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Original Article

Abundance and Serotyping of pathogenic isolates of *Bacillus thuringiensis* isolated from Ajloun Forests

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Abstract

The investigation of *Bacillus thuringiensis* in 16 forest soil samples at Ajloun Northren Jordan involved the isolation of 23 isolates toxic to the third instar dipteran larvae of *Drosophila melnogaster*. The highest viable count of *B. thuringiensis* was in Ebeen forest soils (14.24 x 107 CFU/g) and the lowest viable count was in Rasoun forest soils. The lethal concentration (LC50) of *B. thuringiensis* isolates indicated a variation in their toxicity to *D. melnogaster*. Serotyping of the 23 toxic isolates revealed that they belonged to 5 serotypes, including; *israelensis, kenyae, kurstaki, malaysiensis,* and *morrisoni*. Serotype *israelensis* was the most dominant. It was observed that isolates producing spherical parasporal crystals were the most abundant in the forest soils. The mortality of most isolates against *D. melanogaster* larvae at a constant dose of crystal-spore suspension was ≥50% after 36 h of incubation. Hydrolytic activities of *B. thuringiensis* isolates recovered from forests were varied due to differences in their enzymes productivity. Most isolates have CMCase, pectinase, amylase, lipase, and gelatinase activity. Maceration activity of the isolates to potato was more frequent than that to carrot. Therefore, forest environment can be categorized as a rich source for *B. thuringiensis* isolates that can be used in biological control and plant residue biodegradation. As a result, *B. thuringiensis* recovered from forests can be used to increase soil fertility and to enhance plant growth as well as productivity.

Keywords: *thuringiensis; Forest; Parasporal; Larvicidal; Maceration*

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore forming bacterium that is able to produce proteinaceous parasporal crystals during sporulation. The parasporal crystals exhibited a wide range of toxicity against different insect orders [Diptera, Lepidoptera and Coleoptera] and other invertebrates [8, 19]. *B. thuringiensis* is a widespread bacterium in different habitats including; soil, grain dusts, diseased insect larvae, and sericulture environments-soil being the principal source of novel *B. thuringiensis* isolates [7, 21]. The differentiation of *Bacillus thuringiensis* isolates by the determination of the H-antigen has been used to group isolates into a number of serotypes [5].

The demand for using biological products instead of chemical products as an insecticide led to an increase in the use of *B. thuringiensis* formulations in the environment in order to protect against crop and forest insects. However, little is known about the ecology of this bacterium because of its widespread distribution. Forest soil samples in south Sweden were rich in *B. thuringiensis* more than soil samples collected from cultivated areas [12]. In this study, the abundance, distribution, diversity, serotyping, parasporal crystal shapes, larvicidal activity against *D. melnogaster*, and enzymetic productivity as well as maceration activity of *B. thuringiensis* isolated from forests were investigated.

MATERIAL & METHODS

Samples

A total of 16 soil samples were collected from 3 forests at Ajloun in Northern Jordan, namely Eshtafena [6 samples], Ebeen [6 samples], and Rasoun [4 samples]. Soil samples were collected from the top 10 cm depth after removal of the above 3-5 cm surface soil. Each soil sample was thoroughly mixed and sieved through 2 mm pore size sieve.

Isolation of bacteria

Isolation of *B. thuringiensis* was done according to the method of Ohba and Aizawa [16] followed by the method of Travers *et al.* [22]. One gram of each sample was suspended in sterile distilled water and heated at 80 ºC for 30 min. Heat-treated or pasteurized suspension was diluted and plated on nutrient agar and then incubated at 30 ºC for 24 h. Selection of *B. thuringiensis* isolates was done by addition of the pasteurized suspension to 10 ml of LB broth buffered with 0.25 M sodium acetate pH 6.8. The suspensions were incubated at 30 ºC for 4 h and then heated to 80 ºC for 3 min. Suspensions were diluted and plated on T3 medium [22]. Cells were examined under light microscope to observe the parasporal crystals.

