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Ecology, toxicity, and hydrolytic activities of *Bacillus thuringiensis* in forests

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Abstract: The investigation of *Bacillus thuringiensis* (Bt) in 16 forest soil samples from Ajloun, northern Jordan, involved the isolation of 23 isolates toxic to the third instar dipteran larvae of *Drosophila melanogaster* and 7 isolates toxic to the third instar lepidopteran larvae of *Ephestia kuehniella*. The highest viable count of Bt was found in Ebeen forest soils (14.24×10^7 CFU g^{-1}), and the lowest viable count was found in Rasoun forest soils. The lethal concentration (LC_{50}) of Bt isolates indicated a variation in their toxicity to *D. melanogaster* and *E. kuehniella* larvae, with lower LC_{50} values for *D. melanogaster* suggesting that *D. melanogaster* larvae are more susceptible to Bt toxins than *E. kuehniella* larvae. Serotyping of the 23 isolates toxic to *D. melanogaster* revealed that they belonged to 5 serotypes, including *israelensis*, *kenyae*, *kurstaki*, *malaysiensis*, and *morrisoni*. Serotype *israelensis* was the most dominant. The isolates toxic to *E. kuehniella* larvae belonged to serotype *kurstaki* and produced both bipyramidal and cuboidal parasporal crystals. It was observed that isolates producing toxic spherical parasporal crystals were the most abundant in the forest soils. Hydrolytic activities of Bt isolates recovered from forests were varied due to differences in their enzyme productivity. Most isolates had carboxymethylcellulase, amylase, lipase, and gelatinase activity, while pectinase activity was observed in only a few isolates. Maceration activity of the isolates to potato samples was more frequent than to carrot samples. The larvicidal and hydrolytic activities of tested Bt isolates demonstrated that a forest environment can be categorized as a rich source for Bt isolates that can be used in biological control and plant residue biodegradation. As a result, it is expected that Bt recovered from forests can be used to increase soil fertility and to enhance plant growth as well as productivity.

Key words: Ecology, enzyme, forest, larvicidal, maceration, *thuringiensis*

1. Introduction

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming, naturally occurring bacterium. This bacterium is capable of producing proteinaceous parasporal crystals during sporulation (De Respinis et al. 2006; Konecka et al. 2007; Soberon et al. 2007; Bizzarri and Bishop 2008). These parasporal crystals often contain the δ-endotoxin proteins that exhibit a wide range of insecticidal activity against agriculturally and medically important insect pests of several orders, including Lepidoptera, Trichoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga, and noninsect invertebrates including nematodes, mites, and protozoa (Feitelson 1993; Konecka et al. 2007; Soberon et al. 2007; Cinar et al. 2008). This makes Bt a promising microbial agent for control of insect pests in agriculture, forestry, veterinary, and public health management (Schnepf et al. 1998). δ-Endotoxin proteins consist of 2 classes, Cry and Cyt (Dronina et al. 2006; Soberon et al. 2007), and fall into 32 sets (Crickmore et al. 2012). The bacterium Bt is widespread and can be isolated from natural habitats such as soil, insect habitats, insect larvae, and grain dusts (Dulmage and Aizawa 1982; Smith and Couche 1991). A useful classification of Bt isolates was developed on the basis of H-flagellar antigens by de Barjac and Bonnefoi (1973). Currently, Bt strains are classified into 86 serovars using this H-antigen method (Lecadet et al. 1999).

The demand for using biological products instead of chemical products as insecticides has led to an increase in the use of Bt formulations in the environment to protect against crop and forest insects. However, little is known about the ecology of this bacterium in forests, but it was found that forest soil samples were richer in Bt than soil samples collected from cultivated areas (Landen et al. 1993). Therefore, the present study was established to investigate the abundance, distribution, and diversity of Bt in local forests. In addition, forest Bt isolates were classified by serotyping, and the larvicidal activity against *D. melanogaster* and *E. kuehniella* was determined. To evaluate the effects of Bt on soil fertility and plant growth

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in forest, the maceration activity and the enzymatic productivity of forest Bt isolates were examined in this work.

2. Materials and methods

2.1. Samples

A total of 16 soil samples were collected from 3 forests in Ajloun in northern Jordan, namely Eshtafena (6 samples), Ebeen (6 samples), and Rasoun (4 samples). Soil samples were collected by scraping off surface material with a spatula and then about 500 g was obtained from 3–5 cm beneath the surface. Each soil sample was thoroughly mixed and sieved through a sieve with 2-mm pore size.

