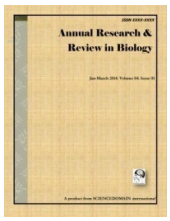

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Environmental Distribution, Frequency and Toxicity of *Bacillus thuringiensis* in Syria

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Authors' contributions

This work was carried out in collaboration between all authors. Author MM has designed and conducted the study and wrote the first draft of the manuscript. Author MA has supervised the whole work. Author FAZ has read and edited the manuscript, improved the English language and made the instructions for authors. Author KA has helped in the experiments related to the toxicity of Bt against the insect pest. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Bacillus thuringiensis* is distinguished by its production of proteinaceous parasporal inclusion bodies during sporulation, which when ingested by susceptible insects, are activated in the midgut into toxins. *B. thuringiensis* can be isolated from numerous sources; however, there has been no recorded isolation of *B. thuringiensis* strains from Syria. Therefore, this study aimed at investigating the distribution, frequency and toxicity of *B. thuringiensis* isolated from different ecosystems in Syria.
Study Design: This study describes the first isolation of the entomopathogenic bacterium, *Bacillus*

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thuringiensis in Syria.
Place and Duration of Study: Scientific Agriculture Research Center in Lattakia, Syria, 2011-2012.
Methodology: *B. thuringiensis* was isolated from soil samples collected from twenty different ecosystem-sites. The characterization of *B. thuringiensis* isolates was performed using morphological examination of colony morphology and formation of parasporal inclusion bodies. The crystal morphology of *B. thuringiensis* was observed by scanning electron microscopy. A bioassay was done to test the efficacy of *B. thuringiensis* against *Galleria mellonella*.
Results: Forest, beach and cultivated soils had more *B. thuringiensis* strains than uncultivated and interior arid soils. The frequency of *B. thuringiensis* was partially dependant on organic matter and pH content of the soil. A total of 65% of the isolates was found to be toxic to *G. mellonella.* The most toxic isolate of *B. thuringiensis* was obtained from cultivated area and produced bipyramidal, cuboidal and rectangular inclusions.
Conclusion: The comparison of the distribution of *B. thuringiensis* in different ecosystems might lead to a better understanding of the ecology of this organism. It is widespread in diverse environments including beaches and uncultivated soils. The presence of organic matter increases the population of *B. thuringiensis* in soils, suggesting that the bacteria have parasitic and saprophytic properties that let them to multiply both inside and outside insects' bodies.

Keywords: Bacillus thuringiensis; parasporal inclusion bodies; ecosystems; Galleria mellonella; biological control; Syria.

1. INTRODUCTION

Bacterial microflora of insects is rich and diverse, containing both Gram-positive and -negative bacteria. *Bacillus thuringiensis* is a Gram-positive, motile and spore-forming bacterium. It can be distinguished by its production of one or more proteinaceous parasporal inclusion bodies (crystal proteins) during sporulation. These crystal proteins are protoxins, which when ingested by susceptible insects (larvae), are activated in the midgut into toxins called delta-endotoxins with molecular masses ranging between 25 and 140 kDa [1]. *B. thuringiensis* strains differ not only in their activity toward different insect species but also in the relative potency of their delta-endotoxins [2]. The toxicity of the strains within the subspecies varies widely. Some of the strains exhibited toxicity to Lepidoptera, Diptera, and Coleoptera as well as to plant parasite nematodes [3-8]. Most strains of *B. thuringiensis* are specific largely to lepidopteran insects and frequently have parasporal inclusion bodies of bipyramidal shape [9]. For example, the greater wax moth *Galleria mellonella,* a destructive pest of beehives and honeycombs, is most susceptible to the subspecies *galleriae*, with other subspecies causing only moderate to low activity [10]. This has led to commercial preparations of *B. thuringiensis* for the biological control of insects of agricultural and industrial importance [11]. These compounds now account for 80-90% of all biological pest control agents sold worldwide.

Bacillus thuringiensis can be isolated from numerous sources including soils [12-14], stored products [9], grain dust [15], plant surfaces [16], diseased insect larvae [1], sericulture environments [17] and insect rearing facilities [18].

