ISOLATION, TOXICITY AND MOLECULAR CHARACTERIZATION OF NATIVE *Bacillus thuringiensis* ISOLATES FROM EGYPTIAN SOIL

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Bacillus thuringiensis (*BT*) is known to have pathogenic effects against several insects, worms and their effects on humans and beneficial insects are negligible (Sanchis and Bourguet, 2008). The toxicity of *Bt* is due to its ability to produce crystalline inclusions (Cry toxins) during sporulation, These inclusions are composed of proteins (Cry proteins) or δ-endotoxins which are highly toxic to a wide variety of insect pests and some invertebrates (Chattopadhyay *et al*., 2004; Vilas-Bôas *et al*., 2007). Recently, Cry toxins were classified based on their primary amino acid sequences, and more than 500different *cry* gene sequences have been classified into 67 groups (Bravo *et al*., 2011). During vegetative growth of *BT*, a number of pesticidal proteins unrelated to Cry proteins are produced by some strains (Mesrati *et al*., 2005). These toxins are named VIPs that do not form parasporal crystal proteins and are apparently secreted from the cell into culture medium. These proteins have shown toxicity against certain important lepidopteran pests like black cut worm (*Agrotis ipsilon*) and cotton leaf worm (*Spodoptera littolaris*), which could not be curbed earlier by the crystal toxins. Moreover, some of the lepidopteran insects that are susceptible to the Cry toxin at microgram level could be killed completely by the VIPs at nano gram level (Doss *et al*., 2002). Due to their high specificity and safety of *BT* to most non-target organisms and to the environment. The crystal proteins are preferred and widely used as an alternative to chemical pesticides in pest management strategies against insect pests of agricultural crops. At the same time, the wide spread use of *Bt* (Crystal Protein) in pest control has led to the potential for development of resistance by target organisms to *Bt* toxins (Liu *et al*., 2001; Sayyed *et al*., 2004). So, this has necessitated new isolation and novel strain that could to overcome the serious problem of evolved resistance by insects to the pesticidal activity of *BT* protein contributing to insect pests control and limit the use of toxic chemicals hazardous to the environment (Kamel *et al*., 2010). Therefore, the present study was aimed to search in the Egyptian soil for novel active isolates of *BT* that may be more efficient in using their toxin against insect pests of agricultural crops as cotton leaf worm and investigate their *Cry* and *Vip* genes using PCR techniques to determine the distribution of C*ry* and *Vip* genes within a collection of the studied isolates. In addition,

SDS-PAGE crystal protein and plasmid profiles were also studied in these isolates.

MATERIALS AND METHODS

1. Materials

1.1 Sample collection

Thirty three Soil samples were collected from nine locations in Egypt around Alexandria city and extending from Westward till Sewa Oasis where the samples were collected (Fig. 1) during 2006 season, these locations differed in their vegetation and cropping patterns as shown in Table (1). They were taken by scraping off soil surface with sterile spatula and 10 cm below the surface. 1kg/sample was placed in sterile plastic bags, covered with sterile cheesecloth and stored at 4°C. Two strains of *Bt kurstaki* (K) and *neoleonensis* H24a (N) were obtained from the Department of Entomology, Faculty of Agriculture, Alexandria University and Department of Biological control, Agriculture research center, respectively and used as standard (negative control) since their toxicity against this insect is well known.

1.2 Cotton leaf worm (Spodoptera littoralis)

Cotton leaf worm (*S littoralis*) was provided by the Department of Entomology, Faculty of Agriculture, Alexandria University. The artificial diet was prepared according to Shorey and Hale (1965). It was used to evaluate toxic potential of the studied isolates.

2. Methods

2.1 Isolation of bacterial strains

One gram soil sample was suspended in 20 mL of nutrient broth in a test tube (50 mL). Then heated to 80°C for 10 min to remove non-spore forming bacteria found in the soil. The samples were diluted 100-fold, and 200 μL of diluted sample was spread on a nutrient agar plate. After that, left to dry and incubated at least 3 days at 30°C.

2.2 Viability of bacterial spores and microscopic examination

The viability of bacterial spores was determined as describe by Zweig (1963). 1mLof bacterial culture was mixed with about 9 mL of sterile water. Two serial dilutions (10^{-3} and 10^{-5}) were prepared from this suspension as describe by Baker (1962) and Collin (1964). Dishes contain numbers of colonies ranged from 30-300 were studied. Ten single colonies with morphology similar to *Bt* were randomly selected from each plate after 72 h, stained with crystal violet and examined for the presence of endospores and parasporal inclusion bodies under phase contrast and light microscopes.

2.3 Insect toxicity assays

The toxicity of the studied isolates was screened on 1st instars neonate larvae of *S littoralis*. Three dilutions (1, 0.75 and 0.5 mL.) and fifteen 1st instars larvae of *S littoralis* were used for each dilution for studying the toxicity of protein crystals

and vegetative insecticidal proteins from the isolates. The LC_{50} values were determined for Cry and Vip extracts using probit analysis without correction, since the non treated controls gave mortality percentages less than 20% (Abbott, 1925).

