ACTA ZOOLOGICA BULGARICA

Applied Zoology
Research Article

Acta Zool. Bulg., 75 (2), June 2023: 265-272

Published online 15 May 2023
https://www.acta-zoologica-bulgarica.eu/2023/002643

Effects of Spinosad and *Bacillus thuringiensis kurstaki* on *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae): Adults' Fertility, Fecundity and Cuticular Hydrocarbons

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Abstract: This study aimed to evaluate the effect of two bacterial preparations: Spinosad and *Bacillus thuringiensis kurstaki* (*Btk*), which were applied at low concentrations (20 µg/l and 23 mg/l, respectively) on L4 larvae of the *Culex pipiens*. This application was used to assess their effects on mortality of the larvae, reproduction and cuticular hydrocarbons in adults resulting from this treatment. The results showed that both bacteria caused a death rate higher than 80 %, and the sub-lethal doses, despite being less toxic to mosquitoes, caused disturbances in the fecundity and fertility of the adults emerging from treated larvae. The use of sublethal doses of Spinosad induced a quantitative increase in cuticular hydrocarbons at a rate of 36 % to 42 % in treated males and females, respectively. Furthermore, the sublethal dose of *Btk* causes a reduction in the cuticular hydrocarbons by 59–64 % in both sexes. The results also show that there are no significant differences in the amounts of cuticular hydrocarbons in treated males and females. The results show that the two bacteria are promising as bio-insecticides against *C. pipiens* and could be good alternatives to chemical pesticides.

Key words: *Btk, Culex pipiens*, cuticular hydrocarbons, fecundity, fertility, Spinosad

Introduction

Mosquitoes of the genus *Culex* Linnaeus, 1958 can transmit human and animal diseases such as filariasis, encephalitis caused by viruses (Chikungunya virus, Japanese encephalitis virus, Venezuelan equine encephalitis and the Zika virus) and West Nile fever. Over 120 million people are now infected with filariasis and 40 million of them are severely disabled. Among these species, *Culex pipiens,* which is cosmopolitan and plays the role of a major vector in the transmission of several diseases, it a considerable nuisance to human populations exposed to its nocturnal bites in most African countries (Subra 1981). Currently, there is no specific treatment and only preventive vaccines are available to combat some of

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these diseases. To ensure better intervention, while preserving the natural environment as much as possible, new preventive methods and new products are constantly being tested. Consequently, to contribute to sustainable environmental management, the implementation of new mosquito control alternatives is further encouraged (Benhissen 2016).

Insecticides of biological origin are reasonable alternatives to chemical insecticides. The arrival of entomopathogenic products (bacterial strains) such as *Bacillus thuringiensis kurstaki* (*Btk*) and spinosad (Sp) led us to believe for several years that the problem of insect resistance to insecticide treatment was going to be quickly solved due to the variety of toxins produced by these entomopathogens (causing the intoxication and then the death of the insect). These new biological molecules with toxic effects were introduced to the market a few years ago. Spinosad results from the fermentation in the bacterium *Saccharopolyspora spinosa* (Mertz & Yao 1990). This bioinsecticide is classified as a reduced-risk product because of its unique mode of action and its high specificity in targeting harmful insects. Moreover, the reason for major interest in spinosad is its low toxicity on mammals, fish, birds, beneficial insects and humans (Thompson & Hutchins 1999).

Bacillus thuringiensis kurstaki (*Btk*) is a bacterium that lives naturally in soils, recognised by its protein crystal included in the cytoplasm (0-endotoxin), which is toxic to mosquito larvae (Hougard et al. 1983).

In insects, cuticular hydrocarbons (HCs) are also used for taxonomic purposes (Phillips et al. 1988) and play a major role in the biology and behaviour of insects (Saïd et al. 2005). Cuticular hydrocarbon analysis has been applied as a tool for the separation of closely related mosquito species by comparing major chromatographic peaks of *Anopheles maculipennis* (Phillips et al. 1990). Several studies on the HCs of different insect species demonstrated the existence of a quantitative difference in HCs according to age and sex, and the absence of a qualitative difference (Sharma et al. 2012). Some authors have observed qualitative differences between the two sexes as in *D. melanogaster* (Cobb & Jallon 1990), *Laupala locusts* (Mullen et al. 2008), *Chrysomya rufifacies* larvae (Zhu et al. 2006), *Teleogryllus oceanicus* (Thomas & Simmons 2009) and *Riptortus pedestris* (Yoon et al. 2012).

