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Isolation and Insecticidal Activity of *Bacillus thuringiensis* Strains Obtained from North Sinai, Egypt

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ABSTRACT

Although *Bacillus thuringiensis* (*B.t.*) is the most popular bacterium used in the biological control of insect pests , still there is a pressing need for isolating *B.t.* strains of new host range and/or better activity . This work was carried out to search for *B.t.* isolates in the unique ecosystem of North Sinai – Egypt.Thirty-six soil samples were collected to represent different locations and specifications. Soil samples were analyzed for total and spore counts and for occurrence and incidence of *B.t.*. The obtained isolates were classified as to their varieties using biochemical tests. The insecticidal activity of the isolated *B.t.* strains was tested against the larvae of both the cotton-leaf worm *Spodoptera littoralis* (Boisd.) and the mosquito *Culex pipiens* L... Polymerase chain reaction (PCR) was used for the detection of both the lepidopteran-toxin gene (*Cry I)* and the dipteran-toxin gene (*Cry IV)* .The results indicated that the ratio of spores to total bacterial counts ranged between 0.9–35.7 %, depending on the soil state and condition. Using acetate selection, 21 *B.t.* strains were obtained from 7 of the tested samples and comprised 3.8% of the investigated *Bacillus* isolates . *B.t.* was found only in the cultivated soils and its presence was in high percentage among loamy ones .The 21 obtained *B.t.* strains were found to be of 8 different biochemical profiles. Nine isolates were found active against the larvae of *Spodoptera littoralis* only (mortality $\geq$ 10%), and one strain showed dual activity against the larvae of both *S.littoralis* and *C.pipiens*. The LC_{50} values of the ten lepidopteran-active strains ranged between 0.2 – 12.2 x 10^7 spore/cm^2 of the diet surface. PCR results matched the bioassay in three of the ten lepidopteran-active isolates, but did not predict the potency of the dipteran-active strain. These results may lead to the possibility of the presence of new toxin gene(s) and/or silent one(s), leaving bioassay to be the most reliable tool for detecting the bioactivity of *B.t.* strains.

Key Words: Isolation, Insecticidal Activity, *Bacillus thuringiensis*, North Sinai, Egypt.

INTRODUCTION

Bacillus thuringiensis (*B.t.*) is one of the best known and most widely used microbial control agents against many important insect pests. Extensive studies about its delta-endotoxin crystals , mode of action , safety to mammals and non-target organisms have been realized (Faust and Bulla,1982; Luthy *et al.*,1982 ; Margalit and Dean,1985; Ceron *et al.*,1995; Aranda *et al*, 1996; Bernstein *et al.*,1999 ; Kim *et al.*,2000 and Nunez *et al.*,2001).

Although there are many B.t.-based products in the world market, there are non-stop attempts towards isolating *B.t.* strains bearing new host range and/or higher activity. This has lead researchers to search in virgin and unique habitats (Ibarra *et al.*, 2003). Thousands of *B.t.* strains have been isolated and tested all around the world, but little information about its distribution, incidence and ecology is available (Dulmage and Aizawa, 1982 and Saleh, 1990).

Many approaches were proposed for classifying *B.t.* to its varieties, but, yet, there is no one comprehensive scheme to be followed for that task. Biochemical testing is the simplest and cheapest method for identification of microorganisms, but molecular biology has provided many informative tools, among which PCR has proved to be an excellent way for detection of identified *B.t.* genes (Ibarra *et al.*, 2003).

The search for native *B.t.* strains-with activity against the agricultural key pest "*Spodoptera littoralis* (Boisd.)" (Lepidoptera) and/or the disease-vector "*Culex pipiens* L." (Diptera) - conducted in this study - focused on a virgin Egyptian ecosystem (*i.e.*, North Sinai) to give some information about the diversity and ecology of *B.t.* in that unique habitat . Biochemical and genetic tools were applied to characterize the obtained strains, and biological assessment was conducted to evaluate their actual potency.

MATERIALS AND METHODS

Thirty-six soil samples were collected from North Sinai, representing different types, conditions and treatments (Table 1). Samples from cultivated soils were taken from plant rhizospheres. All soil samples were air-dried and mixed thoroughly for analyses. Total and spore counts were conducted by surface inoculation on nutrient agar plates (in triplicates).

For isolation of *Bacillus thuringiensis* (*B.t.*) strains, one gram of each soil sample was added to acetate buffered L-broth (in flasks). The mixture was shaken for 4 h at 250 rpm and 30°C, and then heat-treated for 3 min. at 90°C. A diluted sample of the mixture was surfacely inoculated on L-agar and incubated for 24 h at 30°C (Travers *et al.*, 1987). Colonies were picked, transferred to T3-agar and incubated for 24h at 30°C for complete sporulation. Pure cultures were microscopically examined for the presence of crystals (Parry *et al.*, 1983)

Crystal-forming bacilli which produced acid from glucose but no acid from arabinose, xylose or mannitol, hydrolyzed casein, utilized citrate and grew on M9 medium and lecithin plates (regardless lecithinase production) were identified as *B.t.* (Parry *et al.*, 1983 and Harrigan, 1998).