Serology

Serotyping of toxic *Bacillus thuringiensis* isolates was done according to the micromethod of Laurent *et al.* [13]: 90 µl of the bacterial suspensions were taken from 5-8 h culture growing at 30 oC placed in 96-well microplate [U-bottom], 10 µl of two dilutions of each antiserum [1:10 and 1:20] were used, so that final antisera dilutions were 1:100 and 1:200, respectively. In two wells antiserum dilution were replaced by 150 mM NaCl in deionized water as negative controls. The plates were incubated for 75
min at 37 oC, and the agglutination assays under compound microscope [10X magnification] were observed. Positive reactions were visible as a floccular sediment at the bottom of the well and a clear supernatent. Fifty-five antisera that were kindly supplied by Dr. Marguerite-M. Lecadet [Pasteur institute, Paris, France] were used to determine the serotypes of the Jordanian forest isolates.

Bioassay

The toxicity of *B. thuringiensis* isolates was bioassayed against the third instar larvae of *D. melanogaster* according to the method of Karamanlidou *et al.* [11]. Ten third instar larvae were placed into each well of 24-well plates, and 0.3 ml diet homogenate and 0.7 ml *B. thuringiensis* suspension [spore and crystals] were added to each well. The toxicity of each isolate was assayed, in duplicate, for either the original toxin-spore suspension or the diluted preparation. Plates were incubated at 25 ºC for 24 h. Mortality was then scored in comparison with a parallel control with 0.7 ml sterile distilled water instead of toxin. Mortality was observed by viewing the brown midgut of dead larvae under the dissecting microscope. The lethal concentration values that kill 50% of the tested larvae [LC50] were determined by log probit analysis.

RESULTS & DISCUSSION

It was found that *B. thuringiensis* was isolated from 94% of the selected Ajloun forest soil samples suggesting that *B. thuringiensis* is a common microflora in Ajloun forest soils. The high total bacterial and *B. thuringiensis* viable counts in Ebeen forest soil could be due to an optimum survival and enrichment factors in that soil. From this study, it was found that *B. thuringiensis* populations are considerably heterogeneous in terms of colony morphology or diversity, numbers, parasporal shapes, toxicity, and serotypes. This is in agreement with previous observations in other Asian habitats [1, 10, 14, 15, 16].

The index of diversity of *B. thuringiensis* in this study was mostly above 0.50. Therefore, it is comparable with Obeidat *et al.* [15] who reported that the index of diversity in other Jordanian habitats was 0.67. Variation in the LC50 of the isolates is indicatives of their variation in larvicidal activity. This was in accordance with Saadoun *et al.* [18].

The results of this study clearly indicated that forest soils were richer in *B. thuringiensis* than that reported by Obeidat *et al*. [15] in other Jordanian habitats. This finding is in agreement with Landen *et al.* [12] who reported that forest soil samples in south Sweden were richer in *B. thuringiensis* than soil samples collected from cultivated areas. Moreover, it was observed that the toxicity of Ajloun forest *B. thuringiensisi* was higher than that reported by Saadoun *et al*. [18] in other Jordanian habitats. The dominance of spherical parasporal crystals and the *israelensis* serotype was in agreement with the findings of Saadoun *et al.* [18] and Almomani *et al.* [1], respectively.

In the current study, the occurrence of *B. thuringiensis* was investigated in 16 forest soil samples representing 3 forests [Eshtafena, Ebeen,

and Rasoun] at Ajloun in Northern Jordan. The highest estimated viable bacterial count was [18x 109 CFU/g] at Ebeen and the lowest estimated count was at Rasoun [0.53 x 109 CFU/g] [Table 1]. The estimated viable count of spore-forming bacteria was scored after pasteurization step and the viable count of *B. thuringiensis* was scored after acetate selection. [Table 1] showed that the highest estimated viable spore former bacterial count was 18 x 107 CFU/g at Eshtafena and the lowest count was at Rasoun [0.05 x 107CFU/g]. Whereas the highest estimated viable count of *B. thuringiensis* was at Ebeen [14x 107 CFU/g] and the lowest estimated count was at Rasoun.