2.3.1 Cry bioassay

Cry bioassay was preformed according to Kalfon and De Barjac (1985) with some modifications. Bacterial cell suspension were incubated more than 48 h for each bacterial isolate, 100 mL of culture was centrifuged under cooling (4°C) at 3000 rpm for 10 min. Bacterial pellets were resuspended in 5 mL of extraction buffer (Phosphate buffer 0.01 M, EDTA 5 mM, NaCl 0.1% and SDS 2%) for 2h and centrifuged under cooling (4°C) at 6000 rpm for 10 min. Pellets were taken and stirred in 50 mM NaOH for 1h. The insoluble particles were pelleted at 6000 rpm for 10 min and supernatant was stored at -20°C until usage. Mortality was recorded after 48; 72 and 120 h.

2. 3. 2 Vip bioassay

Vip bioassay was performed as describe by Estruch *et al*. (1996) with some modifications. Fresh bacterial culture was grown in PY broth medium for 12 h at 30°C for harvest the vegetative insecticidal proteins. After centrifugation at12,000 xg for 10 min at 4°C, the cell pellet was discarded and the supernatant containing the proteins of interest was retained. Proteins present in the supernatant were precipitated with ammonium sulphate (70% saturation) and collected by centrifugation at 12,000 x g for 10 min at 4°C. The pellet was re-suspended in minimum volume of 20 mM Tris HCl buffer (pH 7.4) and dialyzed overnight at 4°C. The crude extract obtained was subjected to ultra centrifugation at 56,000 x g for 30 min at 4°C to remove traces of suspended matter. The effect of vegetative insecticidal proteins (bacterial extracts not more than 18 h of culturing) was performed by recording the mortality.

2.4 Cry protein analysis using SDS-PAGE

The protein profiles of the studied isolates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as describe by Laemmli (1970). The proteins were separated on a 10% polyacrylamide gel and stained with Coomassie brilliant blue (CBB). Molecular mass of the produced peptide proteins were determined using protein standards (Promega, USA). Intensities of the band were assayed using image analysis software released by Bio-Rad Laboratories Inc., USA (Quantity One[*] 1-D software, version 4.5.2).

2.5 Plasmid isolation

DNA plasmid was isolated according to the method described by Kado and Liu (1981) and separated on 1% agarose. Electrophoresis was carried out at 75 volt for 2 h. The gel was visualized by UV light and photographed.

2.6 DNA extraction and Polymerase chain reaction (PCR)

Genomic DNA was extracted manually *via* applying phenol/chloroform solution technique. General primer gral-cry1 sequences (Bravo *et al*., 1998): (forward) 5'CTGGATTTACAGGTGGGGATAT-3' and (reverse) 5'TGAGTCGCTTCGCATATTTGACT-3' was used to amplify *Cry1* gene as well as V*ip3LB* (*Vip1*) primers (Mesrati *et al*., 2005): (forward) 5'ATGAACAAGAATAATACT-3' and (reverse) 5'TCTATTTGCAGACTTAGCGC-3' was used for V*ip3LB* (Vip1) gene and V*ip3Aa* (Vip2) primer (Selvapandiyan *et al*., 2001): (forward) 5'AGTTTACAAGAAATAAGTGTTA-3' and (reverse) 5'CCTACCATTACATCGTGGAAT-3' was used for amplification *Vip3Aa* (Vip2) gene. PCR was performed in a reaction volume of 25 µL containing 1µg DNA template; 1 µLof 200 pmol/µL of each primer; 12.5 µL of 2x PCR master mix and 9.5 µL of sterilized distilled water. Thermal cycling of PCR reaction (Gene Amp PCR System 9700) was carried out with an initial denaturation at 95°C for 5 min, followed by 34 cycles each at 95°C for 1 min, annealing temperature at 54°C for 1 min. Polymerization temperature was at 72°C for 1 min and final extension at 72°C for 10 min then the samples were held at 4°C. The PCR products were analyzed in 2% agarose gel (GibcoBRL) at 90 Volt for 2 h, bleached in ethidium bromide, bands visualized on a UV Transilluminator and photographed by gel documentation system (Alpha-chem. Imager, USA).

2.7 Statistical analysis

Probability test (Fisher, 1935) was used to estimate the significant differences between the isolates and the reference strains. The probabilities were calculated according to the 2x2 contingency tables using a simple basic computer program suggested by Forbes (1984).

RESULTS AND DISCUSSION

1. Characterizations of B. thuringiensis

Figure (2) showed phase contrast and light microscope photomicrographs of the fixed preparations for five isolates which stained with crystal violet. The results revealed that five isolates as AL3, AL7, AL11, KD2 and KD3 were only identified as *Bt* based on the ability of bacteria to form endospores and parasporal crystals, irrespective of their crystal shapes. These isolates were only distributed in the soil of Alexandria and Kafr -el-Dawar where, AL3 and AL7 isolates showed typical Bacilli characteristics with endospore inclusions under light microscope and gave a scarce number of parasporal crystals under phase contrast microscopy as shown in Figs (2A and 2B). The other three isolates AL11, KD2 and KD3 produced noticeable number of parasporal crystals as the other typical *Bt* characteristics (Figs 2C, 2D and 2E). No significant differences were detected in the average numbers of endospores and

