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FACTORS GOVERNING MOSQUITO SUSCEPTIBILITY TO *BACILLUS SPHAERICUS* STRAINS

By

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العوامل المؤثرة على حساسية البعوض لسلالات البكتريا من نوع باسيلس سفيريكاس

ناديـــة محمـــد لطفي و عادل إبراهيم مردان
هاشم علي عبد الرحمن و نعيمة عبد الرؤوف عبد الرازق

تم اختيار حساسية يرقات أربعة أنـواع من البعوض لثـلاث سلالات من **الباسيلس سفيريكاس** (١٥٩٣ ـ ٤ ، ٢٢٩٧ ، إس . إس ١١) .

وقد دلت النتائج على أن يرقات **الكيولكس بيبتر** كانت أكثرهم حساسية للإصابة بالسلالات الثلاثة يليه كل من **كيوليسيتا لنجريولاتا وأنوفيليس سيرجنتي** وأخيراً **الأييدس كاسبيس** حيث اتضح أن هذا النوع هو أكثر أنواع البعوض مقاومة للإصابة بهذا البكتريم واتضح أيضاً أن مقاومة الإصابة تزداد بازدياد العمر اليرقي للبعوض .

كما وصلت درجة الكفاءة البكتيرية لإحداث الموت لليرقات أقصاها بعد ٤٨ ساعة من نمو البكتريا .

وقد تم مقارنة كفاءة قتل اليرقات لسلالتين من **الباسيلس سفيريكاس** (١٥٩٣ع) ، (٢٢٩٧) باستخدام سلالة (١٥٩٣ ـ ع) وسلالة زرعتا على بعض المنبتات المخلقة صناعياً على أساس قيمة التركيز المحدث للإصابة ٥٠ ، ٩٠٪ للطور اليرقي الثالث (كيولكس ببيتر) فوجد أن إنماء البكتريا على وسط النمو الخاص بالتريبتوز وخلاصة الخميرة قد أدى إلى أعلى معدل إصابة بين اليرقات يليه منبت التريتوز صويا ثم الشوربة المغذية وأخيراً منبت الكازين هيدروليزات .

هذا وقد وجد أن نسبة وفاة اليرقات المعرضة لتركيزات عالية من البكتيريا لعدة ساعات قد تساوت مع تلك التي استمر تعرضها حتى ٤٨ ساعة .

Key Words: Bacillus sphaericus, Bacillus toxins, Entomopathogen, larvicides, Mosquito, Control, *Culex pipiens, Anopheles spp.*

ABSTRACT

Three lyophilized strains of *Bacillus sphaericus* (1593-4, 2297 and SSII) were tested against four different mosquito species. *Culex pipiens* larvae demonstrated the highest level of susceptibility towards the 3 *B. sphaericus* strains followed by *Culiseta longiareoleta, Anopheles sergentii* and then *Aedes caspius.* Tolerance of *C. pipiens* larvae to infection by *B. sphaericus* 1593-4 increased with the increase of larval age. The larvicidal activity increased with the increase of bacterial growth age till 48 hours when it reached complete sporulation. *B. sphaericus* strains 1593-4 and 2297 cultured on tryptose yeast extract medium produced the highest mortality of 3rd instar larvae of *Culex pipiens,* followed by tryptose soy broth media, nutrient broth media and then casien hydrolysate media. Larvae treated with suspensions of bacteria at high concentrations for few hours gave similar mortality levels as when treated for 48 hours.

INTRODUCTION

Among the most promising biological agents for mosquito control is the bacterium *Bacillus sphaericus* Neide. (Mulligan *et al,* 1978) reported excellent strain 1593-4 in small scale field trials, although activity was reduced in polluted water source (commercial dairy sump). The Biological activity of *B. sphaericus* strains was reported to be influenced by many factors some

of which are growth of the pathogen, microbial flora and nutients in mosquito breading sources (Goldberg *et al*, 1974, 1977; Romoska and Pacy, 1979). Therefore, many advances have been made in isolating and producing several strains of *B. sphaericus* with a wide spectrum of activity against larvae of several mosquito species as tested by various workers (Lacey and Singer, 1982; and Wraight *et al*, 1981a). Who tested *B. sphaericus* strain 1593 power (MV-716) and determined the LC95 against 4th instar larvae of *Culex pipiens* Linn. and *C. salinarius* Coquillett to be about 0.2 mg/L. It was also found that older instart larvae were less susceptible and maximum mortality was obtained in 48 hours of exposure. Cheong and Yap, (1985) reported that *B. sphaericus* 1953 had shown a good potentiality as a biocontrol agent for four different mosquito species in Malaysia.