2.2. Isolation of bacteria from forests

Isolation of Bt was done according to the method of Ohba and Aizawa (1986) followed by the method of Travers et al. (1987). One gram of each sample was suspended in sterile distilled water and heated at 80 °C for 30 min. Heat-treated or pasteurized suspension was diluted and plated on nutrient agar, and then incubated at 30 °C for 24 h. Selection of Bt isolates was done by adding 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 °C for 4 h and then heated to 80 °C for 3 min. Suspensions were diluted and plated on T3 medium (Travers et al. 1987). Cells were examined under light microscope to observe the parasporal crystals.

2.3. Serology

Serotyping of toxic *Bacillus thuringiensis* isolates was done according to the micromethod of Laurent et al. (1996): 90 µL of the bacterial suspensions was taken after 5–8 h of culture growth at 30 °C and placed in a 96-well microplate, and 10 µL of 2 dilutions of each antiserum (1:10 and 1:20) were used such that the final antisera dilutions were 1:100 and 1:200, respectively. In 2 wells, antiserum dilution was replaced by 150 mL of NaCl in deionized water, to be considered as the negative controls. The plates were incubated for 75 min at 37 °C. After incubation, positive reactions were observed as floccular sediment at the bottom of the well and a clear supernatant. The serotypes of the forest Bt isolates were determined by the 55 antisera that were kindly supplied by Dr Marguerite M. Lecadet (International Entomopathogenic *Bacillus* Collection Center (IEBC), Institut Pasteur, Paris, France). The reference Bt strains of serotype *israelensis* (IEBC No. T14001) and serotype *kurstaki* HD1 (IEBC No. T03A005) were used as positive controls.

2.4. Bioassay

The toxicity of Bt isolates was bioassayed against the third instar larvae of *D. melanogaster* according to the method of Karamanlidou et al. (1991). Ten third instar larvae were placed into each well of 24-well plates, and 0.3 mL of diet homogenate and 0.7 mL of Bt suspension (spore and crystals) were added to each well. The toxicity of each isolate was assayed in triplicate for either the original toxin–spore suspension or the diluted preparation. Plates were incubated at 25 °C for 24 h. Mortality was then scored in comparison with a parallel control of 0.7 mL of sterile distilled water instead of the toxin. Mortality was observed by viewing the brown midgut of dead larvae under a dissecting microscope. For *E. kuehniella*, the toxicity assay was performed in triplicate according to the method of Obeidat et al. (2004). The mortalities were corrected according to Abbott's formula (Abbott 1925) and the median lethal concentration (LC_{50}) values that killed 50% of the tested larvae were determined by log-probit analysis.

2.5. Maceration activity

The maceration activity of potato and carrot was performed according to the methods of Perombelon et al. (1979) and Delpech (2000). Potato tubers and carrot slices approximately 20–30 mm thick were surface sterilized by soaking for 10 min in 6% sodium hypochlorite, and then rinsed 3 times with sterilized distilled water. Two pieces from each plant were placed on sterilized filter papers in petri dishes. One of them was inoculated with Bt and the other was without inoculation, serving as a negative control. Filter papers were wetted with a sufficient amount of sterile distilled water, without excessive free water. The upper surface of each plant piece was lightly blotted with filter paper to remove any excess moisture before inoculation. A 50 µL solution containing 10^5–10^6 CFU mL^{-1} of each isolate was spread over the plant surfaces in the dishes, while 50 µL of sterile distilled water was spread on the negative control slices. Plates were incubated at 27 °C and frequently observed for maceration development after 3, 4, and 5 days. Three replicates were used for the maceration test. Maceration activity was assessed from the texture of the inoculated tissues with an inoculation loop. Positive results were estimated through tissue softening, whereas hard tissues under the same conditions were estimated to be negative.