There has been no recorded isolation of *B. thuringiensis* strains from Syria. Therefore, this study aimed at investigating the distribution, frequency and toxicity of *B. thuringiensis* isolated from different ecosystems in Syria.

2. MATERIALS AND METHODS

2.1 Soil-Sample Collection

Two Kilo grams of soil sample were collected from twenty locations in Syria with different ecosystems, including cultivated fields planted with vegetables, fruit trees, crops and ornamental plants (14 samples). Soil samples were also collected from forests (9 samples), uncultivated natural areas (2 samples), beaches (4 samples) and from two uncultivated interior arid areas: Nabek and Deraa (4 samples) (Table 1). Soil samples were placed in an ice box, transferred to the laboratory and stored at 4℃ until the *B. thuringiensis* strains were isolated. The percentage of organic matter was determined by using an oven at 550℃ for 3 h, and the pH was determined for each soil sample by using pH/mv

Paqualab Meter. The soil analysis was carried out in the Scientific Agriculture Research Center in Lattakia.

2.2 Isolation of *Bacillus thuringiensis*

The isolation of the spore-forming *B. thuringiensis* was performed according to the method of Ohba and Aizawa [13] and Travers et al. [19]. Briefly, 1 g of each soil sample was suspended in 10 mL of sterile distilled water and mixed vigorously by vortexing for 1 min. The suspension was heated in a water bath at 80ºC for 30 min to exclude the vegetative structures of bacteria. One mL of each suspension was added to 10 mL of LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl and 1 mL 1 N NaOH buffered with 0.25 M Sodium acetate, pH 6.8). Sodium acetate selectively inhibits the germination of *B. thuringiensis* spores. The inoculated broth was incubated at 30ºC for 4 h followed by heating at 80ºC for 3 min to eliminate the growing cells of other spore-forming bacteria. The suspensions were diluted ten-fold, one hundred-fold and one thousand-fold and streaked onto T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate pH 6.8 and 0.005 g of $MnCl_2$). The colonies that formed were randomly picked and left to sporulate at 30°C for seven days. Cultures were then examined for the presence of spores and parasporal inclusion bodies.

2.3 Characterization of *Bacillus thuringiensis*

2.3.1 Crystal morphology

The characterization of presumptive *B. thuringiensis* isolates was performed using morphological examination of colony morphology and formation of parasporal inclusion bodies, which were the criteria used to confirm isolates as being *B. thuringiensis* [19]. Colonies of 7-day-old cultures of *B. thuringiensis* grown on T3 medium were used to study the morphology of parasporal inclusions by smear preparations stained with carbol fuchsine using light and phase contrast microscopes [20].

2.3.2 Scanning electron microscopy

The crystal morphology of a few selected *B. thuringiensis* isolates with parasporal inclusion bodies were cultured in 10 mL of T3 medium and incubated for 7 days at 30°C. Samples were centrifuged at 5000 rpm for 15 min. Pellets (spores and parasporal inclusion bodies) were washed in 2 mL of sterile distilled water and centrifuged at 5000 rpm for 5 min. Washes were repeated twice. The pellets were resuspended in 2 mL of sterile distilled water. The spore and crystal mixtures (10 µl) were placed on an aluminum grid and dried in a desiccator. The samples were coated with 20 nm of platinum for 3 min and observed by Inspect-F-50 scanning electron microscopy at 3 kV.

2.3.3 Insect rearing

Insects were bred on artificial food containing baby cereal (Tamilac), clear honey, glycerin, yeast powder and water at a ratio of 33:23:21:15:8 in plastic cups. They were bred at 30°C and 70% RH in darkness [21]. Seven-day-old third instar larvae of *Galleria mellonella*, bred from 0-24 h old egg masses at 3ºC, were used for the bioassays [22].