The objective of the present experiments was to evaluate the potential efficiency of certain *B. sphaericus* strains by defining their pathogenicity against certain mosquito species.

MATERIALS AND METHODS

Three lyophilized *B. sphaericus* strains were bioassayed against 3rd instar larvae of four different mosquito species: *Culex pipiens*, *Anopheles sergentii*, *Aedes caspius* and *Culiseta longiareolata*. The tested *B. sphaericus* strains were 1593-4 (RB80-Institute-Pasteur), 2297 (SPH-84-Institute-Pasteur) and SS-II (Dulmage-Brownsville Texas, USA). Individual larvae were treated with a fixed dose of each strain. Each treatment had 3 replicates (20 larvae/replicate) and each experiment was repeated three times. Mortality was recorded after 48 hours from treatment. The pathogensis of bacterial mosquito species pathogen, *Bacillus sphaericus* was investigated through a series of experiments as follows:

1. Larval age

Culex pipiens was chosen as a test species due to its high susceptibility to the selected *B. sphaericus* strains. Different larval instars of *Culex pipiens* were treated with different *B. sphaericus* strains. This was done with laboratory reared *C. pipiens* larvae. The assays were conducted according to the method described by Goldberg *et al*, (1974).

2. Bacterial growth age

The development of toxic ingradients of *B. sphaericus* strains as a mosquito larval pathogen in correlated with the growth age as in almost all pathogenic bacteria. The two bacterial strains (1593-4 and 2297) were grown in nutrient yeast extract broth for different incubation periods, (2, 6, 24, 48 and 72 hours) at 27°C. Bacteria at each growth age were bioassayed against 3rd instar larvae of *C. pipiens* at a concentration of 0.01 mg/litre.

3. Cell-spore mixture and spores alone

Toxicity of spores alone and a mixture of spores and vegetative cells was compared, third instar larvae of *C. pipiens* were treated with a bacterial suspension after being boiled for 30 min. (to kill the vegetative cells). Others were treated with bacterial suspension without boiling. The latter suspension contained a mixture of spores and vegetative cells. Mortality readings were recorded 24, 48 and 72 hours after treatment.

4. Nutritive media

To evaluate the influence of nutritional elements on the toxicity of *B. sphaericus*, the two *B. sphaericus* strains (1593-4 and 2279) were grown on 4 different media and then bioassayed using 3rd instar larvae of *C. pipiens*. The four tested media were (Tryptose soy broth + agar); (Trypose = yeast extract + agar); (Casein hydrolysate + yeast extract + agar); and (Nutrient agar). These media were prepared according to the method of Kalfon *et al*, (1983).

5. Exposure period

Third Instar larvae of *C. pipiens* were treated with bacterial suspension of *B. sphaericus* 1593-4 after different exposure periods (1, 2, 4, 6, 24 and 48 hours) inorder to evaluate the influence of the exposure period on larval mortality. Different numbers of larvae were bioassayed as previously described using four bacterial concentrations (0.1, 0.5, 0.01 and 0.05 mg/L.). At each exposure period the tested larvae were exposed to the bacterial suspension for each period of time and were then transferred to distilled water after being washed with water. Mortality readings were taken after 48 hours.

All the experiments were carried out under static conditions. The average percentage mortality was corrected when mortality among the check group exceeded 5% using Abbott's formula.