B.t. isolates were classified as to their biotypes according to seven biochemical reactions; *i.e.*, fermentation of mannose, salicin and sucrose, production of urease and lecithinase , hydrolysis of starch and utilization of esculin (Harrigan , 1998) .

The obtained isolates of different *B.t.* biotypes were all subjected to PCR for detection of lepidopteran (*CryI*) and dipteran (*CryIV*) toxin genes (Carozzi *et al.*, 1991 and Ahmed ,1994) . PCR products were analyzed by

Table (1): Descriptions and specifications of soil samples.

Soil type	Soil condition	Vegetation	Location	N0.
Sand	Uncultivated	Plane desert	Naga Abu- Arad	11A
,,,	,,,	,,,	El- Fita	11F
,,,	,,,	,,,	Naga El- Kawadees	11K
,,,	,,,	Wild plants	Bir- Lihfen	21B
,,,	,,,	,,,	El- Fita	21F
,,,	,,,	,,,	El- Towyl	21T
,,,	Cultivated	Barley	Shadida	31S
,,,	,,,	Okra	Naga El- Lifetat	31L
,,,	,,,	Barley	Naga El- Kawadees	31K
,,,	,,,	Plum	El- Korea	41E
,,,	,,,	Olive	El- Towyl	41T
,,,	,,,	Olive	El- Fita	41F
Sandy loam	Uncultivated	Plane desert	Bir- Lihfen	12B
,,,	,,,	,,,	Om El- Rowisat	12M
,,,	,,,	,,,	El- Makdba	12D
,,,	,,,	Wild plants	Hasana	22H
,,,	,,,	,,,	Village of El-Torkomania	22V
,,,	,,,	,,,	Mountain of El-Torkomania	22N
,,,	Cultivated	Barley	Wady El-Fath and El-Masagid	32W
,,,	,,,	,,,	El- Fita	32F
,,,	,,,	,,,	Naga El- Kawadees	32K
,,,	,,,	Plum	Naga El- Kawadees	42K
,,,	,,,	Olive	El- Korea	42E
,,,	,,,	Almond	Shadida	42S
Loam	Uncultivated	Plane desert	Wady El- Mazer	13R
,,,	,,,	,,,	Village of El-Torkomania	13V
,,,	,,,	,,,	Mountain of El-Torkomania	13N
,,,	,,,	Wild plants	El- Korea	23E
,,,	,,,	,,,	Naga El- Lifetat	23L
,,,	,,,	,,,	Naga Abo- Arad	23A
,,,	Cultivated	Barley	Village of El-Torkomania	33V
,,,	,,,	,,,	Wady El- Mazer	33R
,,,	,,,	Tomato	Naga El- Kawadees	33K
,,,	,,,	Olive	Hasana	43H
,,,	,,,	Plum	Village of El-Torkomania	43V
,,,	,,,	Olive	Wady El- Mazer	43R

Table (2): Primers used in PCR screening*.

Primer	Sequence	Standard and Gene	Nucleotides
Lep I A	5' CCGGTGCTGGATTTGTGTTA 3'	*B.t. kurstaki Cry*IA(b)	310-330
Lep I B	5' AATCCCGTATTGTACCAGCG 3'	*B.t. kurstaki Cry*IA(b)	780-800
Lep 2 A	5'CCGAGAAAGTCAAACATGCG 3'	*B.t. kurstaki Cry*IA(b)	2158-2178
Lep 2 B	5' TACATGCCCTTTCACGTTCC 3'	*B.t. kurstaki Cry*IA(b)	3046-3066
Dip 1 A	5' CAAGCCGCAAATCTTGTGGA 3'	*B.t. israelensis Cry*IV	2551-2571
Dip 1 B	5' ATGGCTTGTTTCGCTACATC 3'	*B.t. israelensis Cry*IV	3328-3348
Dip 2 A	5' GGTGCTTCCTATTCTTTGGC 3'	*B.t. israelensis Cry*IV	740-760
Dip 2 B	5' TGACCAGGTCCCTTGATTAC 3'	*B.t. israelensis Cry*IV	2010-2030

*After Carozzi *et al.* (1991).

agarose gel electrophoresis for 2 h at 60 volts. The banding was visualized at short UV light (Sambrook *et al.*, 1989) . The primers used represented 8 different oligonucleotides specific to *CryI* and *CryIV* classes of toxin genes. The used primers' nucleotide sequences, corresponding genes and location on the gene are listed in Table (2).