In the present study, the delayed effects of *Btk* and spinosad on the reproduction and the development of mosquitoes treated with sub-lethal concentrations were studied. A quantitative and qualitative analysis of HCs of adult males and females of treated *C. pipiens*, will be useful to determine the content of HCs of the species and to observe the disturbance generated on the composition and the structure of the cuticle during the biological stage of treated mosquitos.

Materials and Methods

Insects

Culex pipiens is a cosmopolitan species, which is widely distributed in tropical and subtropical regions. It plays a role of a major vector in the transmission of filariasis (Subra 1981). Its life cycle lasts between 10 to 14 days and it includes four stages: eggs, larvae, pupae and adults. The pre-imaginal stages (eggs, larvae and pupae) are adapted to the aquatic lifestyle whereas the imaginal stage (imago) is aerial (Carnevale et al. 2009). The insects used in this study were obtained from a mass breeding of gravid adults collected in the region of Annaba (eastern Algeria). Breeding was maintained in laboratory conditions at 25 ± 2°C, and 70 to 85 % humidity with a scoot phase of 12:12. A mixture of biscuit and dried yeast provides nutrition for the larvae while the adults feed on raisins.

Insecticides

Spinosad was first isolated from soil samples in 1988 (Thompson & Hutchins 1999). It is a fermented product derived from the mixture of two toxins: spinosyn A and D (Thompson et al. 1997). Solid and pale grey, spinosad is characterised by the smell of stagnant water. Its chemical formula is $C_{41}H_{65}NO_{10}$ (spinosyn A) or $C_{42}H_{67}NO_{10}$ (spinosyn D) (Thompson et al. 2000).

Bacillus thuringiensis kurstaki (*Btk*) is a Gram-positive bacterium, which has the particularity of synthesising a protein crystal of a cubic or bi-pyramidal structure during sporulation. The crystals have a larvicidal activity on different insect species. Poisoning manifests itself very quickly in the intestine by paralysing of the digestive tract (Sanchis et al. 1995). For this study, we used a commercial product, in the form of a powder containing 23000 IU.

Experiments

The adults emerging from the treated larvae by Spinosad and *Btk* were isolated and separated into pairs (male and female) in cages (20 x 20 x 20 cm) with containers of water. After mating, the number of eggs laid by each female was counted. We kept these eggs in the laboratory until the hatching of the

Table 1. The number of couples used for each treatment

Couples	Treatment	Total adults
10 couples	Spinosad	20 adults (10 males and 10 females)
10 couples	Btk	20 adults (10 males and 10 females)
20 couples	Control	40 adults (20 males and 20 females)

Table 2. The hydrocarbons (*n*-alkanes) and their designation

Hydrocarbon	Designation
Octadecane: $C_{18}H_{38}$	C18
Heneicosane: $C_{21}H_{44}$	C21
Docosane: $C_{22}H_{46}$	C22
Tricosane: $C_{23}H_{48}$	C23
Tetracosane: $C_{24}H_{50}$	C24
Pentacosane: $C_{25}H_{52}$	C25
Hexacosane: $C_{26}H_{54}$	C26
Heptacosane: $C_{27}H_{56}$	C27
Octacosane: $C_{28}H_{58}$	C28
Nonacosane: $C_{29}H_{60}$	C29

first stage larvae (L1). The same study was carried out on control adults (Table 1).

For extraction of cuticular hydrocarbons, the adults (N=80, 40 males and 40 females) resulting from the treatment of the larvae with sub-lethal concentrations of spinosad (20 µg/l) and *Btk* (23 mg/l), were sexed as soon as they emerge. They were isolated in a glass tube closed by a foam stopper and containing a piece of cotton soaked in water to maintain humidity. After 24 hours, the individuals were killed by exposure to low temperatures (at -20°C) and subjected to hydrocarbon extraction separately with 500 µl of distilled pentane, to which 100 ng of octadecane (C18) had been added as an internal standard. After five minutes at room temperature, the solvent was removed using a syringe and put into a clean tube. The extracts obtained were stored in the freezer (-20°C) until their analysis by gas chromatography. The same protocol was applied on control adults (N= 40: 20 males and 20 females).