The total number of isolated *B. thuringiensis* was 39 out of them 23 were pathogen to the third instar larvae of *D. melanogaster* with 59% as shown in [Table 2].

The highest diversity of bacterial colonies was found in Eshtafena [7 different morphological colonies] whereas the highest diversity of *B. thuringiensis* colonies was found in Ebeen [4 different morphological colonies] [Table 3]. The index of diversity of B. *thuringiensis* was ranging from 0.00 to 1.00 [Table 3].

The toxicity of *B. thuringiensis* was determined against the third instar larvae of *Drosophila melanogaster* by calculation of the LC50[Table 3]. Isolate 71 serotype *israelensis* from Ebeen showed the lowest LC50 4.45 indicating its high larvicidal activity. As shown in [Table 4], serotyping of the 23 toxic isolates subdivided them into 5 serotypes [*israelensis, kenyae, kurstaki, malaysiensis,* and *morrisoni*]. [Table 4] showed that most of the isolates produced spherical parasporal bodies and most of them were cross reacted with *israelensis* serotype.

CONCLUSION

The present study reveals vegetation diversity and species richness along the altitudinal gradient ranged from 900 to 3000 m at GarhiDopatta Hills. Species diversity and richness values were high in the tree layer in the middle part of the altitudinal gradient. It decreases both towards upper and lower altitude, which was due to deforestation, human interaction, encroachment pressure, low number of species and soil erosion. There is great need of reforestation in the area. Alternate sources of fuel must be provided to local inhabitants to minimize the pressure on wealth of wild plants.[Habib, Malik et al.]

The distribution and abundance of desert plant communities were examined in the lower reaches of the Tarim River, southern Xinjiang. Eighteen plant species were collected at 18 sites along a sequence of increasing ground-water depths in six transects. Except for Tamarix ramosissima and Populus euphratica, which were distributed across nearly all sites, most of the investigated species had low frequency of occurrence. Correspondence analysis [CA] of the 18 species revealed a separation of growth forms into distinct groups corresponding to different ground-water levels. Canonical correspondence analysis [CCA] of the 18 species and seven environmental variables would suggest a major botanical gradient exists relating to ground-water depth and secondary gradients exists that include soil moisture, pH and to a lesser extent alkalinity and mineralization[Zhang, Chen et al. 2005].

Ephemeral plants are a special type of plants living in an environment which is rainy in early spring and xerothermic in summer. In Gurbantunggut Desert, they are mainly distributed in the southern part, playing an important role in the maintenance of desert ecosystem stability and in sand-fixation By adopting stratification sampling, this paper investigated the diversity feature of ephemeral plants at 35 sites covering a total area of 3.86×104 m2 in the southern Gurbantunggut Desert. A total of 93 ephemeral species were recorded, belonging to 24 families and 74 genera, among which, Chen opodiaceae, Compositae,Cruciferae, and Leguminosae were the dominant families, accounting for 19.35%,17.20%,11.83%,and 9.68% of the total species, respectively. In terms of life form, ephemeral plants, annuals with long period of nourishment, and trees and shrubs accounted for 53.76%, 22.58%, and 16.13%, respectively. In terms of importance value, ephemeral plants accounted for 45.73%, while trees and shrubs accounted for 30.93%. [Liu, Liu et al. 2011]

REFERENCE

Almomani, F., Obeidat, M., Saadoun, I., Meqdam, M. (2004). Serotyping of Bacillus thuringiensis isolates, their distribution in different Jordanian habitats and pathogenicity in Drosophila melanogaster. World J. Microbiol. Biotechnol. 20, 749-753.

Atlas, R.M., Parks, L.C., Brown, A.E. (1995). Laboratory Manual of Experimental Microbiology. Mosby-Year Book, Inc, St Louis.

