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Toxicity of Local *Bacillus thuringiensis* Isolates Against *Drosophila melanogaster*

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Abstract: A survey for *Bacillus thuringiensis* was conducted in 12 different local Jordanian habitats. A total of 80 isolates were identified as *B. thuringiensis* and were found to produce 3 different classes of parasporal crystal inclusions; spherical, bipyramidal and bipyramidal and cuboidal parasporal crystals. Spherical inclusions were the most common crystals. *B. thuringiensis* was more diverse in olive pomace, grain dusts and soil habitats that mixed with animal manure than other habitats. The 50% lethal concentration (LC_{50}) of the bacterial suspensions (spores and toxin) to *Drosophila melanogaster* larvae and adults varied from 4.60 to 8.65 and from 7.10 to 43.24, respectively. Of the 80 tested *B. thuringiensis* isolates, 24 were exhibited dual toxicity against both larvae and adults of *D. melanogaster*. *D. melanogaste* female adults were found to be more susceptible to *B. thuringiensis* parasporal crystals than male adults.

Key words: *Bacillus thuringiensis* · *Drosophila melanogaster* · spherical · bipyramid · lethal concentration

INTRODUCTION

The preference of using biological pesticides over chemical pesticides has been widely accepted in different parts of the world for many reasons. Unlike chemical pesticides, biological pesticides are more safe agents, because they are degradable and have a high level of safety for non-target organisms (humans, animals and fish) in addition to their host specificity [1, 2]. Another important advantage of the biological pesticides is their lower resistance in the target pest populations [1]. Biological pesticides are becoming important factor in crop and forest protection and in insect vector control. These pesticides are natural, disease-causing microorganisms such as viruses, bacteria, fungi and portozoans that infect or intoxicate specific pest groups [1, 3].

Different Bacillus species have been isolated and commonly recognized as definite insect pathogen [4]. The most recognised species are; *B. popilliae*, *B. lentimorbus*, *B. larvae*, *B. thuringiensis* and certain strains of *B. sphaericus*. The greatest successes in microbial pesticides have come from uses of *B. thuringiensis* strains such as B. t. israelensis and B. t. kurstaki which are toxic against Diptera and Lepidoptera, respectively [3].

B. thuringiensis is Gram-positive, spore forming and rod-shaped bacterium that has an ability to produce, during sporulation, parasporal crystal inclusions of insecticidal proteins (known as Cry proteins or δ-endotoxins) [5, 6].

A large number of *B. thuringiensis* isolates are screened annually to identify new strains with increased levels of insecticidal activity and a broader spectrum of insect pests [7]. Many *B. thuringiensis* isolates are recovered from numerous sources, including; soils, grain dust, diseased insect larvae and sericulture environments. Soil has been the principal source of novel *B. thuringiensis* isolates [8, 9]. Identified strains of *B. thuringiensis* from soil samples, plant surfaces, dead insects and stored grains showed a wide range of specificity against different insect orders (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera or Mallophaga and Acari) and other invertebrates (Nemathelminthes, Platyhelminthes and Sarcomastigorphora) [10, 11].

The main objectives of this study are to study the abundance and distribution of *B. thuringiensis* isolates in different habitats of Jordan and to determine their toxicities against *Drosophila melanogaster*.

MATERIALS AND METHODS

Sample collection: A total of 40 different samples were collected from 12 different Jordanian habitats,

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Table 1: Recovery of *B. thuringiensis* isolates from different Jordanian habitats and determination of their crystal morphology

Habitat	Sample No.	No. of different *B. thuringiensis* colonies	Crystal morphology[a]
Olive-cultivated soil	1	1	1S
	2	1	1S
	3	2	1S, 1C+BP
	4	1	1S
Olive pomace	5	3	3S
	6	2	2S
	7	2	1S, 1C+BP
	8	3	3S
	9	2	1S, 1C+BP
Grain dusts	10	1	1BP
	11	5	4S, 1C+BP
	12	1	1C+BP
	13	4	2S, 1BP,1C+BP
	14	1	1S
Animal manure mixed soil	15	1	1S
	16	2	1S, 1C+BP
	17	4	1S, 2BP, 1C+BP
	18	4	3S, 1C+BP
Decomposed animal mixed soil	19	4	1S, 3C+BP
	20	3	3S
Bee wax Car station soil	21	3	3S
	22	2	1S, 1C+BP
	23	0	-
	24	1	1S
Slaughter houses soil	25	4	3S, 1BP
	26	4	3S, 1BP
	27	1	1S
	28	2	1S, 1BP
Waste collection pond	29	2	2BP
	30	2	2S
Waste water treatment mixed soil	31	1	1BP
	32	1	1S
	33	1	1S
	34	1	1S
Dam sediment soil	35	2	2S
	36	3	3S
Industrial-byproducts mixed soil	37	1	1C+BP
	38	1	1S
	39	1	1S
	40	0	-
Total	40	80	56S, 10BP, 14C+BP