2.3.4 Bioassay

The purpose of this preliminary bioassay was to show that these new isolates were toxic to insects that are known to be susceptible to *B. thuringiensis* and to subsequently identify the entomocidally potent *B. thuringiensis* isolates. Isolates with crystals were cultured in 10 mL of T3 medium and incubated at 30°C for 7 days with continuous shaking at 250 rpm until bacterial cells were lysed [9,19]. The *B. thuringiensis* suspensions were tested for their toxicity to third instar larvae of *G. mellonella.*

For the bioassay, original toxin-spore bacterial suspensions were prepared and homogenized in a glass homogenizer. Two milliliter of each bacterial suspension were used by replacing of the water component in food used to breed *G. mellonella*, mixed for the best distribution of bacteria and spread in a small Petri-dish. Five third instar larvae of *G. mellonella* were kept in a small Petri-dish containing food contaminated with *B. thuringiensis*. The pathogenicity of each isolate was assayed in four replicates compared to a control, which was prepared with 2 mL of sterile distilled water, and with the reference strain *B. thuringiensis* subsp. *kurstaki* used instead of toxin. After incubation for 4-7 days at 30°C, live or dead larvae could be usually recognized by identifying the brown midgut of dead larvae under the dissecting microscope. Mortality was daily recorded for 7 days.

The viable count of *B. thuringiensis* in the diverse ecosystems was analyzed by MSATC statistical

analysis. In addition, correlation between the viable count of *B. thuringiensis* and both percentage of organic matter and pH was calculated using Spearman's correlation method [23].

3. RESULTS

3.1 Distribution of *B. thuringiensis*

The results indicated that *B. thuringiensis* was found in all selected sites in the Syrian coastal plain and interior arid areas. *B. thuringiensis* strains were highly abundant in all soil samples collected from a wide variety of environments. The soil samples were divided into different groups based on plant communities (Table 1). The highest average viable count of *B. thuringiensis* was found in the forest community (18.2 X 10^5 CFU/g), followed by beach (14.8 X 10^5 CFU/g) and cultivated areas (8.1 X 10^5 CFU/g). In contrast, the uncultivated natural areas (2.8 X 10^5 CFU/g) and interior arid areas (1.2 X 10^5 CFU/g) had the lowest CFU/g (Fig. 1). The organic matter percentage averaged 4.72% (range: 0.27-11.37%), and the recorded pH was 7.52 (range: 6.89-8.41) for the different soil samples. The viable count of *B. thuringiensis* was positively correlated (0.58) with the percentage of organic matter and negatively with pH (-0.37). This means that the viable count of *B. thuringiensis* increased moderately when the percentage of organic matter increased, while it decreased when the pH decreased but with little effect on *B. thuringiensis* population in the soil. Thus, the correlation is well explained because one is positive (0.58) and the another is negative (-0.37).

3.2 Crystal Morphology

In present study, 219 out of 225 (97%) isolates were found to contain proteinaceous parasporal inclusion bodies with a variety of shapes and sizes using light and phase contrast microscopy. These isolates were identified as *B. thuringiensis* (Figs. 2 and 3). Scanning electron microscopy revealed that the parasporal inclusion bodies of some *B. thuringiensis* isolates were different depending on the strain, including bipyramidal, cuboidal, rectangular and spherical inclusions (Fig. 4).

3.3 Bioassay

The results clearly demonstrated that 143 out of 219 (65%) *B. thuringiensis* isolates are toxic to third instar larvae of *G. mellonella,* and 76 isolates (35%) are rot toxic to the pest. The toxic isolates caused mortalities ranging from 0.0 to 40%. The most toxic isolate, with 40% mortality, was obtained from soil taken from under greenhouse-tomato in Banyas. This was followed by two isolates with 35% mortality from soil used to cultivate olives in Demsarkho and 30% mortality caused by isolates obtained from soil used to cultivate citrus in Um-Tyoor as well as tomato-cultivating soil in Banyas (Table 2).