protein crystals between the isolates and the reference strains except AL3 isolate which illustrated significant differences in the average numbers of protein crystals as shown in Table (2). These results indicated that AL7, AL11, KD2 and KD3 isolates were presumptively confirmed as *Bt* and can be studied as a local strain of this bacterium. Previous studies suggested that *Bt* can be identified by conventional methods like morphological and microscopic characters (Keshavarzi, 2008). In addition, these isolates obtained from the soil characterized with dense cropping as Alexandria and Kafr -el-Dawar. In contrast, no possible *B. thuringiensis* was identified from the other locations with specific limited vegetation and mostly characterized as calcareous soils as Urabi; El-Agami; Janaklies; Burg - el - Arab; Mareena; Mersa Matrouh; and Sewa. The present results are in accordance with Thaphan *et al.* (2008) and Raymond *et al.* (2010) who reported that the properties of the soil could affect the abundance and distribution of *Bt*. The presence of *Bt* population in the soil samples was affected negatively by soil sand percentage and this may because it contains a smaller amount of nutrient and water. Vilas-Bôas and Lemos (2004) stated that the recovery of the bacterium is more frequently from agricultural soils and to some degree from urban soils, however in beach soil and desert sands the incidence of this bacterium may be very low or absent and this is probably explained by the variation of the biotic and abiotic factors in these different soil habitats and their possible effects on the distribution of *B. thuringiensis*.

2. Bioassay of insecticidal potentialities

According to the LC_{50} values and mortality percentage of cotton leaf worms after the treatment with bacterial Cry protein extracts, it can be noticed that the two reference strains are highly toxic to cotton leaf- worm since they showed the lowest LC_{50} values of 0.192 and 0.242, respectively with their mortality percentages more than 75%. The most effective isolate against cotton leaf worms was AL11, KD3, KD2 and AL7 isolates in order which recorded LC_{50} values of 0.313, 0.5710, 0.708 and 0.908, respectively with mortality percentage around 50%. So, these isolates can be considered as moderately toxic. On the other hand, the isolate AL3 illustrated very high LC_{50} value (1.342) and turned to be non toxic because it showed mortality percentage less than 25% comparing with the two N and K reference strains as presented in Table (3) and illustrated by Fig. (3).

Table (4) and Fig. (4) showed bacterial cultures mainly in the log phase of growth that were used as the source of vegetative proteins (VIPs) to test their toxicity against cotton leaf worm. Among the five isolates used, only three isolates KD3, KD2 and AL11 showed the lowest LC_{50} values of 0.111, 0.222, 0.244, respectively, with mortality percentages more than 75%. These isolates can be considered effective against Cotton leaf worms (*Spodoptera litoraltis*). The reference strain "K" as well as isolates AL3 and AL7 showed differential toxicity and they can be considered as moderately tox-

ic because their LC_{50} values ranging from 0.49 to 0.98. However, "N" strain recorded the highest LC_{50} (1.1769) value with mortality percent less than 25% accordingly this isolate can be considered not toxic. The present results indicated that the two isolates of Kafr - el-Dawar (KD2 and KD3) and a certain extent Alexandria isolate (AL11) are promising as local strains of this bacterium since, they manifested high toxicity of vegetative insecticidal proteins (VIP) and displayed moderately toxic against cotton leaf-worm due to their insecticidal crystal proteins (Cry proteins). This may point to the potential of these isolates as bioinsecticide against cotton leaf worm (*S. litoraltis)*. The quantitative and qualitative difference in production and composition of protoxin of *B. thuringiensis* isolates is postulated by various investigators, Chenot and Raffa (1995) and Kashyap and Amla (2007) reported that the larval stage and the LC_{50} values reflect that more than one protoxin is present and they are efficiently processed within the gut to provide sufficient active toxin molecules in larvae.

3. Analysis Insecticidal proteins of bacteria (Cry protein) using SDS-PAGE

Surface protein profile analysis is used in the determination of similarity between bacterial strains (Costa, 1992). Comparison of the protein patterns had been reported to be useful in evaluation of the relationship between *Bt* isolates (Swiecicka and De Vos, 2003). In the present study, two reference strains were used. The first was *Bt* var. *kurstaki* (K)

that is known to have at least Cry I (135 kDa) and Cry II (70 kDa) polypeptides. The other reference, *i.e.* *Bt* var. *neoleoninsis* (N) that is known to have the same *cry* genes in addition to a *cyt* genes coding for hemolytic and cytolytic toxins but with very limited insecticidal activities (Kashya and Amla, 2007). Figure (5) shows photograph and diagrammatic drawing representing the SDS-PAG patterns of the present five isolates and the two reference strains. The results show strain "K" revealed 8 bands in total. Among these bands, 4 main bands with molecular mass of 135, 70, 28 and 10 kDa were observed. The 135 kDa band was assumed to be Cry 1 protein and 70 kDa can be resembled Cry 2 protein .The reference strain "N" illustrated similar total number of bands but it was characterized with a band with a molecular mass of about 80 kDa that is most probably corresponding to the Cyt polypeptide. Isolates KD2, KD3 and AL11 showed similar profiles as those of the "K" strain. Isolate AL7 showed less numbers of bands with lower molecular mass of polypeptides. In contrast, isolate AL3 did not display any noticeable bands when compared with the references or the other isolates.