RESULTS

The obtained results (Fig. 1) showed that *Culex pipiens* larvae were highly susceptible to the three tested bacterial strains as compared with the larvae of the other three tested mosquito species. *Culiseta longiareolata* come next to *Culex pipiens* followed by *Anopheles sergentii* while *Aedes caspius* was the least affected species. Regarding the virulence of the three bacterial strains no difference was detected between strains 1593-4 and 2297 concerning their effect on each tested mosquito species. Strain SSII-1, on the other hand, included significantly less mortality than both 1593-4 and 2297 to all species. There was no significant difference in the susceptibility of the 1st and 2nd instars or the 3rd and 4th instars. Similar results also occurred in casses of *B. sphaericus* strains 2297 also it was less effective than strain 1593-4 on the different larval instars of *C. pipien*. The pathogenicity of the tested *B. sphaericus* strains (1593-4 and 2297) was evaluated at different bacterial growth ages by exposing 3rd instar larvae of *C. pipiens* to a fixed concentration (0.1 mg/L.) at each bacterial growth age.

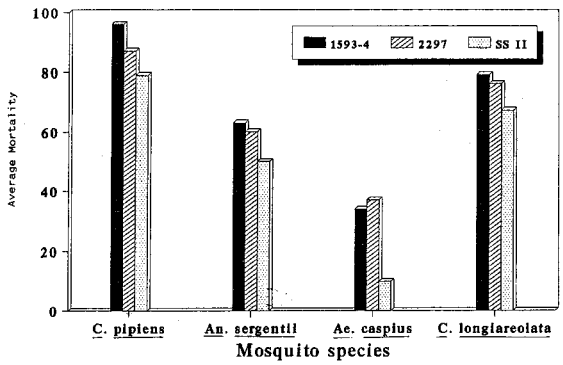

Fig. 1: Comparative mortality of 3rd instar larvae of 4 mosquito species exposed to 3 strains of *B. sphaericus* (conc. 1 mg/L) for 48 hours.

Results of testing the age factor in relation to the larvicidal action of *B. sphaericus* (1593-4) showed a significant relationship. The response of age reached its maximum with 1st and 2nd instarts of the tested larval, while the 3rd and 4th ones were less responded. According to the data presented in (Table 1) it could be pointed out that there was a progressive increase in the mortality as the culture age increase in both tested bacterial strains up to 48 hours. The highest mortality was reached after complete sporulation, in the case of *B. sphaericus* strain 1593.

Table 1

Toxicity of different growth ages of *B. sphaericus* (strains 1593-4 and 2297) (0.1 mg/L-lyophilized material) against 3rd instar larvae of *C. pipiens* after 48 hours of exposure.

Culture age in hours	Larval Mortality									
	Strain 1593-4					Strain 2297				
	R1	R2	R3	M%	Check	R1	R2	R3	M%	Check
2	15	12	13	66.66	0	10	9	11	50.00	1
6	13	14	16	71.66	0	12	15	13	75.00	1
24	20	16	18	90.00	1	18	16	18	86.66	0
48	19	20	20	98.33	1	18	20	18	93.00	0
72	20	20	18	96.66	0	20	18	20	96.66	1

Results obtained from testing the infectivity of cell-spore mixture and spores alone to *C. pipiens* larvae were presented in (Table 2). It was clear that larvae treated with a mixture of vegetative cells and spores showed higher and more rapid mortality than those treated with spores alone. This was evident in all tested strains. Strain 1593-4 induced the highest and relatively short lethal period, followed by strain 2297 and lastly SSII.

Table 2

Infectivity of 3 strains of *Bacillus sphaericus* cells-spores mixture and spores alone at (conc. 0.1 m/l) using 3rd instar larvae of *Culex pipiens.*

B. sphaericus Strains	Average mortality % of Larvae					
	Normal culture			Heated culture		
	24hrs.	48hrs.	72hrs.	24hrs.	48hrs.	72hrs.
1593-4	100.00	—	—	21.66	48.00	100.00
2297	92.33	100.00	—	18.00	42.66	65.33
SSII	71.66	100.00	—	9.66	32.66	73.33
Check	0.00	1.66	3.33	0.00	0.00	0.00

To evaluate the effect of nutritional requirements on sporulation and toxic action of *B. sphaericus* strains (1593-4 and 2297), the bacterium was cultivated on the different nutritive media; tryptose soy broth (A), tryptose yeast extract (B), casein hydrolysate (C), and nutrient broth media (D).