The control treatment showed no effect on the third instar larvae of *G. mellonella,* while the reference strain of *B. thuringiensis* subsp. *kurstaki* showed a low toxicity of 10% mortality. When the crystal morphology of the toxic isolates was determined, it was found that about 30% of the isolates produced bipyramidal and cuboidal crystals. Of the remaining isolates, 14.6% produced bipyramidal crystals, 9.6% produced rectangular, bipyramidal and cuboidal crystals, 4.1% of the isolates produced spherical, bipyramidal and cuboidal crystals, 1.8% produced irregular, bipyramidal, and cuboidal crystals, as well as only 0.5% produced spherical crystals. The most toxic isolate against the third instar larvae of *G. mellonella* was imaged using scanning electron microscopy, and the formation of three parasporal inclusion-bodies was observed. These parasporal inclusions were bipyramidal (Fig. 4A), cuboidal (Fig. 4B), rectangular (Fig. 4C), and spherical (Fig. 4D).

4. DISCUSSION

This study shows that *B. thuringiensis* occurs naturally in diverse ecosystems. This might be due to the ability of *B. thuringiensis* to produce spores that are very hardy in adverse conditions. These spores can tolerate heat up to 80℃ and low temperatures of -20℃ [24]. In addition, the moisture content of the soil has little influence on spore survival; however, previous studies have found that *B. thuringiensis* was able to multiply in sterilized humus poor sand with moisture content over 10%, but that optimum growth was seen at 36% moisture content [25]. *B. thuringiensis* has been also isolated from different soil types in Egypt [26].

In the current study, the highest population of *B. thuringiensis* was found in forest soils due to the high percentage of moisture content and high level of organic matter. In addition, this environment is suitable for insect activity, and chemical pesticides are not present for the control of insects unless necessary. This finding is in agreement with Landen et al. [11], who

reported that forest soil samples were richer in *B. thuringiensis* strains than those ones collected from cultivated areas in Southern Sweden. Previous work by Martin and Travers [14] showed that *B. thuringiensis* was found in forest, agricultural land, steppe, urban, tundra, beach and desert soils, with the largest populations in agricultural land, steppe and forest soils. In addition, Obeidat et al. [27] reported that soils contaminated with animal byproducts and decomposed animal bodies were very rich with *B. thuringiensis* due to the high levels of nutrients and insect activity.

Table 1. Environmental distribution of *Bacillus thuringiensis* isolated from soils of different plant communities

Plant communities	Site	No. of samples	Viable count of *B. thuringiensis* x10^5/g	Index diversity [a]	Organic matter %	pH
Forest						
Forest	Frulk	1	25.1	0.96(22/23)	11.37	6.89
		2	34.0	0.95(21/22)	11.30	6.90
		3	16.0	0.94(15/16)	8.56	7.00
Forest	Mashkeeta	4	13.1	1(9/9)	6.85	7.18
Forest	Mraige	5	3.30	1(10/10)	5.48	7.68
		6	26.3	1(4/4)	8.70	7.20
Forest	Hzeereen	7	1.20	1(5/5)	7.70	7.56
		8	15.0	1(2/2)	6.70	7.43
		9	30.0	1(4/4)	6.85	7.61
Average			**18.2**			
Cultivated area						
Citrus	Um-Tyoor	10	2.80	1(6/6)	2.33	7.70
Cherry	Slenfeh	11	20.6	1(8/8)	5.34	7.58
Apple	Doorin	12	3.20	1(8/8)	1.78	7.16
Walnut		13	1.20	1(8/8)	2,09	7.21
Peach	Kbr-Alabed	14	0.30	1(2/2)	3.43	7.39
		15	6.30	1(2/2)	3.40	7.40
Olive	Demsarkho	16	8.20	1(9/9)	9.32	7.16
Olive	AL-Krdaha	17	2.00	1(9/9)	3.01	7.36
Faba- bean	AL-Kkrdaha	18	2.20	1(3/3)	3.23	7.70
		19	1.30	1(1/1)	1.56	7.43
Squash (open field)	Banyas	20	4.20	1(8/8)	7.26	7.77
Tomato	Banyas	21	14.4	1(8/8)	6,50	7.45
Wheat	Jableh	22	25.0	1(10/10)	2.74	7.20
Ornamental plant	Tishreen University	23	21.9	1(5/5)	8.50	7.11
Average			**8.10**			
Uncultivated natural area						
Wild ornamental plant	Al-Hafeh	24	2.00	1(8/8)	3.43	7.40
Grass	Sport city	25	3.50	1(5/5)	3.56	7.65
Average			**2.80**			
Beach						
Beach	Jableh beach	26	7.70	0.86(6/7)	3.30	7.87
Beach	Lattakia beach	27	25.0	0.67(2/3)	1.23	8.41
		28	26.0	1(2/2)	3.40	7.70
Beach	Blue beach	29	0.30	0.67(2/3)	0.27	8.06
Average			**14.8**			
Interior arid areas						
Uncultivated soil	Nabek	30	1.1	1(4/4)	1.23	8.04
		31	1.6	1(4/4)	1.20	8.12
Uncultivated soil	Deraa	32	0.7	1(4/4)	1.45	7.95
		33	1.4	1(4/4)	1.80	7.90
Average			**1.2**			