Spore-crystal water suspensions, of the identified *B.t.* isolates, were prepared and their spore number was determined by plate count method. The suspensions were bioassayed against second- and third-instar larval Lab-colonies of the cotton leafworm *S. littoralis* and the mosquito *C. pipiens* , respectively (Shorey and Hale , 1965; Chapman and Barr , 1969 ; Gerberg *et al.* , 1969; Singh *et al.* , 1972 and Abou Bakr , 1978) . Biological assessment was conducted according to Abou Bakr (1978), Menon *et al.* (1982), Orduz *et al.* (1993) and Barbazan *et al.* (1998).

RESULTS AND DISCUSSION

Distribution of *B.t.* and sporeforming bacilli

Table (3) shows total bacterial and spore counts of the 36 soil samples. Spore counts ranged between 0.9 and 35.7% of the total bacterial counts. The percentage of spores in cultivated soils ranged between 0.9 and 8.1, while in uncultivated areas, the range raised to 7.5-35.7% (Table 4).

Previous studies of different soil samples from different regions indicated that the percentage of spore-formers in various soils depends on the soil state and conditions. The expected high organic matter content and frequent irrigation may explain the high bacterial content and low spore percentage in cultivated soils (Mahmoud, 1955; Moubarek, 1960; Taha *et al.*1965 and Amin, 2000).

The distribution of *B.t.* in soils obtained from different locations in North Sinai is shown in Table (5). Of 36 analyzed soil samples, seven (19.4%) contained *B.t.*, and of 556 *Bacillus* isolates from these soils, only 21 (3.8%) were *B.t.* . Generally, the ratio of *B.t.* to total *Bacillus* isolates in positive samples ranged between 4.4 - 33.3 %. These findings are in line with those reported by Saleh (1990) who showed that 3.36% of the tested bacilli were *B.t.* and the ratio of *B.t.* to total *Bacillus* isolates ranged between 3.13 – 40.63% after acetate selection. In both studies of Egyptian soils, the *B.t.* recovery was much lower than that reported by Travers *et al.* (1987) about the soils in USA.

Occurrence of *B.t.*

B.t. occurred in cultivated soil samples at relatively high percentage (38.9%), while in uncultivated samples no *B.t.* was isolated. Among cultivated soils, sandy and sandy-loam samples had the lowest *B.t.* occurrence (33.3%), while its highest occurrence was presented in loamy soils (50%) (Fig.1). These results indicate that the organism exists in a wide variety of cultivated soils in North Sinai , and matches with what was obtained by Saleh (1990) , and both studies indicate that *B.t.* seems to be more frequently found in cultivated Egyptian soils than in those of the USA (38.9% versus 17%) (DeLucca *et al.*,1981). The obtained results may indicate the relationship between the soil organic matter and/or water content on the one hand and *B.t.* occurrence on the other.

In this study, the ratio of *B.t.* to total *Bacillus* isolates in positive samples ranged between 4.4 to 33.3% which is quite similar to the results of Saleh (1990) where the ratios were 3.13-40.6% and those of DeLucca *et al.* (1981) where the rate was 75%. On the contrary, Travers *et al.* (1987) reported that 20-96% of the bacilli obtained after acetate selection were *B.t.* .

• *Bacillus thuringiensis* biotypes

The 21 obtained *B.t.* isolates were found to be of 8 different biochemical patterns, similar to the profiles previously named by Dr. Phyllis A.W.Martin, Insect Pathology Laboratory, USDA, Beltsville, Maryland, USA. (Personal communication) (Table 6).

The most commonly isolated biotype was *beijing* (8 isolates) and biotype *scanloniensis* was one of the least. This differs from some previous studies of *B.t.* In Egypt, Saleh (1990) found that *israelensis* and *scanloniensis* were the most common isolates, while Aizawa *et al.* (1975) and Ohba *et al.* (1979) found that varieties *sotto, dendrolimus, darmstadiensis* and *morrisoni* were more dominant in Japan. In Yugoslavia, Vankova and Purrini (1979) found that *kurstaki* was the most common, while in the USA, DeLucca *et al.* (1981) reported *kurstaki* and *galleriae* being the most frequently found types of *B.t.* . However , the diversity of varieties found in this study is in line with the findings of Martin and Travers (1989), which revealed that many soil samples contain several varieties and that the distribution of these varieties was unique to these samples .

• PCR product profiles

The PCR product profiles of 21 tested *B.t.* isolates are shown in Fig.(2) and Table (7). Analysis of these profiles showed that 6 isolates (28.57%) resulted in a single DNA fragment of *CryI* gene (0.490 kb) and two isolates (9.5%) produced 0.908 kb DNA fragment only. One of these isolates (no.71) showed both fragments suggesting the presence of a full length *CryI* gene typical for *B.t.* subsp. *kurstaki*. Concerning the isolates bearing only one DNA fragment of *CryI* gene, this could be regarded to the absence of the full length of the gene or the existence of gene(s) with partial homology to *CryI* class of genes. None of the tested isolates produced positive PCR profile characteristic for the presence of the dipteran-specific *CryIV* toxin gene.

