Proceedings of Basic and Applied Sciences
ISSN 1857-8179 (paper). ISSN 1857-8187 (online). http://www.anglisticum.mk
Proceedings of the 1st International Conference on New Horizons in Basic and Applied Science, Hurghada – Egypt, Vol 1(1), 2013.

Isolation and Molecular Identification of Insecticidal Activity Bacillus Thuringiensis Strain Alex-13 Isolated From Different Ecosystems in Egypt against Spodoptera Littolaris

Salah G. Ali*, Hassan A. Ahmed & Usama M. Abdul-Raouf
Botany and Microbiology Department- Faculty of Science, Al-Azhar University, Assiut, Egypt.
*Corresponding author: E-mail: salahbadr68@yahoo.com

Received November 13, 2013; Revised November 14, 2013; Accepted November 15, 2013

Abstract: *The entomopathogenic bacterium Bacillus thuringiensis (Bt) is the most widely used biopesticide. The specific toxic activity to insects and other organisms is related to the presence of crystals that have different morphologies, sizes, numbers and compositions according to the Bt strain. The crystals contain different proteins, which are encoded by distinct Cry (crystal) genes. In this work we isolated 334 colonies from 59 soil samples in 13 local area in Egypt, colonies examination has carried out under microscope for toxin production, during screening of these isolates we found 16 bacterial strains has been identified by using biochemical reactions and insecticidal activity against Spodoptera littolaris in addition to Bacillus thuringiensis wettable powder comparable to standard strain, only one isolate which are considered most potent strain were confirmed by molecular characterization. Scanning electron microscopy observed that the presence of cuboidal crystals in Bacillus thuringiensis Alex-13, and observed bipyramidal crystals in case of Bacillus thuringiensis HD-1, as standard strain. Polymerase chain reactions (PCR) revealed the presence of Cry1-like sequences in addition to standard strain, which confirmed by gel electrophoresis. Also, during the studying environmental and nutritional conditions requirements (data not show). Bioassay recorded that the highest mortality rate by determination of* LC_{50} *for 5 days against Spodoptera littolaris third instars larvae were 69, 86, and 100(%) in case of Bt Alex-13, Bt-HD-1 standard strain and Bt commercial wettable powder, respectively.*

Introduction

Bacillus thuringiensis: Bacillus thuringiensis is a gram-positive, facultative anaerobic bacterium, which produces intracellular and distinctively shaped crystal proteins during its spore-forming period. The crystal proteins, also called insecticidal crystal protein (ICP) (Hofte and Whiteley, 1989). Large-scale screening of *Bacillus thuringiensis* strains over the last decades has been successful in identifying 69 serotypes and 13 sub-antigenic groups, resulting in 82 serovars from more than 22 countries among the 3500, *B. thuringiensis* isolates of the International Entomopathogenic Bacillus Centre (IEBC) collection (Lecadet *et al.*, 1999).

Kronstad *et al*. (1983) utilized intragenic *Eco* RI fragments from a cloned of *Bacillus thuringiensis* var. *Kurstaki* HD-1-Dipel to locate the crystal protein gene in 22 strains representing 14 subspecies. Andrews *et al.* (1987) examined the primary DNA sequence of Cry1A(a) (var. *kurstaki* HD-1-Dipel), Cry1A(b) (var. Berliner 1715), Cry1A(c) (var. *kurstaki* HD-73), and Cry1A(a) (subsp. *Sotto*) and found that var. *kurstaki* (HD-1-Dipel) showed 99% homology to Cry1A(a) (subsp. *Sotto*), 91% to Cry1A(b) (var. *Berliner* 1715), and 85% to Cry1A(c) (var. *kurstaki* HD-73). Amino acid analysis divided Cry genes in half into N terminal and C terminal domains. The N terminal domain can be further subdivided into three subdomains (arranged from N- to C- terminus), a relatively hydrophobic subdomain, a hypervariable subdomain and a toxic boundary subdomain (delimiting the N and C terminal domains),
Bacillus thuringiensis Berliner Bt strains produce crystals containing proteins that are toxic to many important insect pests. The insecticidal activities of the various toxins differ, but they are considered harmless to higher organisms, including people.5, 6 Genes encoding Bt toxins (Cry genes) have been incorporated into many crop plants to render them insect-resistant. Since 1995 such plants have been

commercialized, for example cotton, maize and potato, resulting in efficient control of several insect pests and in large reductions in chemical pesticide use (Schnepf and Crickmore, 1998.