(a); S: Spherical, BP: Bipyramid, C+BP: Cuboid and Bipyramid

including; agricultural lands mainly planted with olive trees (4 samples), olive pomace (5 samples), infected stock foods (5 samples of grain dusts), animal manure-mixed soils (4 samples), decomposed animal bodies-mixed soilss (2 samples), infected bee wax (1 sample), oil-change-car stations (3 soil samples), slaughter houses (4 soil samples), waste collection ponds (2 soil samples), waste water treatment plants (4 soil samples), dam sediment (2 samples) and industrial-byproducts (4 liquid samples) (Table 1). Soil samples were collected by scraping off surface material with a spatula then ~50 g were obtained at 2 to 5 cm below the surface. Samples were placed in plastic bags, transferred to the lab and stored at 4°C.

Isolation of bacilli containing crystalline structure: The isolation was performed according to Ohba and Aizawa [12] and Meadows [13]. In brief, 1 g of each solid sample or 1 ml of each liquid sample was suspended in 10 ml of

sterile distilled water in 20 ml glass-tube and the preparations were mixed vigorously by vortexing for 1 minute. After mixing, the solid matter was allowed to settle out for 2 minutes, then 1 ml of the supernatant was pasteurized at 80°C for 30 minutes in water bath. Aliquots (100 μl) of each tube was plated on nutrient agar plates and incubated at 30°C for 24 h.

Isolation of *B. thuringiensis*: The method described by Travers [14] was followed. Each of the above pasturized suspensoion (900 μl each) was added to 10 ml of LB borth (10 g tryptone, 5 g yeast exrtact, 5 g NaCl, 1 ml 1 N NaOH, buffered with 0.25 M sodium acetate, pH 6.8) in a 125 ml flask. The broth was incubated for 4 h at 30°C with shaking at 200 rpm. Aliquot of 1 ml was then heated to 80°C for 3 min, then undiluted aliquots (100 μl) were plated on modified nutrient agar (20 g nutrient agar and 8 g of tryptose agar). Plates were incubated for 48 h at 30°C. The formed colonies were purified by subculturing on modified nutrient agar plates and checked for crystal production after 48 h of incubation at 30°C. Each formed colony was screened for the presence of endospores and parasporal bodies by staining bacterial smears with carbol fuchsin stain [6] and then examined under light microscope to observe the morphology of the parasporal bodies.

Growth of isolates for bioassay: Isolates with parasporal bodies were picked onto 10 ml of T3 medium (0.3% tryptone, 0.2% tryptose, 0.15% yeast exrtact, 0.0005% $MnCl_2$ and 0.05 M sodium phosphate (pH 6.8)) in 250 ml Erlenmyer flasks and incubated for 7 days at 30°C with shaking at 250 rpm [13, 14]. To remove exotoxin, broths were poured into 10 ml tubes and centrifuged at 5000 rpm for 15 min. Pellets (spores and crystals) were washed three times in sterile distilled water (5000 rmp, 5 minutes each), then the pellets were resuspended in 3 ml of sterile distilled water [15].

Bioassay for *Drosophila melanogaster* larvae: One gram of artificial fruit fly's diet was homogenized in 10 ml of sterile distilled water in an electrical blinder. The toxin-spore suspension was diluted (one fold serial dilution; 10^{-1}) in sterile distilled water. After 8 days of *D. melanogaster* fly's incubation, the adult flies were cleared and the diet containing larvae was floated up by tap water, then floated larvae were collected in sterile petri dishes. According to Karamanlidou [16], 10 third instar larvae were placed into each well of 24-well plates [Corning Laboratory Science Company, USA]; 0.3 ml of the diet homogenate and 0.7 ml of *B. thuringiensis* toxin-spore suspension were added to each well. The toxicity of each isolate was assayed in duplicate. Plates were incubated at 25°C for 24 h then mortality was scored in comparison to parallel control. The mortality was observed under disecting microscope at 10X. The lethal concentration values of toxin-spore suspension that are able to kill 50% (LC_{50}) were determined by probit analysis using SAS software (11).