The chemical analysis of the extracts was carried out by gas chromatography (GC). The chromatograph used was a Varian CP 9000 equipped with a fused silica capillary column of medium polarity (DB 1701.30 mx 0.32 mm internal diameter, film thickness 1µm, Varian), a split-type injector split less (20 ml/min leak for 30 sec) and a flame ionization detector. The carrier gas was hydrogen (velocity, 25 ml/min at room temperature). The temperatures of the injector and the detector were, respectively, 280 and 290°C. The signal was recorded on a PC under Windows, and analysed using the Maestro program (Chrompack). Each analysis was repeated on 20 different samples. The concentration of the various quantified compounds was calculated according to the response factor of the internal standard C18 (Table 2).

Statistical analyses

Calculations were computed using the XLSTAT 2009 software (Addinsoft, New York, NY). A comparison of the means by a Student's t-test and a comparison of the variances (ANOVA with one classification criterion) were performed.

Results

The effect on mortality

The fourth instar larvae of *C. pipiens* were sensitive to both bacteria, which was reflected by more or less high mortality rates depending on the concentrations used and especially depending on the exposure time (Fig. 1). The results showed a low larvicidal activity recorded for low concentrations. With regard to high concentrations, more than 80 % of treated individuals died after 15 days of treatment (Fig. 1), the results also demonstrated an increase in *C. pipiens* larval longevity and growth at various stages.

The effect on the reproduction (fecundity and fertility) of females

The results presented in Table 3 summarize the effect of Spinosad and *Btk* on the fecundity and fertility of females produced by treated larvae. The results showed that the highest number of eggs was recorded for the control females (without treatment), followed by females emerging from larvae treated with spinosad (37.70 ± 27.52 eggs), while the number of eggs laid by females treated with *Btk* was much lower. The latter laid an average of 33.20 ± 5.116 eggs with a minimum of 24 eggs and a maximum of 42 eggs (Table 3).

The eggs from the control females took a longer time to hatch, with an average time of 2.8 ± 0.42 days, and maximum duration of three days, while the minimum duration was two days. The eggs from the females treated with *Btk* took approximately the same time for hatching (2.30 ± 0.48 days). On the other hand, the females treated with spinosad took less time to hatch with an average time of 1.90 ± 1.66 days (Table 3). Regarding the hatching rate of eggs, the control females displayed the highest

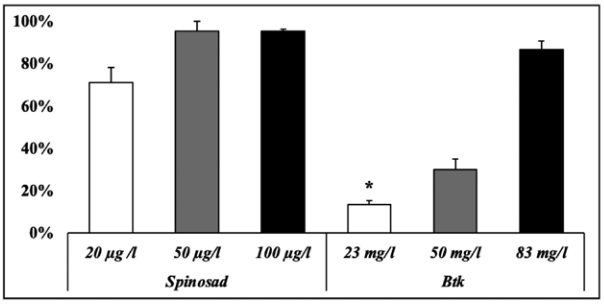

Fig. 1. Mortality rate of *Culex pipiens* larvae treated with spinosad and *Btk*.