The obtained results (Table 3) indicated that both *B. sphaericus* strains were highly affected by the type of media on which they were cultivated concerning their toxicity to *C. pipiens* larvae. On the bases of L_{C50} and L_{C90} values, strain 1593-4 was found to gave higher mortality levels than strain 2297. Medium (B) was found to stimulate both bacterial strains for inducing high mortality. Thus, when *B. sphaericus* strain 1593-4 was grown on this medium, it showed the highest toxicity to *C. pipiens* larvae. Both bacterial strains gave the minimum lethal activity when grown on casein hydrolysate medium (C).

Results on testing the pathogenicity of *B. sphaericus* (1593-4) on *C. pipiens* larvae exposed for different periods ranging from 1 hour to 48 hours were presented in (Table 4). It was found that under all exposure periods, mortality was decreased with the decrease of bacterial concentration. At the same time short exposure periods were needed to cause high mortalities at the higher concentrations. Thus, one hour exposure period was found enough to induct 100% mortality at the highest concentration (1.0 mg/L.). In this case, exposure periods up to 48 hours gave almost the same mortality as did one hour exposure period. At relatively low bacterial concentrations, larval mortality was increased with the increase in the exposure period from one hour up to 4 hours. It was dereased at 6 hours exposure period and increased again with the increase of the exposure period up to 48 hours.

Table 3
Summary of the susceptibility of 3rd instar larvae of *C. pipiens* to strains (1593-4 and 2297) of *B. sphaericus* grown on different cultural media.

Medium Type of media	*B. sphaericus* strain 1593-4		*Bacillus sphaericus* strain 2297	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Tryptose soy broth (A)	2.3×10^4	4×10^6	6×10^5	3×10^8
Tryptose yeast extract (B)	7×10^3	1.5×10^8	1×10^5	3.5×10^8
Casein hydrolysate (C)	5×10^6	6×10^8	2×10^7	5×10^9
Nutrient broth (D)	5×10^4	2.1×10^7	8×10^4	9.5×10^7

Table 4
Mortality of 3rd instar larvae of *C. pipiens* after exposure for different periods to different concentrations of *B. sphaericus* (strain 1593-4).

B. sphaericus concentrations mg/L	Mortality of larvae 48 hours following exposure periods					
	1 hr.	2 hrs.	4 hrs.	6 hrs.	24 hrs.	48 hrs.
1.0	100.00	100.00	96.00	97.30	100.00	100.00
0.5	73.00	82.00	92.00	86.60	89.00	91.00
0.1	27.00	37.00	62.00	49.30	61.00	58.00
0.05	08.16	11.00	34.00	34.00	12.00	21.00
Check	02.00	03.00	02.00	00.00	03.00	00.00

DISCUSSION

The pathogenicity of *B. sphaericus* to mosquito larvae has been studied by testing the susceptibility of the larvae to three different strains of the bacterium. The susceptibility of larvae has been governed by many factors, which mostly related to both the mosquito species and the bacterial strain. Accordingly, the pathogenicity of *B. sphaericus* was estimated by screaning the susceptibility of different mosquito species to different bacterial strains. Results of the experiments indicated high and variable susceptibility levels of the larvae of *C. pipiens, Anopheles sergintii,* and *Culiseta longiareolata.* Larvae of *Ades caspius,* on the other hand, were less susceptible towards all the three tested bacterial strains. This difference in susceptibility has also been reported by Goldberg *et al,* (1974); Ramoska *et al,* (1977) and Balarama, (1980). The susceptibility of insect species than another is probably due to some genetic factors influencing the metabolic process of each species or physiological characteristics such as the pH of gut. These assumptions need further investigations.