[a]*Index diversity: Number of B. thuringiensis isolated/Number of spore-forming bacteria examined*

The high frequency of *B. thuringiensis* from beach soils with low organic matter' contents can be explained by the proximity of the beach to agricultural land, forest and urban areas, which contributes to spreading of *B. thuringiensis* endospores by water, wind and migrating animals [18]. In addition, organisms can be spread via the trade of stored agricultural products, as *B. thuringiensis* is commonly found between countries on the Mediterranean Sea [1] .

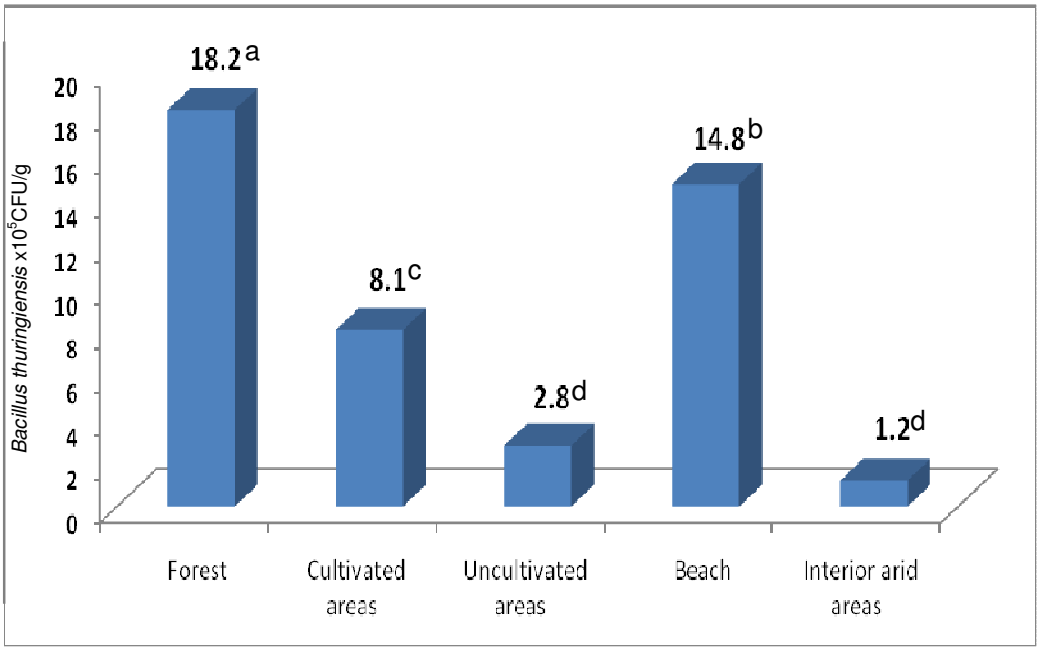

Fig. 1. Distribution of *Bacillus thuringiensis* in diverse ecosystems in Syria

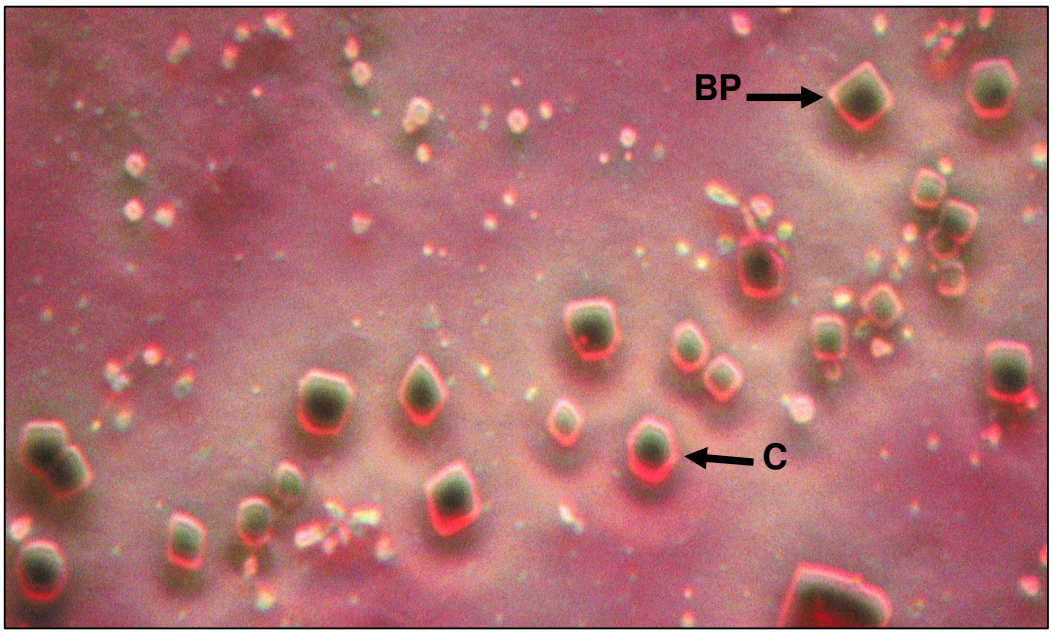

Fig. 2. Phase contrast photograph of sporulated culture of *Bacillus thuringiensis*. The arrows indicate the shape of crystals, BP: bipyramidal, C: cuboidal at 1000x

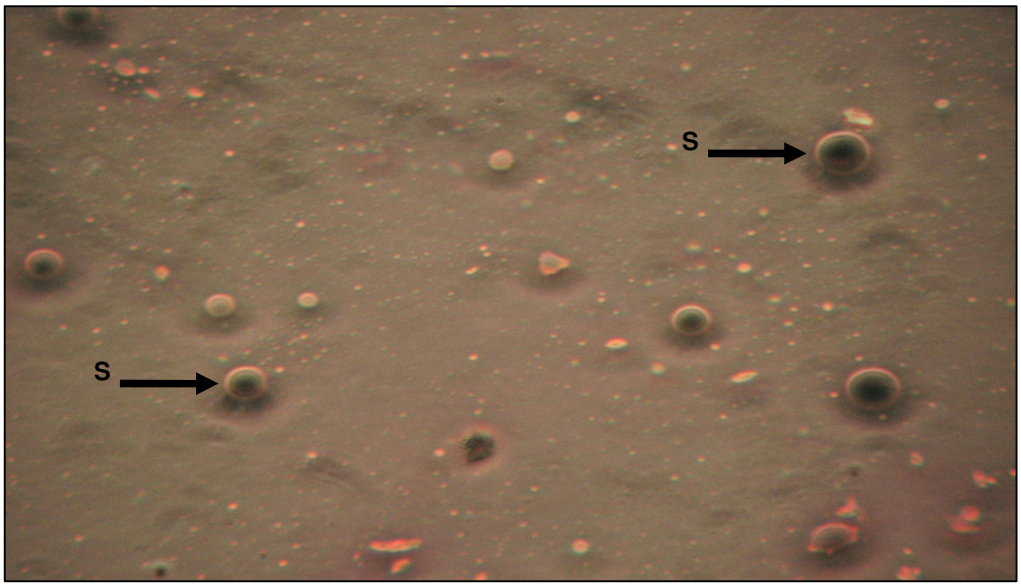

Fig. 3. Phase contrast photograph of spherical inclusions of sporulated *Bacillus thuringiensis* isolate. The arrow indicates the shape of crystals, S: spherical at 1000x