Figure (6) illustrates differences in band intensities of the four main polypeptides (135, 70, 28 and 10 kDa) relative to the reference strain K. The highest intensities was the two known Lepedopetrian toxic polypeptides (135 and 70 kDa) that were recorded for KD2 and KD3. Concerning the reference strain N as well as the isolates AL11 and AL7 were exhibited

lower intensities than the others. While, the isolate AL3 did not show any noticeable intensity in polypeptides because it did not produce any band. The present results suggested that the two KD2 and KD3 isolates are promising novel *Bt* strains whereas, they are capable to produce of CryI and Cry2 polypeptides, in addition to other toxic and non toxic crystal proteins. Furthermore, these two isolates *i.e.* KD2 and KD3 are characterized with their toxic vegetative insecticidal proteins (Vip) production when compared with the other isolates used in the present study. The present results are in accordance with the previous results of Kashyap and Amla (2007) and Fakruddin *et al*. (2012) who obtained 47 isolates from 53 soil samples from diverse agro forest fields in Bangladesh. These isolates generate proteins by 12% SDS-polyacrylamide gel electrophoresis (PAGE) with molecular mass of 10-150 kDa that were almost similar to those of the reference strains of *Bt* subsp. *kurstaki* HD-73, *Bt* subsp. *sotto* and *Bt* subsp. *japonensis*) which confirmed the identity of these isolates as *Bt*.

4. DNA Plasmid profile of the isolates

The two reference strains and the five local isolates except AL3 isolate (Lane 7) showed a molecular weight band corresponding to about 4 kb. Also, the intensity of the separated bands decreased in order of K, N, KD2, KD3 and AL11 respectively as shown in Fig. (7). Based on the present results, it can conclude that the intensity of the separated plasmids decreased in order this may could reflect

decreasing in plasmid copy numbers; however, this cannot be confirmed due to inaccuracy in loaded samples. Also, the present results displayed that AL3 isolate doesn't have any plasmid and this result confirmed the previous findings that the isolate AL3 wasn't able to reveal any noticeable Cry protein band and its Cry extraction was non toxic against cotton-leaf worm thus, its inefficiency could be attributed to the plasmids missing in it. Gonzazel *et al*. (1984) reported that isolates of the same *Bt* subsp are known to contain Cry protein on their plasmids of low copy number and this causes variation in the plasmid profiles. Attallah *et al*. (2014) found that plasmids with molecular weight ranging between 27344 bp to 3958 bp were observed in sixteen *Bacillus* local strains isolated from different Egyptian soils and the identified strains; (*Bt.* subsp. *Kurstaki* (*Bt.* 40), *B. subtilis* subsp. subtilis strain ATCC 168 (*Bs*) and *B. licheniforms* strain ATCC (14580) were shown at least one plasmid, which some of them have a maximum of seven plasmids. In general, all plasmid patterns are unique to each strain.

5. Polymerase chain reactions (PCR)

5.1 Detection of Cry gene

A band with 550 bp approximately was successfully amplified for *Cry I* gene with the presence of a gral Cry I general primer in all samples except the isolate AL3 failed to produce any band as shown in Fig. (8). The result was in agreement with Bravo *et al*. (1998) who used the same primer for obtaining an amplified

fragment for C*ry I* gene that ranged from 543 to 594 bp in length. Performing PCR analysis with specific C*ry* gene primers could specify this amplicon (Ceron *et al*., 1995). The absence of C*ry I* gene in AL$_3$ genome (Fig. 7, lane 7) confirmed and explained the absence of any Cry protein in this isolate and no insecticidal activity was observed for this isolate against cotton leaf-worm. Similar results were obtained by Zhang *et al*. (2000) who, identified isolates of *Bt.* with no *Cry* gene and no insecticidal activity. In contrast, the reference strain N showed 4 other bands 2200, 1100, 900 and 800 bp in lengths. These bands may reflect the non-specificity of gral cry1 primer, which can detect 25 of the 27 different *cry1* genes (Bravo *et al*., 1998). In addition, many authors identified more than one *cry I*-type gene in the genomes of *Bt.* (Lee *et al*., 2001; Martinez *et al*., 2005). Thus, the presence of more than an amplicon in the genome of N reference strain may be due to the amplification of more than one *cry I*-type gene. Also, this feature may explain the high toxicity of the N strain Cry extract against the cotton leaf-worm. Zhang *et al.* (2000) stated that there is a correlation between the existences of more than one cry*I*- type gene and the high insecticidal effects

