longiareolata* larvae. (LC_{50} and LC_{90}, FL, µg/l)

Instar Larvae	*Culex pipiens*		*Culiseta longiareolata*	
	95% Confidence limit		95% Confidence limit	
	LCL <LC_{50}< UCL (µg/l)	LCL <LC_{90}< UCL (µg/l)	LCL <LC_{50}< UCL (µg/l)	LCL <LC_{90}< UCL (µg/l)
L1	3.7 <5.37 <4.7	5.4 <13.69 < 2.1	3.9 <4.21< 2.5	4.23 < 8.81< 6.50
L2	4.5 <7.69 <3.9	9.7 < 17.78< 6.01	6.3 <7.12< 4.04	5.4 <17 < 7.2
L3	4.1 <11.92 <5.2	16.3 < 38.93< 22.7	6.4 <11.31< 5.5	13.4 < 37.70< 15.2
L4	8.2 <16.21< 7.9	13.1 <75.85< 25.7	11.5 < 23.98< 13.6	19.7 <73.31 <13.7



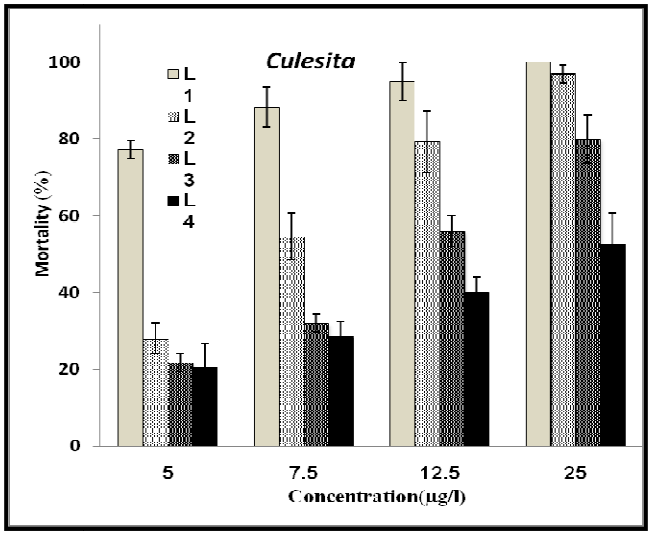


Figure 1: Concentration-response relationship of treatment of the *Bacillus thuringiensis* Vectobac G applied to newly ecdysed larvae of *Culex pipiens & Culesita longiareolata.* (Means ± SD, n = 25)

Longevity of mosquito developmental stages:

Table 2 shows the effect of *Bt.* Vectobac G on the adult longevity of the different developmental stages of *Cx. pipiens* and *Cs. longiareolata* after the treatment with their lethal concentrations (CL_{50} and CL_{90}). Exposure of the fourth instar larvae of both species caused no difference in the duration of the all larval stages except with the LC_{90}. The following pupal stage no difference was recorded in the duration for all concentrations. However the adult longevity of both mosquito species was considerably reduced by the treatment of *Bt.* Vectobac G. The longevity was reduced to 30 days at CL_{50} and 27 days in *Cx. pipiens*. For *Culesita* species the duration was reduced significantly to 29 days at the LC_{50} and to 23 days at CL_{90} whereas the longevity of the control was 32 days.

The Effect of *Bt.* Vectobac G on reproduction was evaluated on different parameters, of the females emerged from the treated fourth instar larvae of *Cx. pipiens* and *Cs. longiareolata* and presented in table 3. Fecundity was highly reduced after the treatment of *Bt.* Vectobac G. The number of eggs laid was inversely proportional to the concentration in treatment. For *Cx. pipiens* the number of eggs laid was reduced from 1332 to 988 and 696 as the concentration was increased, from CL_{50} to CL_{90}. The same observation was recorded for *Cs. longiareolata*, where the egg number decreased from 1675 to 1521 and 1210 under treatment effect of CL_{50} and CL_{90} respectively (Table 3). For the same series of experiments the hatching rates was calculated and showed a significant decrease according to the treatment of *Bt.* Vectobac G (Table 3). The fecundity was also highly reduced after the treatment of *Bt.* Vectobac G. The number of eggs laid was inversely proportional to the concentration in treatment. The number of eggs was reduced from 118 to 14 as the concentration was increased. The fecundity for both species was also affected and a significant decrease was obtained for the two lethal concentrations (Table 3).

Table 2: Effect of *Bacillus thuringiensis* Vectobac G on the longevity of developmental stages; after treatment with the lethal concentrations (CL_{50} & CL_{90}), of 4th instar larvae of *Culex pipiens* and *Culiseata longiareolata*

Means (±Standard deviation) followed by the same letter indicate a significant difference (P<0.05). (n = 25-75)

Longevity of the developmental stages (day)						
Stage	***Culex pipiens***			***Culiseta longiareolata***		
	Control	CL_{50} = 16.21 µg/l	CL_{90} = 75.85 µg/l	Control	CL_{50} = 23.98 µg/l	CL_{90}= 73.31 µg/l
L4	6.50 [a]	6.80	7.33 [a]	5.66[ab]	6	6.66[a]
Pupae	3.53	3.43	3.43	3.30	3.33	3.43
Adult	38.33 [ab]	30.53 [ab]	27.5[ab]	32 [ab]	29[ab]	23.53[ab]