• Insecticidal activity of *B.t.* strains

Qualitative toxicity tests of the 21*B.t.* isolates (Table 8) revealed that ten isolates were found active against second instar larvae of *S.littoralis*. Among these ten cultures, one (no. 141 of biotype *histoplasmosis*) showed moderate activity against second instar larvae of the mosquito *C.pipiens* (40% mortality at a concentration of 4.22×10^7 spore/ml). This was the only dipteran-active strain found. The activity of that strain is to be considered moderate to low, in comparison to eleven Egyptian strains that achieved LC_{50} values ranging between 12.9 and 0.53×10^2 spore/ml (Saleh, 1990). The LC_{50} values of native *B.t.* strains, against mosquitoes, were found 6×10^3 and

Table (3): Total spore counts per gram of dry soil.

Soil sample	CFU/g		Spore %	Soil sample	CFU/g		Spore %
	Total count × 10^5	Spore count ×10^5			Total count × 105	Spore count × 105	
11A	0.52	0.19	35.7	32W	75.6	4.69	6.2
11F	0.26	0.06	22.2	32F	97.0	5.53	5.7
11K	0.80	0.23	28.1	32K	71.1	4.62	6.5
21B	1.67	0.21	12.5	42K	86.1	1.29	1.5
21F	0.94	0.09	9.4	42E	75.4	3.09	4.1
21T	2.00	0.20	10.2	42S	80.7	2.58	3.2
31S	18.1	1.16	6.4	13R	2.27	0.35	15.2
31L	26.2	2.12	8.1	13V	2.81	0.33	11.7
31K	20.7	1.57	7.6	13N	3.26	0.56	17.1
41E	74.1	2.82	3.8	23E	5.02	0.38	7.5
41T	40.4	1.66	4.1	23L	9.51	0.87	9.1
41F	32.6	1.69	5.2	23A	11.1	1.22	11.0
12B	0.91	0.20	22.2	33V	30.4	0.94	3.1
12M	1.73	0.29	17.0	33R	45.6	1.92	4.2
12D	1.84	0.44	24.0	33K	80.1	0.72	0.9
22H	3.24	0.27	8.3	43H	70.5	1.4	2.0
22V	4.11	0.37	9.1	43V	85.1	0.94	1.1
22N	3.06	0.31	10.0	43R	75.6	1.06	1.4

Table (4): The range and average of total spore counts and their ratio in different soil types (counts / g weight).

Soil type	No. of Sample	Total count ×10^5		Spore count × 10^5		Spore %	
		Range	Average	Range	average	Range	Average
Cultivated							
Sand	6	18.1-74.1	35.35	1.16-2.82	1.84	3.8-8.1	5.87
Sandy loam	6	71.1-97.0	80.98	1.29-5.53	3.6	1.5-6.5	4.5
Loam	6	30.4-85.1	64.55	0.72-1.92	1.16	0.9-4.2	2.12
Uncultivated							
Sand	6	0.26-2.00	1.03	0.06-0.23	0.36	9.4-35.7	20.75
Sandy loam	6	0.91-4.11	2.48	0.20-0.44	0.31	8.3-24.0	15.1
Loam	6	2.27-11.1	5.66	0.33-1.22	0.62	7.5-17.1	11.9

Table (5): Incidence of *B. thuringiensis* in North Sinai.

Sample No.	No. of *Bacillus* isolates	*B. thuringiensis*		Sample No.	No. of *Bacillus* isolates	*B. thuringiensis*	
		No.	%			No.	%
11A	20	0	0	32W	20	0	0
11F	15	0	0	32F	5	0	0
11K	10	0	0	32K	16	1	6.3
21B	21	0	0	42K	13	4	30.8
21F	13	0	0	42E	13	0	0
21T	12	0	0	42S	18	0	0
31S	11	0	0	13R	17	0	0
31L	19	5	26.3	13V	9	0	0
31K	12	0	0	13N	11	0	0
41E	18	6	33.3	23E	21	0	0
41T	15	0	0	23L	14	0	0
41F	17	0	0	23A	19	0	0
12B	9	0	0	33V	12	0	0
12M	20	0	0	33R	10	1	10
12D	22	0	0	33K	25	0	0
22H	15	0	0	43H	23	1	4.4
22V	5	0	0	43V	11	0	0
22N	19	0	0	43R	10	3	30