Materials and Methods

Isolation of *Bacillus thuringiensis* with insecticidal activity from Egyptian soil: Collection of soil samples: Soil samples were collected randomly from different locations in Egypt governorates: Assiut Al_Azhar university, Elwasta; El-minia Abokurkas-Mantout,Mallawy; Alexandria Awaied; Kfrel-sheikh Sedi-salem city; Elgharbia Tanta city, Karsa; Cairo Elmarg; Souhag Balasfoura, Dar-Elsalam; Qena Naga Hammady; and Aswan Draow city. The samples were collected in clean plastic bags (about 500 g each) at a depth of 5-10 cm. Soil surface and all other debris were first removed and transferred to laboratory until the isolation procedures for *Bacillus thuringiensis* was conducted**.**

Isolation method: Ten grams of soil sample was added to 100 ml of acetate buffer L-broth in 500 ml Erlenmeyer flask, the mixture was shaken in rotary shaker at 240 rpm, for 4 hours at 30 °C. At the end of the shaking time, samples were heat treated at 80°C for 3 minutes; a serial dilution was made from the heat-treated soil suspension. Under aseptic conditions 0.2 ml of diluted samples (10^{-1} to 10^{-8}) was pipetted onto prepared isolated media (Mannitol-Egg Yolk-Polymyxin B sulfate Agar) using sterile glass spreader, and incubates overnight at 30 °C. The microbial growth was characterized and purified by streaking several times on the isolating medium, and then subcultured on the slants of the nutrient agar (NA) medium.

Molecular characterization of bacterial strains: Isolation of Genomic DNA from cell cultures: The locally isolated *Bacillus thuringiensis* strains were grown for 24 hrs. On LB broth medium at 30°C and carried out is as follow:

1- Prepare the cell suspension, for cells grown in suspension, pellet 5 x 10^6 cells by spinning at 1200 x g in a centrifuge tube. Discard the supernatant, and wash the cells once with PBS, and resuspended cells with 200 µl cold (4°C) PBS.

2- Add 25 µl of OB Protease (D3496) or Proteinase K (D3495) at 20 mg/ml solution, vortex to mix well, and incubate at 65 °C in a water bath for 5 minutes to effect complete lysis.

3- Add 220 µl Buffer BL and vortex to mix, incubate at 70 °C for 10 minutes, a wispy precipitate may form on addition of Buffer BL, but dose not interfere with DNA recovery, and adjust the volume of Buffer BL required based on amount of starting material.

4- Place the column into a second 2 ml tube and wash by pipetting 750 µl of wash Buffer diluted with ethanol, centrifuge at 8,000 x g for 1 min, and again dispose of collection tube and flow-through liquid.

5- Using a new collection tube, wash the column with a second 750 µl of wash Buffer and centrifuge as above. Discard flow-through.

6- Using the same 2 ml collection tube, centrifuge at maximum speed (10,000 x g) for 2 min, to dry the column. This step is crucial for ensuring optimal elution in the following step.

7- Place the column into a sterile 1.5 ml microfuge tube and add 200 µl of preheated (70°C) Elution Buffer. Allow tubes to sit for 3 min, at room temperature. Incubate at 70°C rather than at room temperature will give a modest increase in DNA yield per elution. Alternatively, the second elution may be performed using the first eluate. The expected yield from a 30 mg sample is 8-35 µg genomic DNA, depending on type of tissue, this technique has determined in Molecular Biology unit at Assiut University.