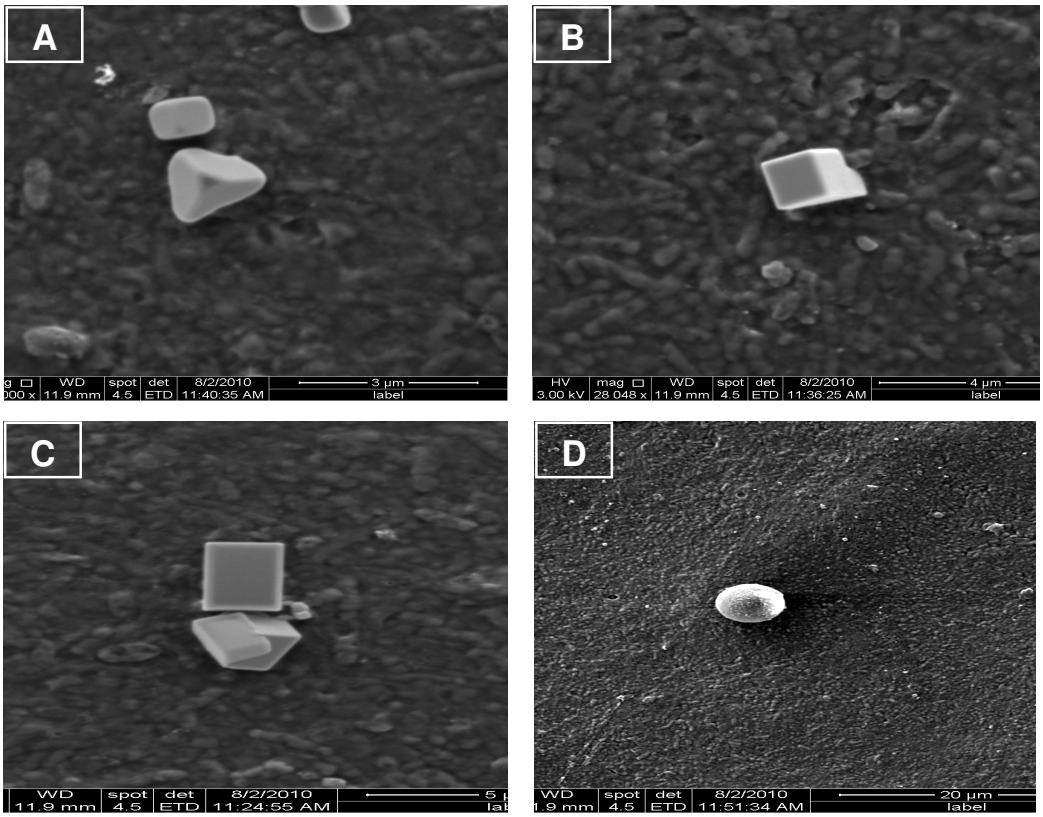

Fig. 4. Scanning electron micrographs of parasporal inclusion bodies of some *Bacillus thuringiensis* isolates. (A): Bipyramidal inclusion (30,000x), (B): Cuboidal inclusion (28,048x), (C): Rectangular inclusion (20,000x) and (D): Spherical inclusion (5,000x)

Table 2. Larvicidal activity of *Bacillus thuringiensis* isolates against third instar larvae of *Galleria mellonella*