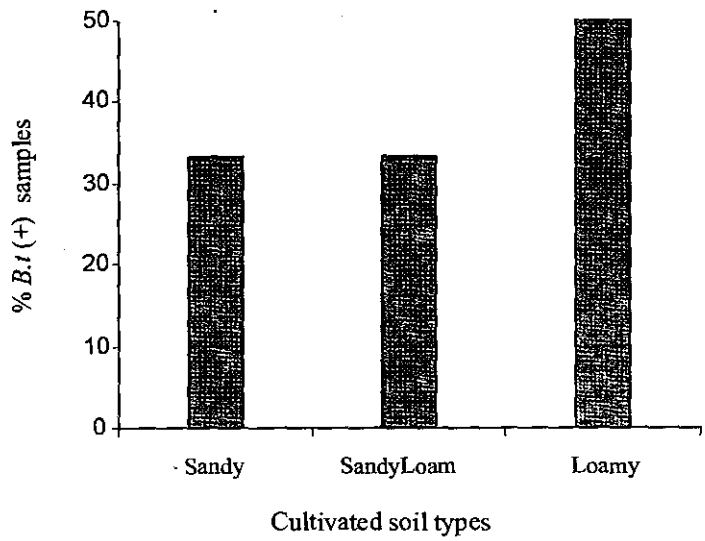

Fig.(1): Distribution of *B.t.* in relation to soils condition .

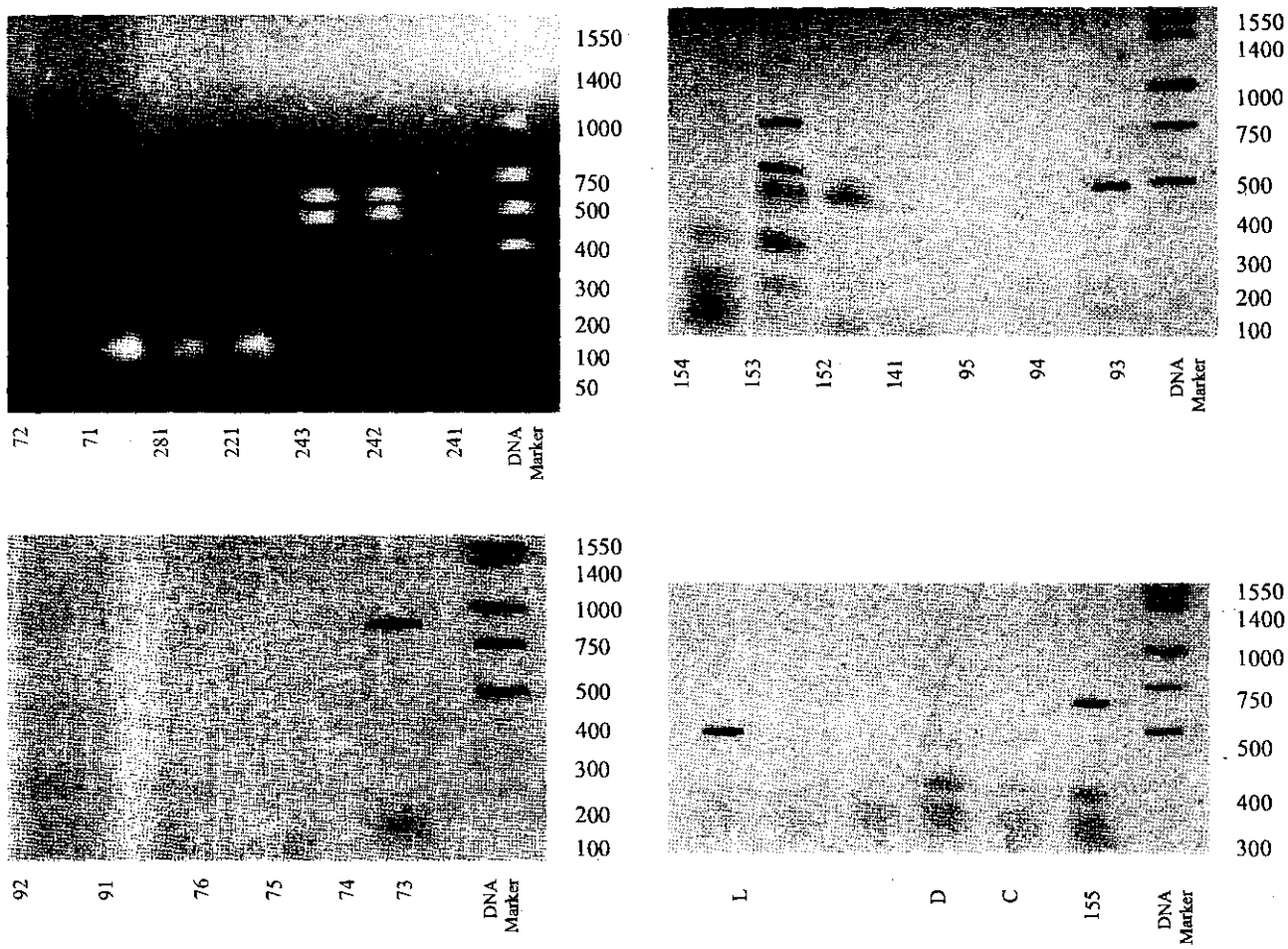

Fig. (2). PCR product profiles of isolated *B.t.*

Table (6): Biochemical patterns of isolated *B. thuringiensis* strains.