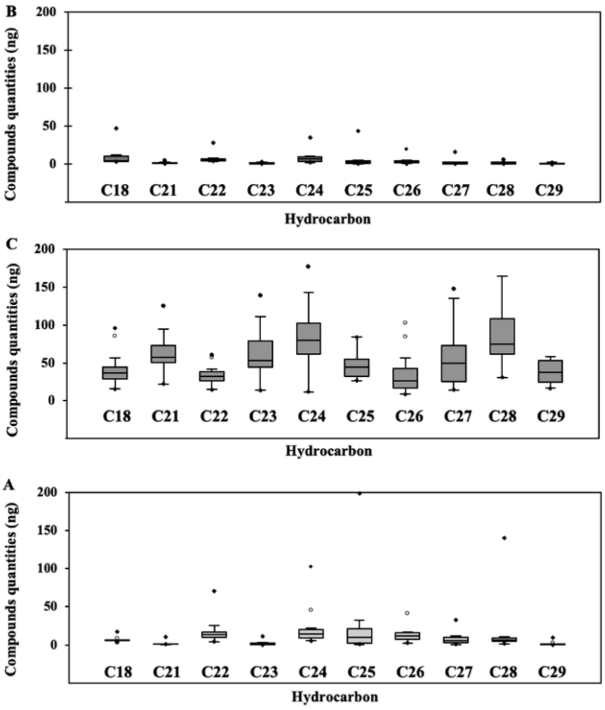

Fig. 2. The effect of spinosad (20 µg/l) and *Btk* (23 mg/l) on the cuticular hydrocarbons of *Culex pipiens* males (A:

number of hatching eggs with a rate of 90 %, while the treated females with spinosad and *Btk* were 55 % and 68 %, respectively (Table 3).

Effect on the cuticular hydrocarbon profile of *C. pipiens* adults

In males (Fig. 2), the treatment with a sub-lethal concentration of spinosad and *Btk* affects the cuticular profiles. From the 10 compounds analysed, a very strong decrease in the total quantity of hydrocarbons was recorded in males treated with *Btk*. The total loss of hydrocarbons represents almost 59 % of the total quantity of the various compounds found in the controls (Fig. 2B). However, for the males treated with spinosad, there was an over-activation of the biosynthesis of all the hydrocarbons. The percentage gain in cuticular hydrocarbons was close to 36 % (Fig. 2C).

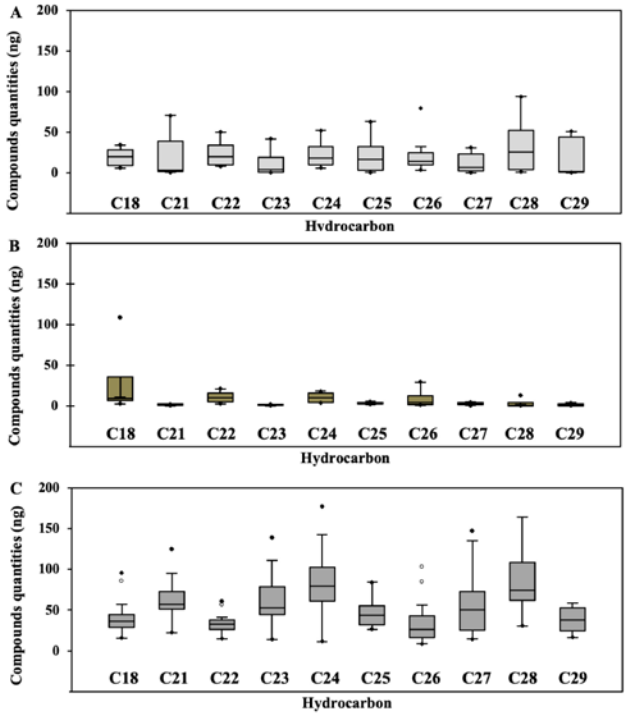

Fig. 3. The effect of spinosad (20 µg/l) and *Btk* (23 mg/l) on the cuticular hydrocarbons of *Culex pipiens* females (A: Controls; B: *Btk*; C: Spinosad).

In females, the treatment of the females with *Btk* and spinosad acts on the cuticular profile as on the males (Fig. 3). A decrease in the amounts of cuticular hydrocarbons was recorded in females treated with *Btk*. The percentage of the total loss of hydrocarbons in females was 64 % (Fig. 3B). The results obtained from the females are similar to those of the males treated with spinosad. The treated females showed an increase in the amounts of cuticular hydrocarbons in comparison to the controls (Fig. 3C). The gain in cuticular hydrocarbons in females (42 %) was slightly greater than in males (Fig. 3C).

The results presented in Table 4 show the differences between the quantities of cuticular hydrocarbons in the males and the females treated with the two products *Btk* and spinosad. The adults of *C. pipiens* exposed to *Btk* had quantities of carbon, which were not significantly different between the two sexes, with the exception of carbon C21 and C26 where significant differences were recorded between the quantity rates (*P*: 0.034*) (Table. 4). Same results were recorded for adults treated with spinosad. According to the static analysis, there were significant differences only between the carbon levels recorded in C22, C29 and C30 with sig-

Table 3. The effect of *Btk* and spinosad on fecundity and fertility of *Culex pipiens* females. **Legend:** Cont: The control females; A: females treated with Sp; B: females treated with *Btk*.; Mean ± SD: mean ± standard deviation; Min: Minimum; Max: Maximum).