Table 3: Effect of *Bacillus thuringiensis* Vectobac G on reproduction of the females emerged from the treated fourth instar larvae of *Culex pipiens* (n= 20 females)

Means followed by the same letter indicate a significant difference (P<0.005)

Treatment	N° egg laid	Hatching rate (%)	Fecundity
Control	1332[a]	96.84[a]	66.6[a]
CL_{50} = 16.21 µg/l	988[ab]	75.40[ab]	49.4[ab]
CL_{90} = 75.85 µg/l	696[ab]	55.02[ab]	34.8[ab]

Table 4: Effect of *Bacillus thuringiensis* Vectobac G on reproduction of the females emerged from the treated fourth instar larvae of *Culesita longiareolata* (n= 20 females)

Means followed by the same letter indicate a significant difference (P<0.005)

Treatment	N° egg laid	Hatching rate (%)	Fecundity
Control	1675[a]	97.41[a]	83.75[a]
CL_{50} = 23.98 µg/l	1521[ab]	81.93[ab]	75.05[ab]
CL_{90} = 73.31 µg/l	1210[ab]	59.92[ab]	60.50[ab]

DISCUSSION

Mosquito control is a vital public-health practice throughout the world and especially in the tropical zones because mosquitoes spread many diseases. In the present context of integrated vector control, due to rapid increase in mosquito resistance and growing public concern over environmental pollution, use of chemical insecticides for mosquito control is no longer encouraged; rather use of effective and eco-friendly alternatives is promoted. A program on biological control of mosquitoes, virulence prospecting and evaluation of new isolates is one of the most important steps taken to determine their effect on target populations, and thereby selecting the most promising ones for producing biological insecticides. Much of the work carried out to evaluate *Bacillus thuringiensis* effects against different natural enemies has been done [23; 8], with particular commercial product and has been developed as an insecticide rather than predators in the laboratory rather than the field [24]. *Bacillus thuringiensis* ssp. *israelensis*(*Bti*) is one of the bacterial biolarvicides, presents an alternative for controlling several mosquito species [25; 26; 27]. *B. thuringiensis* subsp. *Israelensis* produces a component of a spherical parasporal body and composed of four main toxin proteins, Cyt1Aa, Cry47Aa, Cry4Ba, and Cry 11Aa. The primary insecticidal component of the *B. sphaericus* strains used in mosquito control operations is a binary toxin (Bin) [28; 11; 12] produced during sporulation and vegetative stages of the *Bacillus*. Many studies have shown that mosquito larval midgut is the primary target of the binary toxin. After ingestion by susceptible larvae, crystals are solubilised in the midgut and two proteins are released [13; 14], and cleaved by endogenous proteases to form active toxin [15; 16].The efficacy and safety characteristics of this bacterial agent have made it a suitable candidate for large-scale production and field-testing. There is no report of resistance development in vector mosquitoes to this bacterium unlike *Bacillus sphaericus* to which development of resistance is already reported [29; 30]. The toxicity assays conducted under laboratory conditions on *Cx. Pipiens* and *Cs. longiareolata* larvae indicated that *Bt.* Vectobac G exhibited a larvicidal activity when applied to newly ecdysed larvae for 24 h. The same effects were found when the *Bacillus sphaericus* was used against *Anopheles stephensi* [31]. The target mosquito species tested were extremely sensitive to the *Bt.* Vectobac G formulation, with the most sensitive species *Culiseta longiareolata.* Some larvae survived in the short term and continually died through the following larval stages and after emergence. Many factors might Influence such delayed effects, including genetics, the number of bacteria ingested, the degree of larval midgut damage, and the mode of action of the used toxin [32; 8]. However some larval mosquitoes may survive to the treatment restore any more damage caused by the toxin, develop resistance and grow normally. Previous study indicated that some components of Bin toxin synergistically bind to a single class of specific receptors present on larval midgut [33], cause a formation of pores in the epithelial cell membrane through an unknown mechanism [34] and then induce the death of larvae. However, other toxins act by modifying a large array of proteins by its ADP-ribosylation. These modified proteins, being essential for the growth and effect later the development, resulting to in abnormal emergence or death [35].

In the present study**,** *Bt.* Vectobav G treatment reduced the larval duration, non the pupal and introverted the adult emergence. Those treated larvae escaped from mortality showed reduced longevity. The adult which emerged from treated larvae were morphologically normal but showed a great reduction in fecundity. the same results were mentioned when the *Bacillus sphaericus* was tested against malaria vector *Anopheles stephensi* [31]. Many reports show changes in fecundity after treatment with *B. thuringiensis* [36; 37; 38]. Combined treatment of *Bacillus thuringiensis* with neem and pongamia showed an adult mortality and reduction in fecundity in *Culex quinquefasciatus* after the treatment with *B. sphaericus* (GR strain) [39]. From the present study we conclude that *Bt.* Vectobac G proved good larvicidal agent against *Cx pipiens and Cs. longiareolata* larvae in laboratory and also reduced the longevity of different developmental stages, egg productions and fecundity.

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