	Biochemical test													
Biotype	gl	mt	ar	xy	ca	ci	M-9	ma	st	ur	es	sa	le	su
jacksoni	+	-	-	-	+	-	+	+	+	+	-	-	+	+
heimpeli	+	-	-	-	+	-	+	-	+	-	-	-	+	+
racooni	+	-	-	-	+	-	+	+	+	+	+	-	+	+
beijing	+	-	-	-	+	-	+	-	+	+	-	-	+	+
histoplasmosis	+	-	-	-	+	-	+	-	-	+	+	+	+	-
kurstaki	+	-	-	-	+	-	+	-	+	+	+	+	+	-
thermopoliensis	+	-	-	-	+	-	+	-	+	-	-	+	+	-
scanloniensis	+	-	-	-	+	-	+	-	+	+	-	-	+	-

gl: glucose, ca: casein, st: starch, le: lecithinase, mt: mannitol, ci : citrate, ur: urease, su: sucrose, ar: arabinose, M-9: minimal growth medium, es: esculin, xy: xylose, ma: mannose, sa: salicin.

Table (7): PCR results of isolated strains and their insecticidal activity.

Isolate	Bioassay result	Possible activity		PCR product (bp)
		Lep.	Dip.	
71	Lep.	+	-	(490,908), (649)
72	Lep.	-	-	-
73	Lep.	-	-	(649)
74	-	-	-	-
75	-	-	-	-
76	-	-	-	-
91	-	-	-	-
92	Lep.	+	-	(490)
93	-	-	-	-
94	-	+	-	(490)
95	-	+	-	(490)
141	Lep./Dip.	-	-	-
152	Lep.	-	-	-
153	Lep.	-	-	-
154	Lep.	-	-	-
155	Lep.	+	-	(908)
241	-	+	-	(490)
242	Lep.	-	-	-
243	-	-	-	-
221	-	-	-	-
281	-	+	-	(490)

Table (8): Larvicidal effect of *B.thuringiensis* isolates against *C. pipiens* and *S. littoralis*.

Isolate No.	Concentration x 10^7 spore/ml or cm^2	Mortality %	
		C. pipiens	*S.littoralis*
Control	-	0	0
71	3.32	2	10
72	3.62	2	10
73	4.22	2	10
74	6.02	2	0
75	5.57	2	5
76	4.97	2	4
91	5.81	3	8
92	6.52	0	30
93	7.98	0	7
94	2.71	4	0
95	3.01	0	0
141	4.22	40	100
152	2.95	0	90
153	3.21	2	100
154	3.46	2	80
155	3.92	2	100
221	5.93	2	0
241	3.59	0	7
242	3.77	4	30
243	6.17	2	0
281	4.97	0	0

6 x 10^3 and 1-10 x 10^4 spore/ml respectively (Goldberg and Margalit, 1977 and Padua *et al.*, 1980).

Comparative bioassay of the ten lepidopteran-active strains, showed that their LC_{50} values ranged between0.2 and 12.2 X 10^7 spore/cm^2 of the diet surface (Table 9)

The most potent strain was of biotype *histoplasmosis* strain no. 141 (LC_{50} = 0.2X10^7 spore/ml). These results are similar to those reported for var. *kenyae* (ISPC-1 and ISPC-7) and var. *kurstaki* (ISPC-4) that ranged between 2.57-70.4X10^8 spore/cm^2 against *Heliothis armigera* (Kulkarmi and Amonkar, 1988) – both test insects are from the same order and family.

The insecticidal properties of the *B.t.* strains isolated in this work generally confirm the susceptibilities of the two test insects and the preferential toxicity of *B.t.* strains towards insect pests (Davidson,1982 and Andrews *et al*, 1987). The dual activity of some *B.t.* strains against insects of orders Lepidoptera and Diptera was previously reported by Hall *et al.* (1977), Panbangred *et al.* (1979), Ignoffo *et al.* (1980) and Saleh (1990).

Comparing the results of the PCR product profiles to those obtained from the bioassay (Tables 7 and 8) may lead to the possibility of the existence of new toxin gene(s) - for the strains showing entomocidal activity but negative PCR results - on the one hand, and silent toxin gene(s) - for the strains bearing the gene(s) but gave negative bioassay results- on the other .This may indicate that the bioassay would be the most reliable approach for detecting the bioactivity of *B.t.* strains, though PCR could still be an acceptable guideline for tracing the action of *B.t.* isolates.

It is worth to note that the isolates which did not show activity against any of the tested insects, require further investigation for their host spectra and may be of economic and applicable importance.

REFERENCES

Abou Bakr, H. (1978): Biological and Pathological Studies on the American Bullworm *Heliothis armigera* Hubner. M.Sc. Thesis, Faculty of Agriculture, Cairo University, 170 pp.