		Cont	A	Cont	B
Number of eggs	**Mean ± SD**	45.20 ± 9.23	37.70 ± 27.52	46.80 ± 8.43	33.20 ± 5.116
	Min	33	0	33	24
	Max	59	75	59	42
	Tobs	0.82		2.71	
	p	0.43		0.153	
Hatching time	**Mean ± SD**	2.80 ± 0.42	1.90 ± 1.66	2.9 ± 0.57	2.30 ± 0.48
	Min	2	0	2	2
	Max	3	4	4	3
Hatching rate %	**Mean**	91.78	55.25	90.37	68.85
	Min	87.03	0	80	34.28
	Max	98.21	98.07	98.21	91.42

Table 4. Comparison between cuticular hydrocarbon.s of females and males treated with *Btk* and spinosad. Legend: C, rarbon; *Significance.

	Btk				**Spinosad**			
C	**Females (Mean)**	**Males (Mean)**	**F (Observed)**	***p*-value**	**Females (Mean)**	**Males (Mean)**	**F (Observed)**	***p*-value**
C21	32.3775	6.1911	7.4340	0.0343*	28.6181	27.7752	1.8509	0.1952
C22	1.3476	1.7407	1.7503	0.2340	49.9808	81.1318	5.5063	0.0342*
C23	10.9013	10.6165	0.2808	0.6152	29.7223	33.2621	3.5922	0.0789
C24	1.0353	1.2440	0.4523	0.5263	60.8196	30.0052	2.4307	0.1413
C25	10.4661	12.7049	1.6228	0.2498	66.3188	76.1589	2.1574	0.1640
C26	3.4777	12.3485	7.4268	0.0344*	43.4731	23.4556	1.8170	0.1991
C27	9.6374	6.9457	0.7224	0.4280	33.2556	15.0666	0.4472	0.5146
C28	2.9217	1.3301	1.2378	0.3085	50.1981	72.3907	1.0577	0.3212
C29	3.7442	2.4639	2.4638	0.1675	77.9440	159.6373	19.1361	0.0006*
C30	1.4828	1.1002	0.3960	0.5524	31.9365	52.0575	5.6799	0.0319*

Controls; B: *Btk*; C: Spinosad).

nificances *P*: 0.034*, *P*: 0.0006* and *P:* 0.031*, respectively (Table 4).

Discussion

The mosquitoes treated with pathogenic bacteria show a prolonged development and a reduction in the body size of larvae, fecundity, number of gonotrophic cycles and adult longevity (Marina et al. 2003). In our study, treatment with low concentrations of spinosad (20 µg/l) caused a disturbance in fecundity and fertility in adults. The reduction in fecundity and hatching rate in treated females would be under the properties of spinosad, which specifically affects the function of γ-Aminobutyric acid (GABA) and nicotinic acetylcholine receptors of target insects (Salgado 1998). Other results demonstrated that, at sub-lethal doses, spinosad affected the brain of *Oreochromis niloticus* (see Piner & Üner 2014).

Our results have shown that the use of low dose *Btk* (25mg/l) against the mosquitoes studied can cause a decrease in fertility and fecundity. Similar results were reported in *Drosophila melanogaster* – *Btk* acted strongly on the development of *D. melanogaster* larvae, which influenced the number of pupae, blockage of the imaginal moults and malformations of the wings of adults (Habbachi et al. 2014). The same effect was recorded when using chitin synthesis inhibitors against *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* eggs (Vasuki 1990). The results were also supported by tests of azadirachtin, diflubenzuron and pyriproxyfen on eggs of *Aedes albopictus*, *A. aegypti*, *A. atropalpus* and *C. pipiens* (Suman et al. 2013).

Amira (2014) demonstrated that Halofenozide (RH-0345) affects *C. pipiens* fertility. Other results showed that when the toxin was ingested to the desert locust (*Schistocerca gregaria*) at the start of the larval stage, the slowing down of development occurred from this stage and the delay persisted longer as the quantities of the toxin ingested were bigger (Benhissen 2016).