Bayoumi, R.A., Yassin, H.M., Swelim, M.A., Abdel-All, E.Z. (2008). Production of bacterial pectinase(s) from agro-industrial wastes under solid state fermentation conditions. J. Appl. Sci. Res. 4(12), 1708-1721.

Carder, J.H. (1986). Detection and quantitation of cellulase by Congo red staining of substrates in a cup-plate diffusion assay. Anal. Biochem. 15, 375-79.

de Barjac, H., Bonnefoi, A. (1973). Mise au point sur la classification des Bacillus thuringiensis. Entomophage. 18, 5-17.

Delpech, R. (2000). Simple and affordable scientific investigations with the bacterial plant pathogen E.carotovora. J. Biol. Educ. 34, 55-160.

Dulmage, H., Aizawa, K. (1982). Distribution of Bacillus thuringiensis in nature. In: Kurstak, E. (ed). Microbial and viral pesticides. New York, Marcel Dekker, Inc., p. 209-237.

Feitelson, J. (1993). The Bacillus thuringiensis family tree. In: Kim, L. (ed). Advanced engineered pesticides. New York, Marcel Dekker, Inc., p. 63-72.

Gomes, D.J., Hasan, M.F., Rahman, M.M. (2002). Screening for α-amylase producing thermophilic fungi recovered from natural decomposing lignocellulosic materials. Dhaka Univ. J. Biol. Sci. 11(1), 39-48.

Hastowo, S., Lay, B.W., Ohba, M. (1992). Natural occurring Bacillus thuringiensis in Indonesia. J. Appl. Bacteriol. 73, 108- 113.

Karamanlidou, G., Lambropoulos, A., Koliais, S., Manousis, T., Ellar, D., Kastritsis, C. (1991). Toxicity of Bacillus thuringiensis to laboratory populations of the olive fruit fly (Dacus oleae). Appl. Environ. Microbiol. 57, 2277-2282.

Landen, R., Bryne, M., Abdel-Hamead, A. (1993). Distribution of Bacillus thuringiensis strain in southern Sweden. World J. Microbiol. Biotechnol. 10, 45-50.

Laurent, P., Ripouteau, H., Cosmao Dumanior, V., Frachon, E., Lecadet, M. (1996). A micromethod for serotyping Bacillus thuringiensis. Lett. Appl. Microbiol. 22, 259-261.

Martin, P., Travers, R. (1989). Worldwide abundance and distribution of Bacillus thuringiensis isolates. Appl. Environ. Microbiol. 55, 2437-2442.

Obeidat, M., Almomani, F., Saadoun, I. (2000). Diversity of Bacillus thuringiensis in different habitatsof northern Jordan. J. Basic. Microbiol. 40, 385-388.

Ohba, M., Aizawa, K. (1986). Distribution of Bacillus thuringiensis in soils of Japan. J. Invert. Pathol. 47, 277-282.

Perombelon, M.C., Gullings, J., Kelman, J. (1979). Population dynamics of E. carotovora and pectolytic Clostridium spp. In relation to decay of potatoes. Phytopathol. 69,167-173.

Saadoun, I., Al-Momani, F., Obeidat, M., Meqdam, M., Elbetieha, A. (2001). Assessment of toxic potential of local Jordanian Bacillus thuringiensis strains on Drosophila melanogaster and Culex sp. (Diptera). J. Appl. Microbiol. 90, 866-872.

Schnepf, E., Crickmore, N., Rie, J., Lereculus, D., Baum, J., Feitelson, J., Zeigler, D., Dean, D. (1998). Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol. Molec. Biol. Rev. 62, 775-806.

Sigurgísladóttir, S., Konráðsdóttir, M., Jónsson, A., Kristjánsson, J.K., Matthiasson, E. (1993). Lipase activity of thermophilic bacteria from icelandic hot springs. Biotech. Lett. 15(4), 361-366.

Smith, R., Couche, G. (1991). The phylloplane as a source of Bacillus thuringiensis variants. Appl. Environ. Microbiol. 57, 311-315.