Polymerase Chain Reaction (PCR)
PCR amplification of the Cry gene of the local *Bacillus thuringiensis* strains were conducted using two primers, Strep F; 5'- CCGGTGCTGGATTTGTGTTA-3', Strep R; 5'- AATCCCGTATTGTATCAGCG -3' PCR performed for 100 ml reaction volume contained, PCR amplification buffer (MBI Fermentas Inc, Amherst, NY 14226), 200 mmol of each deoxynucleotides, 100 pmol of each oligonucleotide primers and template DNA (0.25 mg of purified DNA from 1 mg/ml of stock solution) 2 different Bt isolates. Standard Bt strain was also used as positive control. Denaturation of template DNA was done for 5 min at 94 °C. After hot start, 2U of Taq DNA polymerase (MBI Fermentas, Amherst, NY 14226) was added. PCR was performed for amplification of Cry 1A (b), Cry III and Cry 1V genes under specific thermal profile as follows: Denaturation at 94 °C for 60 s, annealing at 45 °C for 2 min and polymerization at 72 °C for 3 min for 45 cycles followed by final extension at 72 °C for 10 min. Annealing at 56 °C for 2 min and polymerization at 72 °C for 3 min for 45 cycles followed by final extension at 72 °C for 10 min. Three ml of amplified PCR product was loaded on agarose gel (Bio Rad Laboratories, CA. 94547.) and analyzed after electrophoresis (**Kim *et al.*, 1998**). The remaining PCR product was stored at -20 °C for further work.

Laboratory studies: Tested insects- Cotton leaf worm strain *S. littoralis* (Bosid). The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) obtained as egg masses from the cotton fields at Assiut University, plant protection Department, faculty of agriculture, and transferred directly to laboratory of the botany and microbiology department, faculty of science, AL-Azahr University Assiut branch.

Preparation of *Bacillus thuringiensis* spore-crystal mixtures: *Bacillus thuringiensis* was grown on 400 ml CBI medium in 2 liter Erlenmeyer flasks on a rotary shaker (100 rpm) at 30 °C for 3 days. Upon lysis, vegetative cells, spores and crystals were harvested by centrifugation (3000 rpm) for 15 min, the pelleted particles, mainly consisting of spores and crystal, were resuspended in phosphate buffered saline (PBS).

Purification of crystals: A 200 ml of bacterial suspension precipitate was washed with distilled H_2O three times and once normal saline and resuspended in distilled water. The suspension was shaken vigorously by hand for 5-15 sec. the froth which appears on the surface was held back on filtration with Whatman No. 1 filter paper. Crystals were prepared from the suspension as follows: to 35 ml of the suspension, 30 ml of 1% sodium sulphate and 35 ml of carbon tetrachloride were added and the whole was mixed at 7.000 rpm for 2.5 min, the mixture was allowed to stand for 15 min, and the aqueous phase which contains pure crystals was separated.

Bioassay Experiments: Determination of the toxicity of *Bacillus thuringiensis* commercial wettable powder (W.P) against 3rd instar larvae of *Spodoptera littoralis*.
***Bacillus thuringiensis* (Trade name: ECOTECBIO 10%W.P.)** Biological insecticide from *Bacillus thuringiensis* (Bt) 10% Wettable powder. This compound was produced under license from Agricultural Genetic Engineering Research institute ARC. EGYPT. Rate / fed.200gm/fed. The leaf dipping technique was used to test the efficiency of test compounds (W.P) against the 3rd instar larvae of *S. littoralis*, using avegerage weight of 40 mg. Eight concentrations were prepared from *Bacillus thuringiensis* of (W.P) ranged from (5000, 4000, 2000, 1000, 500, 250, 100 and 50 ppm). Fresh castor bean leaves were dipped in water with 0.1% of Triton x100 for 10 seconds, as standard methods to