Bioassay for *Drosophila melanogaster* adults: One gram of artificial diet was placed in a plastic vial mixed with 4 ml of sterile distilled water and 1 ml of the toxin-spore suspension, 10 males or 10 juvenile females were placed in the vial. After incubation at 25°C, the mortality was examined daily for 5 days.

RESULTS

A total of 38 samples (out of 40) collected from 12 Jordanian habitats (Table 1) were found to contain isolates producing parasporal crystal inclusions with a variety of shapes and sizes. It was found that at least one sample from each habitat contained isolates with parasporal crystals. As shown in Table 1, among the growing bacterial colonies, parasporal crystals formation was evident in 80 colonies which represented a similar morphology to *B. thuringiensis*.

After screening of the isolates for the presence of parasporal crystals, they were grouped into three major classes based on crystal morphology: class 1, Spherical (S); class 2, Bipyramidal (BP); and class 3, Bipyramidal and Cuboidal (BP+C) (Table 1). The isolates that produced spherical crystals were the most common (56 isolates) with at least one isolate was detected in each sample. Only 10 isolates (10_1, 13_3, 17_1, 17_3, 25_4, 26_3, 28_2, 29_1, 29_2 and 31_1) were able to produce bipyramid crystals. The remaining 14 isolates (3_2, 7_2, 9_2, 11_5, 12_1, 13_4, 16_2, 17_4, 18_4, 19_1, 19_3, 19_4, 22_2 and 37_1) were produced both bipyramid and cuboidal crystals (Table 1).

The highest diversity of *B. thuringiensis* colonies was found in sample 11 (5 diverse colonies) from grain dusts (Table 1). Sample 23 (car station soil) and sample 40 (industrial-byproducts) showed no *B. thuringiensis* colonies (Table 1). Olive pomace, decomposed animal bodies mixed soils, animal manure mixed soils and slaughter houses soil showed an increased diversity of *B. thuringiensis* per sample (Table 1).

The toxicity of the local 80 *B. thuringiensis* isolates against *Drosophila melanogaster* (adults and larvae) was

Table 2: Toxicity of *B. thuringiensis* Isolates Against *D. melanogaster* Larvae and Adults

			Larvae		Adults		
Habitat	Isolate[a]	Crystal morphology[b]	LC_{50}[c]	$R^{2(d)}$	LC_{50}	R^2	% of Intoxicated
Olive-cultivated soil	32	BP+C	8.42	0.97	14.52	0.93	100% Female
Olive pomace	71	S	6.48	0.96	9.92	0.98	100% Female
	72	BP+C	7.48	0.98	28.30	0.90	100% Female
	82	S	5.16	0.93	7.28	0.96	100% Female
	91	S	6.41	0.91	19.98	0.92	100% Female
Grain dusts	112	S	6.50	0.90	18.54	0.97	75% Female, 25% Male
	113	S	6.49	0.91	7.12	0.93	50% Female, 50% Male
	114	S	7.22	0.97	35.26	0.90	100% Female
	121	BP+C	6.29	0.97	12.76	0.90	100% Female
	132	S	6.52	0.96	7.10	0.99	50% Female, 50% Male
Animal manure mixed soil	161	S	8.65	0.99	32.66	0.91	100% Female
	162	S	6.53	0.97	8.84	0.98	100% Female
	172	S	4.60	0.94	11.44	0.95	100% Female
	174	BP+C	6.92	0.94	8.72	0.95	50% Female, 50% Male
	184	BP+C	7.16	0.91	43.24	0.90	100% Female
Decomposed animal mixed soil	202	S	5.64	0.91	7.98	0.92	100% Female
	203	S	5.72	0.93	9.72	0.96	100% Female
Bee wax	211	S	5.82	0.93	9.82	0.96	100% Female
Car station soil	221	S	6.19	0.99	16.88	0.93	100% Female
	222	BP+C	6.97	0.98	34.48	0.91	100% Female
Slaughter houses soil	271	S	6.56	0.93	23.76	0.95	100% Male
Waste collection pond soil	302	S	5.89	0.94	8.12	0.98	50% Female, 50% Male
Waste water treatment soil	341	S	6.32	0.99	35.46	0.97	50% Female, 50% Male
Dam sediment soil	361	S	6.76	0.93	10.26	0.91	50% Female, 50% Male