Ahmed, M.A.E. (1994): Cloning and Sequencing of Toxin Gene from a Local *Bacillus thuringiensis* Subspecies. M.Sc. thesis, Faculty of Science, Mansoura University.

Aizawa, K.; Fujiyoshi, N.; Ohba, N. and Yoshikawa, N. (1975): In "*Proc. First Intersect. Cong. I.A.M.S.*" 1974, 2, pp 597- 606, cited by Dulmage and Aizawa,1982.

Amin, A. O. (2000): Biodiversity of diazotrophs associated to natural vegetation of Sinai and their contribution to soil biofertility. M.Sc. Thesis, Faculty of Agriculture, Cairo University.

Andrews, R. E.; Faust, R. M.; Wabiko, H. and Raymond, K. C. (1987): The biotechnology of *Bacillus thuringiensis*. In: "*Critical Reviews in Biotechnology*" (Eds. Stewart and Russel) CRC Press Inc., Florida, *Vol. 6*, pp 163-232.

Aranda, E.; Sanchez, J.; Peferoen, M.; Guereca, L. and Bravo, A. (1996): Interactions of *B. thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda*. Journal of Invertebrate Pathology, 68(3), pp. 203-212.

Barbazan, P.; Baldet, T.; Darriet, F.; Escaffre, H.; Djoda, D.H. and Hougard, J. M. (1998): Impact of treatments with *Bacillus sphaericus* on *Anopheles* populations and the transmission of malaria in Maroua, a large city in a savannah region of Cameroon. Journal of American Mosquito Control Association, 14(1), pp 33- 39.

Bernstein, I. L.; Bernstein, J. A.; Miller, M.; Tierzieva, S.; Bernstien, D.I.; Lummus, Z.; Selgrade, M. J.K.; Doerfler, D.L. and Seligy, V.L. (1999): Immune responses in farm workers after exposure to *B. thuringiensis* pesticides. Environmental Health Perspectives, 107(7), pp.575-582.

Carozzi, N.B.; Kramer, V.C.; Warren, G.W.; Evola, and Koziel, M.G. (1991): Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Applied and Environmental Microbiology, 57(11), pp 3057-3061.

Ceron , J.; Ortiz, A.; Quintero , R.; Guereca , L. and Bravo , A. (1995): Specific PCR Primers directed to identify *Cry I* and genes within a *Bacillus thuringiensis* strain collection Applied and Environmental Microbiology, 61(11), pp.3826-3831.

Chapman, H. C. and Barr, A. R. (1969): Techniques for successful colonization of many mosquito species. Mosq. News, 29(4), pp 532- 535.

Davidson, E. W. (1982): Bacteria for the control of arthropod vectors of human and animal disease, In *"Microbial and Viral Pesticides"*, (Ed. Edward Kurstk), Marcel Dekker Inc., NY, pp 289-315.

DeLucca, A. J.; Simonson, J. G. and Larson, A. D. (1981): *Bacillus thuringiensis* distribution in soils of the United States. Can. J. Moicrobiol., 27, pp 865- 870.

Dulmage, H. T. and Aizawa, K. (1982): Distribution of *Bacillus thuringiensis* in nature. In " *Microbial and Viral Pesticides* ". (Ed. Kurstak), Marcel Dekker, Inc. NY. pp.209-237.

Faust, R. M. and Bulla, L. A. (1982): Bacterial and their toxins as insecticides. In "Microbial and Viral Pesticides". (Ed. Kurstak), Marcel Dekker, Inc. pp.75-208.

Gerberg, E. J. Hopkins, T.M. and Gentry, J. W. (1969): Mass rearing of *Culex pipiens*. Mosq. News, 29(3), pp 382-385.

Goldberg, L. J. and Margalit, J. (1977): A bacterial spore demonstrating rapid Larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univitatus, Aedes aegypti* and *Culex Pipiens*. Mosq. News, 37(3), pp 355-358.

Hall, I. M.; Arakawa, K. Y.; Dulmage, H. T. and Correa, J. A. (1977): The pathogenicity of strains of *B. thuringiensis* to larvae of *Aedes* and *Culex* mosquitoes. Mosq. News, 37(2), pp 246- 251.

Harrigan, W. F. (1998): Biochemical tests for identification of microorganisms, In "Laboratory Methods in Food Microbiology", Academic Press, California, pp 100- 118.

Ibarra, J.E.; del Rincon, M.C.; Orduz, S.; Noriega, D.; Benintende, G.; Monnerat, R.; Regis, L.; de Oliveira, C.M.F.; Lanz, H.; Rodriguez. M.H.; Sanchez, J.; Pena, G. and Bravo, A. (2003): Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. Applied and Environmental Microbiology, 69(9), pp.5269-5274.

Ignoffo, C.M.; Garcia, C.; Kroha , M.J. and Fukuda , T. (1980) : Susceptibility of *Aedes aegypti* larvae to four varieties of *B. thuringiensis*. Mosq News, 4 (2), pp 290-291.