make distribution of *B. thuringiensis* and Talk (used as carrier). Three replicates of each concentration were used, in addition ten larvae comparable to control, the treated leaves were left to dry for 30 minutes before offering to the larvae, the larvae were allowed to feed on treated leaves of each concentration for 48 hrs, and then transported to untreated leaves until the end of larval stage (six instar larvae) under laboratory conditions. Castor bean leaves were dipped in water mixed with 0.1% of Ttriton x100 only used as control. Mortality percentages were calculated after treated times of (24, 48, 72, 96 and 120 hrs). The averages mortality obtained were corrected according to **Abbott's formula (Abbot 1925).** The corrected mortalities at different concentrations were subjected to probit analysis according to **(Finney, 1971)**.

To determine LC_{50}

$$\text{\% corrected mortality} = \frac{(\text{\% mortality in treatment} - \text{\% mortality in control})}{100 - \text{Mortality \% in control}} \times 100$$

Determination of the toxicity of *Bacillus thuringiensis* HD-1 standard strain and Alex-13. Against 3rd instar larvae of *Spodoptera littoralis*. The bacterial strains were grown until sporulation was completed in LB liquid medium on a rotary shaker (200 rpm) at 30°C for 5 days. The culture was centrifuged at 12,000 rpm for 15 min, at 4°C. The pellet (toxins and spores) was washed once after sporulation with 1M NaCl and twice with sterile distilled water, then the pellet was dried, and used for bioassay. The bioassay methods were done as previously mentioned.

***Bacillus thuringiensis* identification:** After incubation in an aerobic atmosphere at 30ºC for 48 hrs, a loopful from each broth was streaked on LB agar plates. Based on colony morphology, a single presumptive Bt colony from each plate was selected and obtained in pure culture on *Bacillus cereus* selective agar plates **(Holbrook and Anderson, 1980).** As definitive criteria for identification, sporulated cultures of presumptive *Bt* isolates were examined by phase contrast microscopy for the synthesis of crystalline parasporal inclusions.

Results

Localities and collection of soil samples

The number of bacterial isolates were ranged from (1-15) per one sample. Three hundred and thirty five (334) strains were isolated from fifty nine soil samples carrying the No. of (1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 56, 57, 58, 59, 60, 62, 63, 65, 66, and 67). A relatively higher value recorded (i.e. 15 isolates) were obtained from soil sample No. 14. Which were collected from Assiut Governorate, Elwasta city. While, the lowest value recorded (i.e. 1 isolate) was obtained from soil samples Nos. (31 and 59). However, there are no isolate strains of bacteria were obtained from soil samples Nos. (3, 6, 19, 40, 53, 61 and 64).

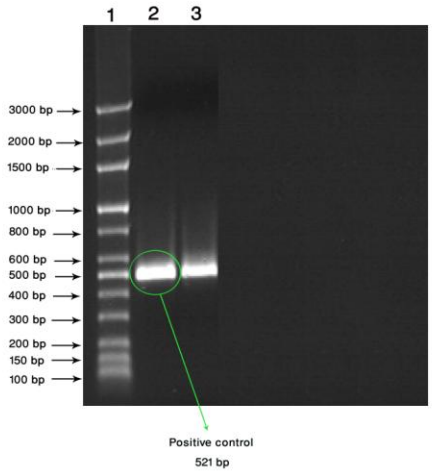
Identification of *Bacillus thuringiensis* strains. Morphological and Physiological Identification of the isolated Bacilli. Microscope examinations of the *Bacillus thuringiensis* strains indicated that the bacteria were gram positive, rod-shape cells containing only one endospore. All the isolates were characterized by the presence of crystals with different sizes (big, medium, and small), and different shapes (irregular, spherical, flat square, and bipyramidal shape). The spore and crystals were present in free or encapsulated form (both inside the sporangium). The sporangium contained only one spore plus

one crystal. The presence or absence of crystals is the major criterion for distinguishing between *Bacillus thuringiensis* and closely related *Bacillus cereus*.Different biochemical tests to identify the bacterial isolates. Were performed as described in Bergey's Manual of Bacteriology (1986) **(Krieg and Holt, 1984, and Staley *et al.*, 1989).** Table (1).