(a); X_y: X; Sample no., Y; Isolate no., (b); S: Spherical, BP + C: Bipyramid and Cuboid, (b); LC_{50}: The lethal, concentration that kill 50%, (c); R^2: Correlation coefficient

examined after mixing the toxin-spore suspension doses with the diet of third-instar larvae or adults. Table 2 shows that 24 *B. thuringiensis* isolates exhibited dual toxicity against both *D. melanogaster* larvae and adults. As indicated in Table 2, at least one *B. thuringiensis* isolate from each selected habitat, except industrial-byproducts mixed soils, was toxic to *D. melanogaster* larvae and adults. Olive pomace, grain dusts and animal manure mixed soil appeared to be the major sources of *B. thuringiensis* isolates that exhibited dual toxicity against both larvae and adults (14 isolates), whereas olive cultivated soils, bee wax, slaughter houses, waste collection pond soils, waste water treatment soil and dam sediment soil had only one toxic isolate to both larvae and adults (Table 2). The most toxic isolates to *D. melanogaster* larvae and adults were 17_2 (LC_{50} = 4.60) from animal manure mixed soil and 13_2 (LC_{50} = 7.10) from grain dusts, respectively. Whereas, the least toxic isolates to larvae and adults were 16_1 (LC_{50} = 8.65) and 18_4 (LC_{50} = 43.24) from animal manure mixed soil, respectively (Table 2).

The morphology of toxic *B. thuringiensis* isolates to *D. melanogaster* larvae and adults from a single habitat was determined (Table 2). Out of the 24 isolates which were toxic to *D. melanogaster* larvae and adults, 18 isolates were able to produce spherical crystals and the remaining 6 toxic isolates (3_2, 7_2, 12_1, 17_4, 18_4 and 22_2) were able to produce both bipyramid and cubiodal crystals (Table 2). None of the isolates with bipyramidal crystals were toxic to *D. melanogaster* larvae or adults. All isolates that were recovered from olive-cultivated soils and with spherical crystals were non-toxic to *D. melanogaster* larvae and adults. However, the other isolates with the same crystal morphology and recovered from different habitats were toxic (Table 2). Results show that isolates of both bipyramidal and cuboidal crystal morphology recovered from bee wax, slaughter houses, waste collection pond, waste water treatment and dam sediment

habitats were non-toxic to *D. melanogaster* larvae and adults. Whereas, the other isolates with the same crystal morphology and recovered from the other habitats were toxic.

The bacterial suspension toxicity (spores and crystals) of each isolate was examined against male and female adults of *D. melanogaster*. Eighteen isolates producing spherical parasporal inclusions were toxic to *D. melanogaster* adults after 24 hours of incubation at 25°C (Table 2). Out of these 18 adult toxic isolates, 11 exhibited an insecticidal activity against females (3 from olive pomace, 1 from grain dusts, 3 from animal manure mixed soils, 2 from decomposed animal bodies mixed soils, 1 from infected bee-wax and 1 from car station soils), one isolate (27_1) obtained from slaughter houses soil was toxic against males (LC_{50} = 23.76) and 6 isolates (3 from grain dusts, 1 from waste collection pond soil, 1 from waste water treatment soil and 1 from dam sediment soil) were toxic to both male and female adults (Table 2). On the other hand, 5 isolates (3_2, 7_2, 12_b, 18_4 and 22_2) producing both cuboidal and bipyramidal inclusions were found to be toxic only to female adults and one isolate (17_4) was toxic against both male and female adults (Table 2). Therefore, 16 isolates were toxic to females, 7 isolates were toxic to both females and males and only 1 isolate was toxic to males.