Kim, H.S.; Yamashita, S.; Akao, T.; Saitoh, H.; Higuchi, K.; Park, Y.S.; Mizuki, E. and Ohba, M. (2000): In vitro cytotoxicity of non-cyt inclusion proteins of a *B. thuringiensis* isolate against human cells, including cancer cells. Journal Applied Microbiology, 89(1), pp. 16- 23.

Kulkarmi, U.V. and Amonkar, S.V. (1988): Microbial control of *Heliothis armigera* (Hb): Part I- isolation and characterization of a new strain of *B. thuringiensis* and comparative pathogenicity of three isolates of *B. thuringiensis* against *H. armigera. Indian* Journal of Experimental Biology, 26(9), pp 703- 707.

Luthy, P.; Cordier, J. and Fischer, H. (1982): *Bacillus thuringiensis* as a bacterial insecticide: Basic considerations and application. In "Microbial and Viral Pesticides " (Ed. Kurstak), Marcel Dekker , Inc ., pp.35-74.

Mahmoud, S.A.Z. (1955): Spore-formers occurring in soils. Their germination and biochemical activity. Ph.D. Thesis, Leeds University, England.

Margalit, J. and Dean, D. (1985): The story of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) J. American Mosquito Control Association, 1(1), pp.1-7.

Martin, P.A.W. and Travers, R.S. (1989): Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol.,55(10), pp.2473-2442.

Menon, K. K. R ; Rao, A. and Amonkar, S. V. (1982): Isolation, identification and toxicity of a spore-bearing *Bacillus* (ISPC-5) from diseased mosquito larvae., Indian Journal of Experimental Biology, 20, pp 312- 315.

Moubarek, M. S. M. (1960): Addition of organic manures to Tahreer soil and their effect on microflora and some plant nutrients. M.Sc. thesis, Faculty of Agriculture, Ain Shams University.

Nunez, Valdez, M.; Sanchez, J.; Lina, L.; Guereca, L. and Bravo, A. (2001): Structural and functional studies of alpha-helix 5 region from *B. thuringiensis Cry1Ab* delta-endotoxin. Biochem. Biophys. Acta, 9, 1546(1), pp. 122-131.

Ohba, M.; Aizawa, K. and Furusawa, T. (1979): Distribution of *B. thuringiensis* serotypes in Ehime Prefecture, Japan. *Applied Entomology Zoology, 14*, pp 340- 345.

Orduz, S.; Restrepo, N.; Diaz, T. and Rojas, W. (1993): Isolation and characterization of thirteen new strains of *Bacillus sphaericus* from Colombia toxic to *Culex quinquefasciatus* larvae. *Memorias, do, Instituto Oswaldo* Cruz, 88(4), pp 629- 632.

Padua, L.E.; Ohba, M. and Aizawa, K. (1980): The isolates of *Bacillus thuringiensis* serotype 10 with a high preferential toxicity to mosquito larvae. J. Invertebr. Pathol., 36, pp180-186.

Panbangred, W.; Pantuwatana, S. and Bhumiatana, A. (1979): Toxicity of *B. thuringiensis* toward *Aedes aegypti* larvae. Journal Invertebrate Pathology, 33, pp 340- 347.

Parry, J. M.; Turnbull, P. C. B. and Gibson, J. R. (1983): *A Color Atlas of Bacillus species*, Wolfe Medical Publications Ltd. England, *6*, p 269.

Saleh, W. D. (1990): Characterization and insecticidal activity of *Bacillus thuringiensis* strains isolated from Egyptian soils. M.Sc. Thesis, Faculty of Agriculture, Cairo University, 124 pp.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989): *Molecular Cloning: A Laboratory Manual*. Second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Shorey, H.H. and Hale, R.L. (1965): Mass rearing of the larvae of nine noctuid species on a simple artificial medium. J. Econ. Entomol., 58, pp 522- 524.

Singh, K.R.P.; Patterson, R.S.; La Brecque, G.C. and Razan, R.K. (1972): Mass rearing of *Culex fatigans*. WHO/VBC/72.386, p26.

Taha, S. M.; Mahmoud, S. A. Z.; El- Damaty, A. and Ibrahim, A. N. (1965): Effect of prolonged use of fertilizers on some chemical and microbiological properties of soil. The 1st Conf. Of Microbiology, U. A. R.

Travers, R. S.; Martin, P.A.W. and Reichelderfer, C.F. (1987): Selective process for efficient isolation of soil *Bacillus* spp. Appl. Environ. Microbiol., 53, (6), pp 1263- 1266.

Vankova, J. and Purrini, K. (1979): Natural epizootics caused by bacilli of the species *B. thuringiensis* and *B. cereus. Z. Angew Entomology, 88*, pp 216- 221, cited by DeLucca *et al.*, 1981.