The virulence of the three bacterial strains is also different. Strain 1593-4 proved to be the most pathogenic one when bioassayed against the 4 tested mosquito species followed by strain 2297 and then strain SSII-I. This observation leads to the idea that bacterial strains of *B. sphaericus* as well as mosquito species appear to be among the main factors for evaluating the pathogenicity of this bacterium. Based on the for mentioned susceptibility tests it becomes clear that larvae of *C. pipiens* can be considered as a model insect host for studying the pathogenicity of *B. sphaericus.* Results of susceptibility experiments of different larval instars clear out high susceptibility level of younger instars than older ones. The sequence of larval mortality was similar when both bacterial strains (1593-4 and 2297) were tested. The high susceptibility observed among young instars may be attributed to their low resistance to the toxic action of the bacterium. This observation can be explained by the difference in the physiological characters between early and late instars due to the development and/or maturation of certain glandular cells, and in late instars, the secreation of certain enzymes due to which tolerance to the bacterial infection may develop (Goldberg *et al,* 1974; Ramoska *et al,* 1977 and Mulla *et al,* 1984). Pathogensis of *B. sphaericus* on mosquito larvae has been known to be mainly dependant on the idea that the multiplication of the bacterium inside the larval gut is essential in causing toxicity which reaches its maximum during sporulation. Our results agree to these findings. Bacterial suspensions prepared from 2-6 hours old cultures (vegetative cells + spores) were found to be relatively less toxic to 3rd instar larvae than older ones (spores) 24-72 hours old cultures. This observation may be explained by that maximum release of toxins which are secreated during

vegetative growth and multiplication is correlated with sporulation. This has been supported by results of another experiments carried out during this study in which whole a culture, i.e. contains spores and vegetative cells, was tested for larval toxicity before and after being boiled for 20 minutes: thus representing spores-cells mixture induced 100% mortality within 24 hours while the larvae exposed to spore suspension reached the same mortalities after 72 hours. These results indicated that spores do not reduce mortality themselves but need to germinate inside larval gut, propagate and develop vegetative cells which consequently induce larval mortality. These findings are also supported by Davidson *et al*, (1975).

The correlation between sporulation and toxicity to mosquito larvae as demonstrated by bioassaying the two *B. sphaericus* strains, after being grown on the 4 different media, clears out a direct relationship between them. The results indicated a relatively high mortality among tested larvae when exposed to both strains cultivated on tryptose yeast extract medium. However, *B. sphaericus* strain 2297 induced a relatively high mortality when grown on tryptose soy broth and nutrient broth media. This may be attributed to the presence of parasporal crystal-like structure nominated as alfa endotoxin by De Barjac and Charles, (1983) and which increases the pathogenicity level of the bacterium. Such parasposral structure develops during sporulation and exist in the sporangium.

Results of exposing 3rd instar larvae of *C. pipiens* to *B. sphaericus* for different time periods ranging from 1-48 hours indicate that, the period of larval exposure to the bacterial suspensions has no significant effect on pathogenicity. This observation may be explained by that inoculum ingested by each larva soon propagates inside the gut and hence induces toxicity leading to larval mortality. The inoculum is not correlated to the toxic units released during its propagation. Accordingly, the surrounding medium of bacterial suspension is not considered toxic by itself, but a source of toxicity after being ingested. Hence the amount of bacteria taken by larvae during the first hour is responsible for the induction of larval mortality through its multiplication inside the gut. Based on the pathogenicity of *B. sphaericus* is governed by many factors which are mostly related to both mosquito species and bacterial strains.

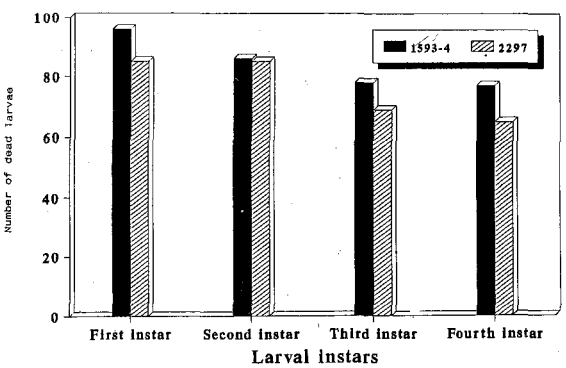

Fig. 2: Mortality of different larval instars of *C. pipiens* by *B. sphaericus* strain 1593-4 and 2297 (conc. 1 mg/L) for 48 hours.

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