2.6. Enzymes determination

The capability of Bt isolates to produce carboxymethylcellulase (CMCase) was studied by culturing these isolates on a carboxymethylcellulose agar medium (Carder 1986). Presence of a clear zone was used to indicate positive CMCase activity. Pectinase activity of Bt strains was determined according to the method of Bayoumi et al. (2008) by culturing the isolates on a pectin agar medium. The developing clear zones after incubation were investigated and taken as criteria for determining the pectinolytic productivity. For gelatinase productivity (Aneja 2003), gelatin agar medium was inoculated with the active cultures and incubated at 37 °C for 48 h and then kept at 4 °C for 2 h. Medium liquefaction indicates positive

gelatinase activity. Lipase hydrolytic activity analysis was performed according to the method of Sigurgísladóttir et al. (1993) by using agar plates, on which olive oil and calcium salt had been mixed. Precipitation of free fatty acids with calcium (white zones) was used as an indicator of hydrolytic activity after 2 h at 65 °C. The forest Bt isolates were tested for amylase activity by employing a zone-clearing technique (Atlas et al. 1995; Gomes et al. 2002) using a starch agar medium.

3. Results

In the current study, the occurrence of Bt was investigated in 16 forest soil samples representing 3 forests (Eshtafena, Ebeen, and Rasoun) in Ajloun in northern Jordan. The highest estimated viable bacterial count was 18.39×10^7 CFU g^{-1} from Ebeen and the lowest estimated count was from Rasoun (0.53×10^7 CFU g^{-1}) (Table 1). The estimated viable count of spore-forming bacteria was scored after the pasteurization step, and the viable count of Bt was scored after acetate selection. Table 1 illustrates that the highest estimated viable spore-forming bacterial count was 18.20×10^5 CFU g^{-1} from Eshtafena and the lowest count was from Rasoun (0.05×10^5 CFU g^{-1}). On the other hand, the highest estimated viable count of Bt was from Ebeen (14.24×10^4 CFU g^{-1}) and the lowest estimated count was from Rasoun.

The highest diversity of bacterial and Bt colonies was found in Eshtafena (7 diverse bacterial colonies and 5 diverse Bt colonies) (Table 2). The index of diversity ranged from 0.00 to 1.00 (Table 2). Thirty-nine isolates were identified as Bt according to the presence of parasporal crystals (Table 2). Only 23 of them were toxic to the third instar larvae of *D. melanogaster*. The toxicity of Bt was determined against the third instar larvae of *D. melanogaster* by the calculation of the LC_{50} (Table 3). Isolate 7_1 (serotype *israelensis*) from Ebeen showed the lowest LC_{50} (4.45), indicating its high larvicidal activity. It was found that 7 Bt isolates, which produced bipyramidal and cuboidal parasporal crystals of the serotype *kurstaki*, exhibited dual toxicity against *D. melanogaster* and *E. kuehniella*. As shown in Table 3, serotyping of the 23 toxic isolates subdivided them into 5 serotypes (*israelensis*, *kenyae*, *kurstaki*, *malaysiensis*, and *morrisoni*). Table 3 shows that most of the isolates produced spherical parasporal bodies, and most of them were cross-reacted with the *israelensis* serotype.

Table 4 illustrates that the hydrolytic activity of the forest Bt isolates was variable. Most of the isolates had CMCase, amylase, gelatinase, and lipase activity. The highest CMCase, pectinase, amylase, and lipase activities were found in isolates 5_1, 6_2, 3_3, and 1_1, respectively (Table 4). Interestingly, those isolates belonged to serotype *israelensis*. On the other hand, it was found that only 8 isolates were pectinase-positive. Maceration activity of the recovered isolates to potato and carrot samples proved that 16 isolates were able to macerate potato and 8 isolates were

Table 1. Viable counts of bacteria, spore-forming bacteria, and *Bacillus thuringiensis* (Bt) in soils of Ajloun forests.

Forest	Sample no.	Viable count of bacteria before pasteurization $g^{-1} \times 10^7$	Viable count of spore-formers after pasteurization $g^{-1} \times 10^5$	Viable count of Bt $g^{-1} \times 10^4$ (%)
Eshtafena	1	2.08	4.18	2.08
	2	6.29	3.98	1.98
	3	1.09	3.02	2.24
	4	14.39	4.95	4.36
	5	3.18	6.34	3.98
	6	6.78	18.20	12.30
Ebeen	7	6.33	9.54	8.08
	8	12.52	16.09	14.24
	9	4.46	2.18	2.36
	10	10.03	5.96	3.94
	11	8.17	13.24	10.12
	12	18.39	8.92	6.96
Rasoun	13	8.11	2.12	2.14
	14	11.24	9.04	5.04
	15	0.89	0.05	0.05
	16	0.53	0.08	0.00

Table 2. Isolation of *Bacillus thuringiensis* (Bt) from soils of Ajloun forests.