Molecular characterization of *Bacillus thuringiensis* isolate

1. Characterization of Cry genes of local Bt isolate: The polymerase chain reaction (PCR) is a procedure that allows rapid determination of the presence or absence of a target DNA sequence. One isolated spore forming strain *Bacillus thuringiensis* Alex-13 in addition to *Bacillus thuringiensis* subsp. *Kurstaki* HD-1, were analyzed using two sets of primers specific for each of these insecticidal groups for lepidopterans. As shown as in Fig. 1. the products 521 bp. for Cry1A and Cry1A in case of *Bacillus thuringiensis* subsp. *Kurstaki* HD-1, while in case of *Bacillus thuringiensis* Alex-13 that give (532 bp). The positive PCR results matched the bioassay in all cases.

Fig. 1: Agarose gel electrophoresis and the presence of amplified PCR products of CryIA gene from local isolates, Lane 1: DNA marker. Lane 2: gene amplified from standard Bt strain *kurstaki* as positive control (521 bp), Lane 3: amplified CryIA gene from *Bacillus thuringiensis* Alex-13 (532 bp),



2. Electron Microscope

Some characteristics (shape and size) of crystals of *Bacillus thuringiensis* isolated strain recovered from local soil samples. The crystal shape and size of these isolate was different according to comparable strain. The isolated strain of *Bacillus thuringiensis* isolate Alex-13 has only one shape of crystal (cuboidal, plate 1), but in case of standard strain of *Bacillus thuringiensis* HD-1, has only one type of crystals (bipyramidal, Plate 2).

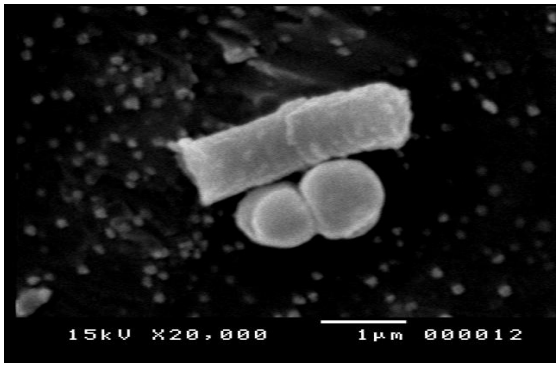


Plate 1: Scanning electron micrograph of crystal and spores of *Bacillus thuringiensis* isolate Alex-13 (X20, 000)

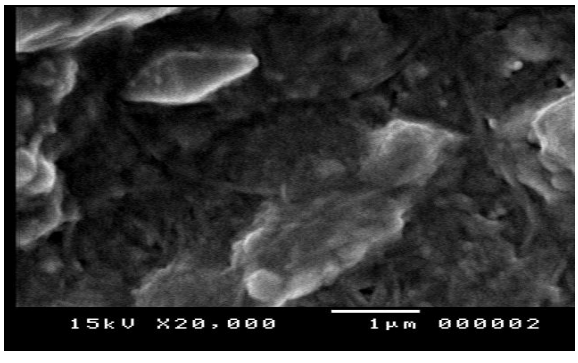
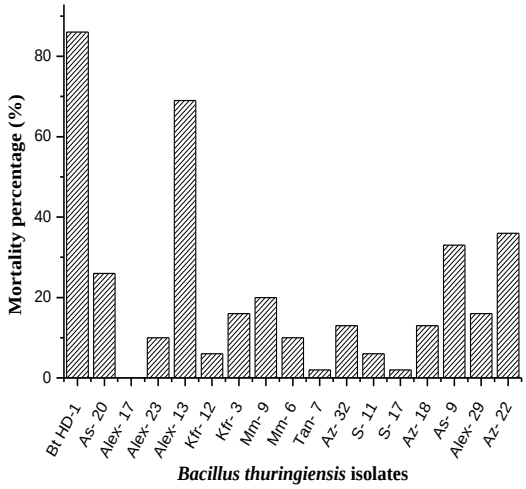