5.2 Detection of Vip genes

Many *Bt.* strains secrete non δ- endotoxins vegetative insecticidal proteins (Vip1, Vip2 and Vip3) during vegetative growth. VIPs represent a new type of insecticidal proteins because they are secret-

ed as soluble proteins, rather than forming crystal inclusions inside the *B. thuringiensis* mother cell (Warren, 1997). In the present study, the genome of the five isolates and the reference strain (K) succeed to amplify only one band with 400 bp approximately in length in presence of specific Vip1 primer that represent *vip3LB* (*Vip1*) gene while, the reference strain (N) failed to produce any amplified fragment with the same primer as shown in Fig. (9). Mesrati *et al*. (2005) obtained a sharp band with 419bp in size coding for *vip3LB* (Vip1) gene. While, Vip2 primer failed to react with any genome of the isolates or reference strains under the present study. It can suggest that the genome of the reference strain N do not comprise the *vip3LB* (Vip1) gene as well as, *vip3Aa* (Vip2) gene do not exist in any genome of the local isolates or reference strains in this study. Osman (2010) stated that the existence reduction of *Vip1/Vip2* genes in *Bt.* is perhaps due to the fact that these two genes have a wider distribution in *B. cereus* than in *Bt.*, However, the insecticidal effects of Vip proteins for the five local isolates and the reference strain K are probably due to the presence and the expression of a vegetative insecticidal gene with high homology to the *vip3LB* gene specially, the high toxicity of VIPs extracts for KD2; KD3 and AL11 isolates that could be attributed to the high expression of this gene. In relation to the reference strain N, the absence of both *vip* genes (*vip3LB* and *vip3Aa*) and their protein in it mostly reflected the non-toxicity of its Vip extracts against cotton leaf-worm. It can conclude from our investiga-

tion that, Egyptian agricultural soils rich with dense cropping are important source for providing a large genetic resource of *B. thuringiensis* strains. KD2, KD3 and AL11 isolates may confer commercial applications in agriculture and may lead the isolates to be identified as potential strains for their use in the development of bioinsecticide to control insect pests in Egypt.

SUMMARY

Thirty three local soil samples were collected from West-ward till Sewa Oasis in Egypt to search novel isolates of *Bacillus thuringiensis* (*Bt*) and evaluate their toxic potentiality to overcome the serious problem of evolved resistance by insects to the pesticidal activity. The first instars larvae of cotton leaf worm (*Spodoptera littoralis*) were used to test their toxic potentiality in the presence of the two standard strains *kurstaki* (K) and *neoleonensis* H24a (N). The results showed that three isolates from Alexandria (AL3, AL7 and AL11) and two isolates from Kafr - el-Dawar (KD2 and KD3) were presumptively confirmed as *Bt*. by morphological and microscopic characters. The treated larvae with Insecticidal crystal protein (ICP) exhibited mortality percentage around 50% for all isolates except AL3 isolate. The vegetative insecticidal proteins (VIPs) of KD3, KD2 and AL11 isolates revealed mortality percentages more than 75%. While, AL3 and AL7 isolates showed differential toxicity. Crystal proteins analysis by SDS-PAGE showed KD2, KD3 and AL11 isolates gave similar profiles as those of "K" strain which was characterized with 135 kDa and 70 kDa bands. AL3 isolate failed to show any noticeable bands. According to their plasmids patterns, 4 kb was shown in all isolates and the reference strains except AL3 isolate. *Cry1*, *Vip1* and *Vip2* genes of the isolates was detected by polymerase chain reaction (PCR). The results indicated that one band with 550bp in size was present in all isolates except the AL3 isolate. *Vip1* primer succeeds to amplify a band 400 bp in size in all isolates and "K" strain. *Vip2* primer failed to react with any genome of the studied isolates or reference strains. This study suggested that KD2, KD3 and AL11 isolates may lead to be identified as potential strains of *Bt*. for their use in the development of bioinsecticide to control insect pests in Egypt.

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Table (1): Cropping patterns of different locations in Egypt where soil samples were collected.

location	Abbreviation	No. of samples	Sample type
Alexandria	AL	14	Different seasonal crops
Kafr -el- Dawar	KD	3	Different seasonal crops
Urabi	UR	3	Olive arcades
El -Agami	AG	1	Fig field
Janakleis	JA	1	Grape yard
Burg – el – Arab	BA	8	Different fruit arcades
Mareena	MA	1	Ornamental garden
Marsa Matrouh	MM	1	Olive arcades
Sewa	SE	1	Olive arcades

Table (2): Average numbers of endospores and parasporal crystals per field for the five isolates (AL3; AL7; AL11; KD2 and KD3) and the two reference strains (*B. thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *neoleonensis*).

Isolates	Mean spore number per microscopic filed ± standard error	Mean crystal number per microscopic filed ± standard error
K	23.12 ± 2.08	11.36 ± 0.56
N	22.43 ± 1.87	9.14 ± 0.54
AL3	23.53 ± 2.15	3.29 ± 0.37*
AL7	22.81 ± 1.98	8.36 ± 0.63
AL11	24.08 ± 2.23	10.21 ± 0.58
KD2	22.27 ± 2.62	8.66 ± 0.72
KD3	22.78 ± 1.79	8.43 ± 0.54

* Significant at $p < 0.5$ K = *B. thuringiensis* var. *kurstaki*
N = *B. thuringiensis* var. *neoleonensis*

Table (3): Represents LC50 values for Cry extracts of the two reference bacteria (*B. thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *neoleonensis*) and the five isolates (AL3, AL7, AL11, KD2 and KD3).