DISCUSSION

In the present study, the occurrence of *B. thuringiensis* was investigated in 40 samples representing 12 different habitats in Jordan. It was found that *B. thuringiensis* was present in 95% of the selected samples, suggesting that *B. thuringiensis* was highly frequent in the habitats of Jordan. This is in agreement with Martin [17] finding who found that 94.3% of samples of Asian soils rich in *B. thuringiensis*. On the other hand, 63% of the tested colonies after acetate selection were able to give parasporal bodies and show similar phenotypical characteristics to *B. thuringiensis*.This result was comparable with Martin [17] finding who found that 78% of all colonies examined formed parasporal bodies. Soil samples from Asia were the most rich in *B. thuringiensis* over all locations of the world [17, 18], this is may be due to soil types or due to geographical differences

Data show that the most common Jordanian *B. thuringiensis* isolates were those that are able to produce spherical inclusions. These isolates were found in 87.5% of *B. thuringiensis* producing samples. Thus, there is a wide distribution of spherical inclusions producing *B. thuringiensis* in habitats of Jordan over the other crystal forms. Other workers [17] have isolated *B. thuringiensis* from several locations in Eastern Asia and they found that isolates with bipyramidal crystals and spherical crystals were the most common, respectively. Jordanian habitats have not been included in these studies. The differences in the parasporal crystals shape distribution might be related to the sample location and habitat that may enhance the growth of certain *B. thuringiensis* isolates that are able to produce that specific crystal morphology and might be due to genetic variation that caused by the difference in the environmental conditions.

The highest abundance and diversity of *B. thuringiensis* were found in olive pomace, grain dusts, animal manure mixed soils, decomposed animal bodies mixed soils and soils of slaughter houses. This abundance may be due to high levels of insect activity and optimum enrichment in these soils. The increased potentials for plasmids transfer among bacterial strains in such habitats may explain this diversity. This is in agreement with other workers findings [13, 17, 19].

As an achievement of this study, the spherical inclusions producing isolates were screened for their toxicity against dipteran insect *D. melanogaster* which are more susceptible to spherical inclusions rather than other crystal forms [12]. Drosophila were chosen for the toxicity bioassays because *B. thuringiensis* is not normally toxic to insect larvae that live in the soil [17], but it is toxic to insects that have aerial or water-borne larvae [17]. In this study, 24 *B. thuringiensis* isolates were found to be toxic to *D. melanogaster* larvae and adults with 75% of these toxic isolates produced spherical crystals and 25% produced both cuboidal and bipyramidal crystals. Ohba and Aizawa [12] have suggested a possible relationship between the shape of the parasporal bodies and the toxicity. They found that most of their toxic strains contained spherical bodies; this suggestion was in agreement with the findings of this study. Other reports [13, 16, 17] showed that isolates with cuboidal crystals exhibited an insecticidal activity against diptera, whereas the isolates that produced bipyramidal inclusions exhibited only an insecticidal toxicity against lepidoptera.

It was found that grain dusts were one of the major sources of toxic *B. thuringiensis* isolates to *D. melanogaster*. The reason for this abundance is may be due to a long time of storage of the grains that allows exposure to insects and build up of *B. thuringiensis* from a wide variety of sources. *B. thuringiensis* is known to present naturally on the plant material or from soil

contamination, thus there is a regular enrichment of *B. thuringiensis* isolates on the stored grains. This is in agreement with Meadows finding [13].

Results show that isolate 17_2 is the most toxic to *D. melanogaster* larvae. However, the least toxic isolate to the same insect species is 16_1. Both isolates were recovered from animal manure mixed habitats, but from different locations and different types of manure. Variation in isolate toxicity may be due to difference in host specificity [1, 20] or may be due to difference in environmental conditions that lead to genetic variation.

Interestingly, results indicated that 30% of the Jordanian *B. thuringiensis* isolates were toxic to *D. melanogaster* adults with 67, 4 and 29% of them were toxic to females, males and both males and females, respectively. This is the first study that demonstrates the toxicity of *B. thuringiensis* against *D. melanogaster* adults. Generally, it was found that these isolates were more toxic to adult females rather than adult males. This may be due to sexual variation and differences in feeding activity which is at higher level for female adults. Moreover, *D. melanogaster* larvae were more susceptible to *B. thuringiensis* toxins than adults because larval stage intoxicated at lower LC_{50} values. Studies of Karamanlidou and co-workers [16] indicated that *B. thuringiensis* isolates exhibited a wide spectrum of toxicity against insect's larvae rather than adults, they suggested that this may be due to the difference in the modes of feeding; the action of toxin after feeding; and higher concentrations of the toxin being delivered to the larvae.

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