Forest	Sample no.	No. of different bacterial colonies	No. of different Bt colonies	No. of toxic Bt isolates	Index of diversity[a]
Eshtafena	1	6	5	1	0.83
	2	5	3	0	0.60
	3	3	3	3	1.00
	4	4	2	3	0.50
	5	2	2	2	1.00
	6	7	3	3	0.43
Ebeen	7	4	3	1	0.75
	8	2	1	2	0.50
	9	5	4	0	0.80
	10	4	3	1	0.75
	11	6	3	3	0.50
	12	3	3	2	1.00
Rasoun	13	3	1	0	0.33
	14	2	1	0	0.50
	15	2	2	2	1.00
	16	2	0	0	0.00
Total	16	60	39	23	

[a]Index of diversity: diversity of Bt colonies / diversity of total bacteria colonies.

Table 3. Parasporal morphology, serotyping, and larvicidal activity of forest *Bacillus thuringiensis*.

Forest	Isolate no.[a]	Parasporal morphology[b]	Serotype	LC_{50}[c]	
				D. melanogaster	*E. kuehniella*
Eshtafena	1_1	S	*israelensis*	5.41	-
	3_1	S	*malaysiensis*	6.28	-
	3_3	S	*israelensis*	6.32	-
	3_4	BP+C	*kurstaki*	5.09	6.56
	4_1	S	*kenyae*	6.49	-
	4_2	BP+C	*kurstaki*	6.38	7.62
	4_3	S	*morrisoni*	5.49	-
	5_1	S	*israelensis*	5.26	-
	5_3	S	*israelensis*	5.34	-
	6_1	BP+C	*kurstaki*	5.36	6.83
	6_2	S	*israelensis*	5.20	-
	6_3	BP+C	*kurstaki*	4.53	6.28
Ebeen	7_1	S	*israelensis*	4.45	-
	8_1	S	*kenyae*	6.28	-
	8_4	BP+C	*kurstaki*	6.57	8.26
	10_1	S	*israelensis*	6.15	-
	11_1	S	*israelensis*	6.36	-
	11_2	BP+C	*kurstaki*	6.62	7.48
	11_3	S	*morrisoni*	5.46	-
	12_1	S	*israelensis*	5.56	-
	12_3	BP+C	*kurstaki*	6.52	8.63
Rasoun	15_1	S	*israelensis*	6.32	-
	15_3	S	*israelensis*	6.30	-

[a]X_y: X is the sample number; y is the isolate number. [b]S: spherical; BP: bipyramidal; C: cuboidal. [c]LC_{50} = log (spore concentration mL^{-1}).

Table 4. Hydrolytic and rotting activities of forest *Bacillus thuringiensis* isolates.

Isolate (serotype)	CMCase (mm)	Pectinase (mm)	Amylase (mm)	Lipase (mm)	Gelatinase	Rotting of potato	Rotting of carrot
1_1 (*israelensis*)	-	-	10	12	-	+	-
3_1 (*malaysiensis*)	14	-	15	10	+	+	-
3_3 (*israelensis*)	13	-	16	8	+	+	+
3_4 (*kurstaki*)	15	-	12	-	+	+	-
4_1 (*kenyae*)	-	-	10	10	-	+	-
4_2 (*kurstaki*)	-	-	8	8	+	+	-
4_3 (*morrisoni*)	-	7	-	10	+	-	+
5_1 (*israelensis*)	16	9	10	9	+	+	+
5_3 (*israelensis*)	-	-	-	6	+	-	-
6_1 (*kurstaki*)	10	-	8	8	+	+	-
6_2 (*israelensis*)	12	10	8	7	-	+	+
6_3 (*kurstaki*)	7	-	6	-	+	+	-
7_1 (*israelensis*)	14	-	-	-	-	-	-
8_1 (*kenyae*)	7	-	8	8	-	+	+
8_4 (*kurstaki*)	-	8	10	-	+	+	+
10_1 (*israelensis*)	-	7	10	6	+	+	+
11_1 (*israelensis*)	-	8	-	6	-	-	-
11_2 (*kurstaki*)	10	-	-	8	+	-	-
11_3 (*morrisoni*)	-	6	6	7	-	+	-
12_1 (*israelensis*)	9	-	-	6	+	-	-
12_3 (*kurstaki*)	-	-	-	-	+	-	-
15_1 (*israelensis*)	9	-	8	-	+	+	-
15_3 (*israelensis*)	10	9	10	6	+	+	+