Bacillus thuringiensis subspecies	Dilution									LC_{50}
	1 ml			0.75 ml			0.5 ml			
	T	S	S%	T	S	S%	T	S	S%	
K	45	5	11.11	45	13	28.89	45	16	35.56	0.242
N	45	1	2.22	45	9	20.00	45	14	31.11	0.192
AL3	45	30	66.67	45	33	73.33	45	40	88.89	1.342
AL7	45	24	53.33	45	19	42.22	45	34	75.56	0.908
AL11	45	17	37.78	45	19	42.22	45	21	46.67	0.313
KD2	45	7	15.56	45	26	57.78	45	29	64.44	0.708
KD3	45	17	37.78	45	19	42.22	45	24	53.33	0.571

T: Total number of treated larvae   S: total number of surviving larvae
S%: percentages of surviving larvae

Table (4): Represents LC50 values for Vip extracts of the two reference bacteria (*B. thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *neoleonensis*) and the five isolates (AL3, AL7, AL11, KD2 and KD3).

Bacillus thuringiensis subspecies	Dilution									LC_{50}
	1 ml			0.75 ml			0.5 ml			
	T	S	S%	T	S	S%	T	S	S%	
K	45	5	11.11	45	7	15.56	45	25	55.56	0.496
N	45	25	55.56	45	30	66.67	45	33	73.33	1.177
AL3	45	5	11.11	45	19	42.22	45	27	60.00	0.620
AL7	45	22	48.89	45	26	57.78	45	28	62.22	0.986
AL11	45	2	4.44	45	3	6.67	45	17	37.78	0.244
KD2	45	0	0.00	45	5	11.11	45	15	33.33	0.222
KD3	45	4	88.89	45	16	35.56	45	13	28.89	0.111

T: Total number of treated larvae   S: total number of surviving larvae
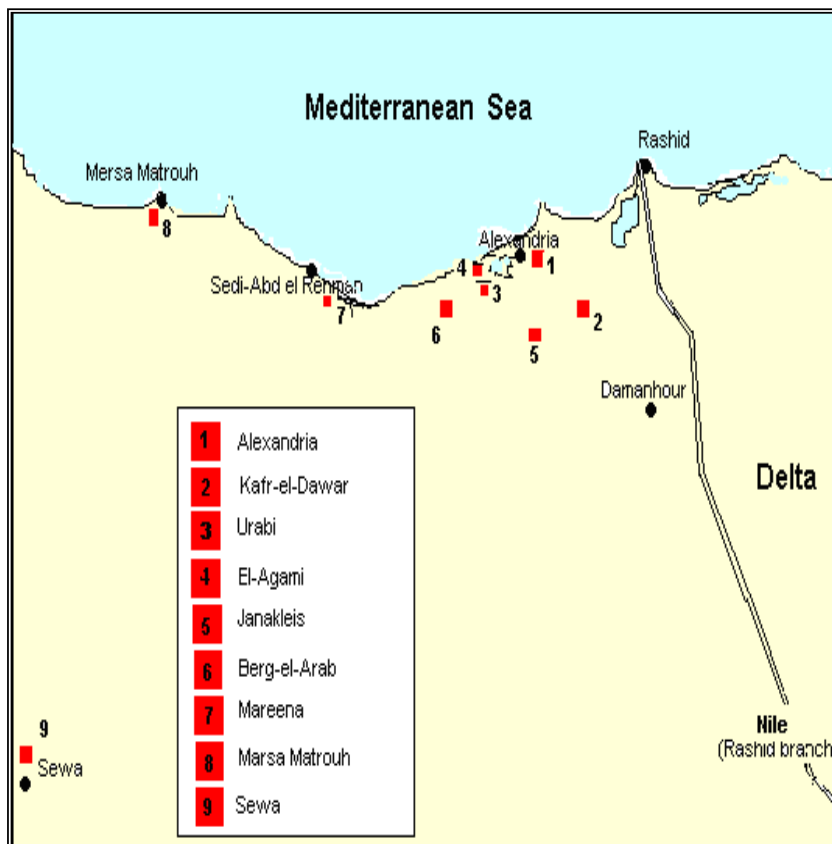S%: percentages of surviving larvae

Fig. (1): Map of the North-western coast of Egypt, showing the locations of the sites from which the soil samples were collected. Red squares indicate sites from which toxic *Bacillus thuringiensis* was isolated