Location	Type of field	No. of tested isolates	Mortality (%) (mean of four replicates) 5%	10%	15%	20%	25%	30%	35%	40%
Furulk	Forest	58	17	11	6	2	2			
Mashkeeta	Forest	9	3	1						
Mraige	Forest	14	6	3						
Hzeereen	Forest	12	7	1	1	1				
Jableh	Wheat	10	1	2						
Um- Tyoor	Citrus	6	1	1	1	1		1		
Kbr- Alabed	Peach	3	1	1		1				
Doorin	Apple	8	2	1	1	1	1			
Doorin	Walnut	8	3	1	1					
Slanfeh	Cherry	8	1	1	1					
Demsarkho	Olive	8	1			1			2	
AL- Krdaha	Olive	9	2	1	1	1				
AL- Krdaha	Faba-bean	4	1	1		1	1			
Banyas	Sequach	8	4	3	1					
Banyas	Tomato	8	3		2		1	1		1
Tishreen University	Ornamental plant	5	1	3	1					
A l- Hafeh	Wild ornamental plant	8	2	1	4					
Sport city	Grass	4	1		1	1				
Lattakia beach	Beach	4	1	2						
Jableh beach	Beach	6	1	2	1					
Blue beach	Beach	2	1							
Nabek	Uncultivated	8	4							
Deraa	Uncultivated	8	1							
Reference strain *B.t.kurstaki*		2		2						
Total		**219**	**65**	**36**	**22**	**10**	**5**	**2**	**2**	**1**

This study shows that Syrian soils are very rich in *B. thuringiensis*. The high levels of organic matter and the neutral pH of soils from different locations in Syria were optimal for the growth of *B. thuringiensis*. This is in agreement with the study of Saleh et al. [28], who found that *B. thuringiensis* spores were able to germinate and compete successfully with other soil microorganisms in neutral soil pH (6.3) and in the presence of proteinaceous amendments. Martin and Travers [14] reported that *B. thuringiensis* occurs everywhere in the world and from every kind of soil, especially soil samples collected in Eastern Asia, where *B. thuringiensis* was the most abundant. The frequency of recovery of *B. thuringiensis* in Syria was 97% of the tested soil samples. Martin and Travers [14] reported that the frequency of *B. thuringiensis* is 85% in Asia. In Canada, *B. thuringiensis* was distributed in soils at a frequency of 26-39% [29]. In New Zealand, the frequency of *B. thuringiensis* was 70% [30], whereas the frequency in Jordan was 63% [31]. The variation in the frequency of *B. thuringiensis* among soils collected from different locations might be due to differences in soil types, geographical differences, the soil sample micro-environment, the isolation method and genetic variation.

Most *B. thuringiensis* strains isolated from Syrian soil samples were found to be toxic to many insects [32]. *B. thuringiensis* isolates with elevated levels of insecticidal activity against *G. mellonella* were isolated from cultivated environments, which are preferred by lepidopteran insects [9,14]. In contrast, *B. thuringiensis* isolated from soil collected from beach and uncultivated environment was relatively less toxic to *G. mellonella*. These environments are less preferred by lepidopteran insects. This is in agreement with the study of Xavier et al. [33], who reported that the environmental distribution and insecticidal specificity of *B. thuringiensis* strains is based primarily on the prevalence and abundance of the target insects in a particular environment and

the *cry* gene content of the *B. thuringiensis* strains. However, the results of this study showed that five of the *B. thuringiensis* isolates with the highest toxicity produced bipyramidal inclusions; the most toxic produced bipyramidal, cuboidal and rectangular inclusions, two produced both bipyramidal and cuboidal inclusions, and the remaining two isolates produced bipyramidal inclusions. This suggests a possible relationship between the shape of parasporal inclusion bodies and the toxicity, i.e. strains with bipyramidal crystals exhibiting toxicity toward Lepidoptera [17]. The non-toxic isolates obtained in this study might be toxic to other insects. Thus, further investigations are needed to study the effect of these isolates on other insects' orders, as these isolates might exhibit more toxic potential for other pests' suppression.

5. CONCLUSION

In conclusion, the comparison of the distribution of *B. thuringiensis* in different ecosystems might lead to a better understanding of the ecology of this organism. It is widespread in diverse environments including beaches and uncultivated soils. The *B. thuringiensis* isolates from diverse environments have different insecticidal activities. The occurrence of *B. thuringiensis* depends on many factors, i.e. organic matter in the soil and the pH. The presence of organic matter increases the population of *B. thuringiensis* in soils, suggesting that the bacteria have parasitic and saprophytic properties that let them to multiply both inside and outside insects' bodies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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