able to macerate carrot. All of the amylase-positive isolates were able to cause potato rotting. Most of the pectinase-positive isolates (6 out of 8 isolates) were able to cause the rotting of carrot. Isolates 3_3, 5_1, 6_2, 10_1, and 15_3 belonging to serotype *israelensis* exhibited a broad spectrum of hydrolytic and rotting activities (Table 4); isolates 5_1 and 15_3 were positive for all tested hydrolytic and rotting activities, whereas isolates 3_3, 6_2, and 10_1 were positive for the performed rotting and hydrolytic activities except for pectinase, gelatinase, and CMCase, respectively.

4. Discussion

Bt was isolated from 94% of the selected forest soil samples, suggesting that Bt is a common microflora in forest soils. The high total bacterial and Bt viable counts in Ebeen forest soil could be due to optimum survival and enrichment factors in that soil. From this study, it was found that Bt populations are considerably heterogeneous in terms of colony diversity, numbers, parasporal shapes, toxicity, and serotypes. This is in agreement with previous observations in other Asian habitats (Ohba and Aizawa 1986; Martin and Travers 1989; Hastowo et al. 1992; Obeidat et al. 2000; Almomani et al. 2004; Ammouneh et al. 2011).

The index of diversity of Bt in this study was mostly above 0.50. Therefore, it is comparable with the findings of Obeidat et al. (2000), who reported that the index of diversity in other Jordanian habitats was 0.67. Variation in the LC_{50} of the isolates indicates the variation in larvicidal activity. This was in accordance with the findings of Saadoun et al. (2001).

The results of this study clearly indicated that forest soils were richer in Bt than the soils of other habitats, as reported by Obeidat et al. (2000). This finding is in agreement with that of Landen et al. (1993), who reported that forest soil samples in southern Sweden were richer in Bt than soil samples collected from cultivated areas. Moreover, it was observed that the toxicity of forest Bt was higher than that reported by Saadoun et al. (2001) for other

Jordanian habitats. The dominance of spherical parasporal crystals and the *israelensis* serotype was in agreement with the findings of Saadoun et al. (2001) and Almomani et al. (2004), respectively.

It was found that Bt isolates toxic to the dipteran larvae of *D. melanogaster* have lower LC_{50} values than those toxic to the lepidopteran larvae of *E. kuehniella*, suggesting that *D. melanogaster* larvae are more susceptible to Bt toxins than *E. kuehniella* larvae. This difference in the susceptibility could be due to the fact that Bt toxins are host-specific (Sanchis et al. 1996). Moreover, Karamanlidou et al. (1991) proposed that the specificity of Bt toxins might vary due to the difference in the target insect species, which differ in their susceptibility to the mode of action of such toxins or due to the insect gut conditions.

Several previous studies (Karamanlidou et al. 1991; Meadows et al. 1992; Saadoun et al. 2001; Obeidat et al. 2004) suggested a possible relationship between the shape of the parasporal crystals and the toxicity. For instance, Bt isolates containing spherical or cuboidal crystals exhibited toxicity to Diptera, whereas the isolates that produced bipyramidal crystals exhibited only a toxicity to Lepidoptera. In accordance, it was found in the present study that Bt isolates producing spherical crystals were only toxic to the dipteran insect *D. melanogaster*, while Bt isolates that produced both cuboidal and bipyramidal crystals of the serotype *kurstaki* exhibited dual toxicity toward *D. melanogaster* and the lepidopteran insect *E. kuehniella*.

It was noticed that amylase-producing isolates were able to cause potato rotting, suggesting that potato rotting is due to amylase activity. A positive correlation between pectinase activity and carrot rotting can also be established because most of the pectinase-producing isolates were able to cause the rotting of carrot slices.

The larvicidal activity and hydrolytic activity of the recovered isolates suggest that forests are a rich source for insecticidal Bt isolates that have significant biodegrading activity toward plant residues, which may lead to an increase in soil fertility. Therefore, forest Bt can be applied to forests or to other plant environments to end up with 2 possible agricultural benefits: insecticidal and degradation activities that enhance plant growth and productivity.

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