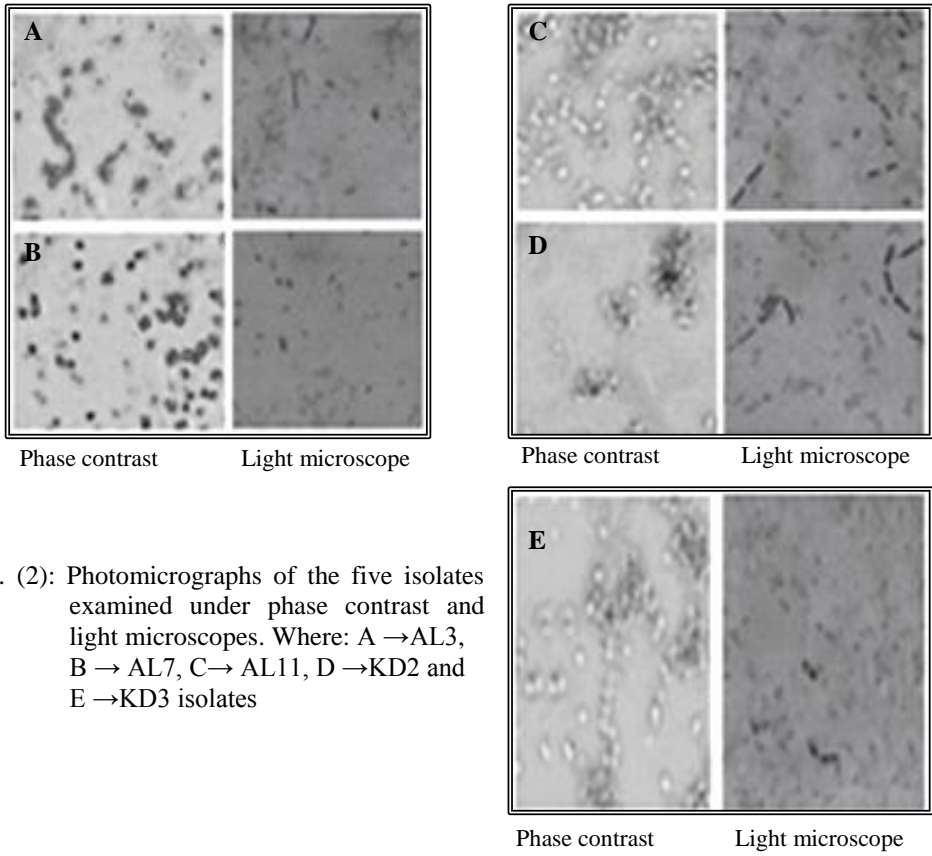Phase contrast   Light microscope   Phase contrast   Light microscope

Fig. (2): Photomicrographs of the five isolates examined under phase contrast and light microscopes. Where: A →AL3, B → AL7, C→ AL11, D →KD2 and E →KD3 isolates
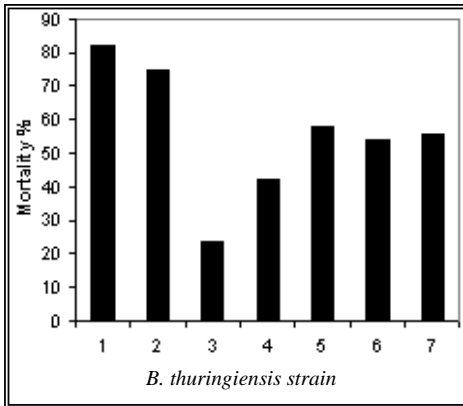
Phase contrast   Light microscope

Fig. (3): Mortality percentages of cotton leaf-worm (*Spodoptera litoraltis*) after treatments with Cry bacterial protein extracts of the two reference strains and the five *B* isolates. 1: K, 2: N, 3: AL3, 4: AL7, 5: AL11, 6: KD2 and 7: KD3.
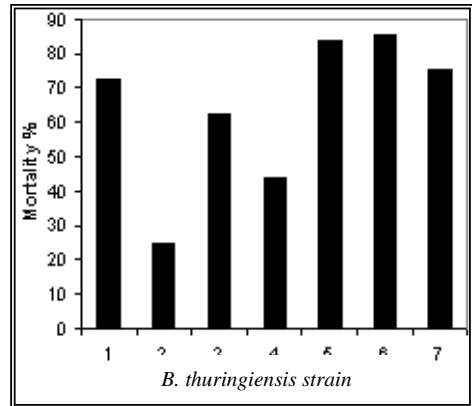
Fig. (4): Mortality percentages of cotton leaf-worm (*Spodoptera litoraltis*) after treatments with VIP bacterial extracts of the two reference strains and the five *B* isolates. 1: K; 2: N; 3: AL3; 4: AL7; 5: AL11; 6: KD2 and 7: KD3.
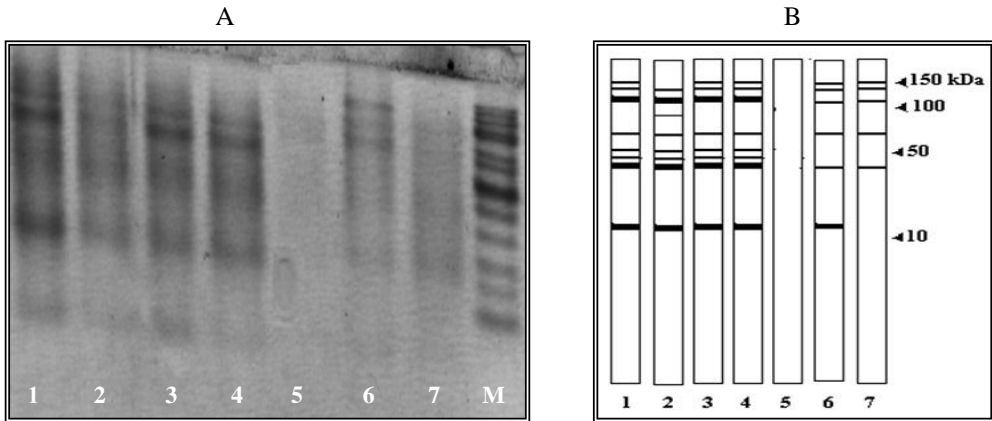
Fig. (5): Representing a photograph (A) and diagrammatic drawing (B) of Cry protein profile by SDS-PAGE for the five isolates and the two reference strains. Where: (1) K strain *B. thuringiensis* var, *kurstaki* and (2) N strain "*B. thuringiensis* var. *neoleonensis*", (3) KD2, (4) KD3, (5) AL3, (6) AL11, (7) AL7 isolates and (M) marker protein.
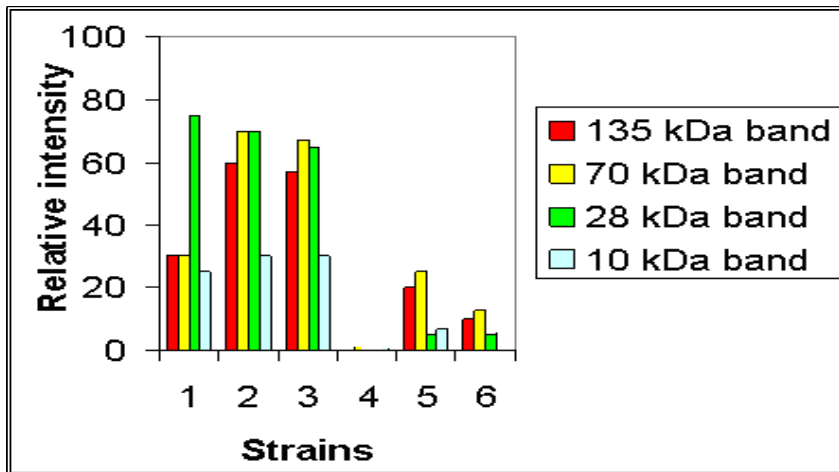