As in many insects, cuticular waxes protecting the individual from external environment (temperature, humidity) contain many compounds with long carbon chains: cuticular hydrocarbons (HCs); their functions are to prevent desiccation (Urbanski et al. 2010) and to contribute to chemical communication (Howard & Blomquist 2005). It is also suggested that hydrocarbons are not merely passive protectors but play a major role in mate recognition and selection as well as in population divergence (Anyanwu et al. 2000). In some insects, part of male recognition is related to the detection of specific hydrocarbon compounds and other components of the cuticular lipid layer (Peshcke 1987).

The chemical characterization of HCs from adults of *C. pipiens* indicated the presence of n-alkanes belonging to a chain of 21 to 30 carbons in length. Similar HCs have been determined in adults of *A. aegypti* (Horne & Priestman 2002), *A. albopictus* (Kruger & Pappas 1993), *C. quinquefasciatus* (Chen et al. 1990) and *Anopheles gambiae* (Caputo et al. 2007). The characterisation of HCs from *A. aegypti* revealed the presence of 78 compounds and only 42 of them have been identified (Horne & Priestman 2002). HCs have also been studied in species of Orthoptera (Thomas & Simmons 2009, Bounechada et al. 2011), beetles (Golebiowski et al. 2008), Hymenoptera (Choe & Ramirez 2012) and Diptera (Claude 2007).

The use of Spinosad at a low dose (20 µg/l) against the mosquitoes revealed that this toxin caused a strong increase in the quantities of all the hydrocarbons. Thus, the quantity of HCs increased from 15 µg to appr. 159 µg in males and from 33 to 146 µg in females. Furthermore, a previous study showed that, in the larvae of *C. pipiens,* RH-0345 caused an increase in HCs of treated larvae compared to control larvae (Amira 2014). Similar results were reported by Habbachi (2013) who showed the quantitative reduction of cuticular hydrocarbons of *Bllatella germanica* adults during the application of low doses of spinosad.

The use of Btk (23mg/l) caused disturbance of the cuticular profile. Lereclus & Chaufaux (1986) showed that the crystals of the bacterium ingested by insect larvae were rapidly hydrolysed and the toxin then caused paralysis of the digestive tract, thus the insects died of toxemia or septicemia in the days following the treatment. Habbachi (2013) also demonstrated the effect of Btk on the cuticular hydrocarbons on adults of *B. germanica*.

In this study, the results showed only quantitative differences in the hydrocarbon profiles of *C. pipiens* treated adults. A significant increase in the amounts of HCs was found in adults under the effect of Spinosad for both sexes, whereas *Btk* causes significant decrease in HCs for treated individuals.

The quantitative differences observed between the HCs of treated adults and the control series reflect some modifications in the biosynthesis of hydrocarbons (Mullen et al. 2008). Straight-chain alkanes and n-alkenes are formed through the elongation of fatty acyl-CoAs, which are converted by decarboxylation to hydrocarbons, which are with one carbon less in the chain (Howard & Blomquist 2005). Moreover, the rate of HCs changes with geographic, climatic, environmental and genetic variations (Khidr et al. 2013), the presence of endoparasites (Trabalon et al. 2000), ovarian activity or development (Izzo et al. 2010) and reproduction (Foitzik et al. 2011).

Conclusion

The effects of sublethal doses of a bioinsecticide are important, especially those disrupting traits related to insect reproduction such as mate-finding, sex ratio and egg fertility. Under laboratory conditions, we demonstrated that when spinosad and *Btk* were ingested by the larvae, this causes disturbances in the fecundity and fertility of adults resulting from the treated larvae. Additionally, the use of sublethal doses of Spinosad induced an increase in cuticular hydrocarbons of treated adults. At the same time, the sublethal dose of *Btk* causes a quantitative reduction in the levels of cuticular hydrocarbons. This study demonstrated that Spinosad and *Btk* are promising as larvicides against *Culex pipiens* and they could be alternatives to chemical pesticides because preserving human health and the environment.

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Received: 20.04.2022
Accepted: 25.11.2022