Travers, R., Martin, P., Reichelderfer, C. (1987). Selective process for efficient isolation of soils Bacillus spp.. Appl. Environ. Microbiol. 53, 1263-1266.

Table 1. Abundance and distribution of Bacillus thuringiensis (B.t) isolates at Ajloun forests soil

Forest location	Sample number	Viable count of bacteria before pasteurization at nutrient agar /g x10^9	Viable count of spore-former/after/pasteurization at nutrient agar/g x10^7	Viable count of *B.t.* /g x10^7	*B.t %*
Eshtfena	1	2	4	2	1.00
	2	6	4	2	0.33
	3	1	3	2	2.00
	4	14	5	4	0.28
	5	3	6	4	1.33
	6	6	18	12	2.00
Ebbeen	7	6	9	8	1.33
	8	12	16	14	1.16
	9	4	2	2	0.50
	10	10	6	4	0.40
	11	8	13	10	1.25
	12	18	9	7	0.39
Rasoun	13	8	2	2	0.25
	14	11	9	5	0.45
	15	0.89	0.05	0.05	0.05
	16	0.53	0.08	0.00	0.00

Table 2. Number of identified *Bacillus thuringiensis*, No. of pathogenic *Bacillus thuringiensis* and % of pathogenic isolates in each sample

Location	No. of samples	No. of identified (*B.t*)	No. of pathogenic (*B.t*)	% of pathogenicity
Eshtafena	1	2	1	50
	2	1	0	0
	3	4	3	75
	4	3	3	100
	5	2	2	100
	6	5	3	60
Ebbeen	7	3	1	33
	8	4	2	50
	9	1	0	0
	10	2	1	50
	11	4	3	75
	12	3	2	66
Rasoun	13	1	0	0
	14	1	0	0
	15	3	2	66
	16	0	0	0
Total	16	39	23	59

Table 3. Diversity of *Bacillus thuringiensis* (*B.t*).

Location	No. of samples	No. of different colonies morphology on nutrient agar	No. of different (*B.t*) colonies on selective medium	Index of diversity*
Eshtafena	1	6	5	0.83
	2	5	3	0.60
	3	3	3	1.00
	4	4	2	0.50
	5	2	2	1.00
	6	7	3	0.43
	7	4	3	0.75
	8	2	1	0.50
Ebbeen	9	5	4	0.80
	10	4	3	0.75
	11	6	3	0.50
	12	3	3	1.00
Rasoun	13	3	1	0.33
	14	2	1	0.50
	15	2	2	1.00
	16	2	0	0.00
Total	16	60	39	

(*) Index of diversity is: The diversity of *B. thuringiensis* colonies / diversity of total bacteria colonies

Table 4. larvicidal activity, Parasporal morphology, and serotyping, of isolated *B. thuringiensis*

Forest	Isolate.No	Parasporal morphology	Serotype	LC50
Eshtafena	11	S	*israelensis*	5.41
	31	S	*malaysiensis*	6.28
	33	S	*israelensis*	6.32
	34	PB+C	*kurstaki*	5.09
	41	S	*kenyae*	6.49
	42	PB+C	*kurstaki*	6.38
	43	S	*morrisoni*	5.49
	51	S	*israelensis*	5.26
	53	S	*israelensis*	5.34
	61	PB+C	*kurstaki*	5.36
	62	S	*israelensis*	5.20
	63	PB+C	*kurstaki*	4.53
Ebeen	71	S	*israelensis*	4.45
	81	S	*kenyae*	6.28
	84	PB+C	*kurstaki*	6.57
	101	S	*israelensis*	6.15
	111	S	*israelensis*	6.36
	112	PB+C	*kurstaki*	6.62
	113	S	*morrisoni*	5.46
	121	S	*israelensis*	5.56
	123	PB+C	*kurstaki*	6.52
Rasoun	151	S	*israelensis*	6.32
	153	S	*israelensis*	6.30

a. Xy: X is the sample number; y is the isolate number.
b. S: Spherical; BP: Bipyramid; C: Cuboid.
c. LC50 = log (spore concentration/ml).