Fig. (6): Intensities of the main polypeptides (135; 70; 28 and 10 kDa) separated by SDS-PAGE relative to those of the reference strain "K" "*B. thuringiensis* var. *kurstaki*"where: (1) Strain "N" "*B. thuringiensis* var. *neoleonensis*" (2) KD2, (3) KD3, (4) AL3, (5) AL11 and (6) AL7 isolates.

Fig. (7): Plasmid profile of the five isolates and the two reference strains. Where: (1) K strain *B. thuringiensis* var, *kurstaki* and (2) N strain *"B. thuringiensis* var. *neoleonensis"*, (3) KD2, (4) KD3, (5) AL11, (6) AL7, (7) AL3 isolates and (M) DNA marker (2000-100 bp).
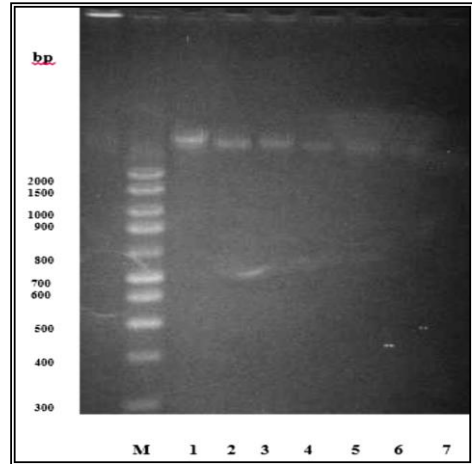

Fig. (8): PCR product of the five *B. thuringiensis* isolates and the reference strains using gral Cry 1 primer. (M) DNA marker (3000-100 bp); (1) strain N *"B. thuringiensis* var. *neoleonensis"*, (2) strain K, *"B. thuringiensis* var. *kurstaki"*, (3) KD2, (4) KD3, (5) AL11, (6) AL7 and (7) AL3 isolates.
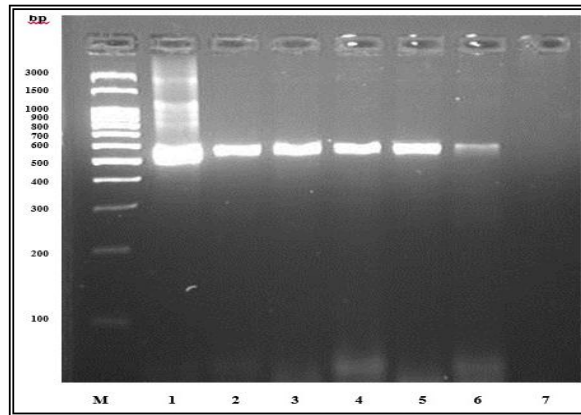

Fig. (9): PCR product of the five *B. thuringiensis* isolates and the reference strains in presence vip1 primer. (M) DNA marker (3000-100 bp). (1) Strain N *"B. thuringiensis* var. *neoleonensis"*, (2) strain K *"B. thuringiensis* var. *kurstaki"* (3) KD2, (4) KD3, (5) AL11, (6) AL7 and (7) AL3 isolates.