Plate 2: Scanning electron micrograph of crystal and spores of *thuringiensis* subsp. *Kurstaki* HD-1 (X20, 000)

Preliminary screening of *Bacillus thuringiensis* strains: Among the collected soil samples, 16 strains (As 20, As 9, Alex 17, Alex 13, Alex 23, Alex 29, Kfr 12, Kfr 3, Az 32, Az 18, Az 22, S 11, S 17, Mm 9, Mm 6 and Tan 7) found to contain *Bacillus thurngiensis* crystal producing strains as judged by microscopic observations. These isolates were bioassayed for insecticidal activity at (4000 ppm) against *Spodoptera littoralis* larvae, comparable to standard strain. The insecticidal potencies of these selected *B. thuringiensis* isolates are summarized in Fig.3. The highest insecticidal potencies against *S. littoralis* larvae were obtained with spore-crystal complex of *Bacillus thuringiesis* Alex- 13, where mortality percentage reached 69%, while the mortality percentage of *Bacillus thuringiesis* HD-1, pointed out 86% .Three strains (As- 20, As- 9 and Az- 22) has shown a middle mortality rates ranged from 26-36%, the other strains has shown less mortality rates ranged from zero-20%. The difference in mortality percentage of *Bacillus thuringiesis* isolates could be attributed to the type of delta-endotoxin crystal protein itself present in each Bt isolate, or could be due to the sensitivity of the digestive system of the insect (epithelial cell membrane of the mid gut) to reach with or to be damage by endotoxin **(Jaquet *et al.*, 1987).**

Fig. 3: Toxicity of *Bacillus thuringiensis* isolates against *Spodoptera littolaris* 3rd instar larvae



Insect bioassay: Toxicity of *Bacillus thuringiensis* strains against to 3rd instar larvae of *Spodoptera littorals.* The values of LC_{50}. and LC_{90} of the tested isolate with their fiducially limits, slope of Log. Concentrations probit lines compared with standard isolate and commercial natural toxin product are show the bioactivity of *B. thuringiensis* HD-1, and *Bacillus thuringiensis* Alex-13 against the 3rd instar larvae of the cotton leaf worm ***S. littoralis*** under laboratory conditions by using leaf dipping technique. In general, data show the superiority of *Bacillus thuringiensis* wettable powder activities than other tested strains in their toxicity and their acute affect, which appeared after 1 day, the commercial wettable powder insecticide was rapid and highly toxic. The given result show that the mortality of *Bacillus thuringiensis* commercial wettable powder reached 100%, and LC_{50} values daily are (4201.1, 324.3, 240.1, 196.8 and 196.8 ppm) respectively until the fifth days. The toxicity of other different tested strains was differed according to the structure and their source, the mortality of *B. thuringiensis* HD-1, was recorded at 86% and LC_{50} values are (7370.3, 2871.7, 2275.3, 1166.9 and 749.5 ppm) respectively. On the other hand, the local isolate of *Bacillus thuringiensis* Alex-13 pointed out 69% and recorded LC_{50} values last three days of (2.6600, 5356 and 3109 ppm).

Table 1. Biochemical characteristics of *Bacillus thuringiensis* **detalosi** strain comparable to standard strain:

Biochemical reaction	Standard strain HD-1	Isolated strain Alex-13
Gram stain	Bacilli +Ve	Bacilli +Ve
Spore stain	+Ve	+Ve
Anaerobic growth	+Ve	+Ve
Parasporal crystals	+Ve	+Ve
Gelatin liquefication	+Ve	+Ve
Catalase activity	+Ve	+Ve
Acid production from Glucose		
Galactose	+Ve	+Ve
Mannitol	–Ve	–Ve
D-xylose	–Ve	–Ve
Hydrolysis of Casein	+Ve	+Ve
Starch	+Ve	+Ve
Growth in. 3% NaCl	+Ve	+Ve
5% NaCl	+Ve	+Ve
7% NaCl	+Ve	+Ve
10 % NaCL	- Ve	- Ve
Temperature. 30 °C	+Ve	+Ve
40 °C	+Ve	+Ve
50 °C	- Ve	- Ve
Nitrate reduction	+Ve	+Ve
Indole production	–Ve	–Ve
Motility	+Ve	+Ve
VP reaction	+Ve	+Ve
Urea Hydrolysis	–Ve	–Ve
Triple Sugar Iron Agar	K/A	K/A
Citrate utilization	+Ve	+Ve
Sugar fermentation (lactose)	- AG	- AG

-Ve = Negative, +Ve = Positive, K = alkaline, A = Acid, AG = Acid and gas.

Discussion

Isolation of *Bacillus thuringiensis* from soil: This study reported that one isolate were identify as *Bacillus thuringiensis* strain Alex-13 compared with *Bacillus thuringiensis* sub spp. *Kurstaki* HD-1 as standard strain. In this respect, **Martin and Travers (1989)** reported the serovar *kurstaki* as the most common type in Asia, whereas the serovar *israelensis* was most common in Europe and the United States. Similarly, **Mizuki *et al.*, (1999)**, found the serovar *kurstaki* to be the predominant on the phylloplanes in Japan. Another study from Asia also reported *israelensis* and *kurstaki* as the most frequent types in Jordanian habitats **(Al-Momani *et al.*, 2004). Santiago Alvarez and Quesada Moraga, (2001)** reported that the bacterial formulations registered as bioinsecticides in Spain are based mainly (around 80%) on subspecies *kurstaki*, of which they have isolated only two strains from the Canary and the Balearic Archipelago, respectively. In addition, *Bacillus cereus* is a ubiquitous soil bacterium near neighbor to Bt **(Han *et al.*, 2006)**. And other spore-forming bacteria. Other authors have also found that the concentration of Bt in certain kind of soils seemed to be very low. **Delucca *et al.*, (1981).** Pointed out that it is possible to find only one Bt for every two thousand *Bacillus* isolates.

Characterization of *Bacillus thuringiensis* strains: Novel toxins may be recognized among these isolates by characterization of the proteins detected on gels and high pressure liquid chromatography or microscopically by the shape of their crystals, but cryptic genes that are not expressed will be discovered by genetic approaches **(Aronson, 1994 & Masson *et al.*, 1998).**

A) Phenotypic characterization for isolated strains.

1- Phase contrast Microscope: Examinations of the *Bacillus thuringiensis* indicated that the bacteria were gram positive, rod-shape cells containing only one endospore. The isolated strain was characterized by the presence of the crystals with different sizes (big, medium, and small), and only one shape (cuboidal).

B) Biochemical characterization of the isolated strains: The present results show that the isolated strains of Bacillus spp. Have ability to hydrolyse gelatin. It could degrade glucose, sucrose and maltose. No degradation of xylose, starch, mannitol and lactose was observed. These result similar to **(Seleena *et al.*, 1995).** These properties are similar to those reported for *B. thuringiensis* serovar. *israelensis* except that (*Bti*) produced arginine dihydrolase **(de Barjac and Frachon 1990).**

C- Molecular characterization and Identification of isolated strains:1- Characterization of Cry genes of local Bt isolates: The amplification of Cry 1A gene recorded at 521 bp for Cry1A in case of *Bacillus thuringiensis* sub spp. *Kurstaki* HD-1, while *Bacillus thuringiensis* Alex-13 that give 532 bp The characterization of Bt collections had been successful in determining the environmental role of Bt and establishing the distribution of the Cry genes among isolates **(Ceron *et al.*, 1995; Bravo *et al.*, 1998; Hongyu *et al.*, 2000; Uribe *et al.*, 2003; Gao *et al.*, 2008 & dos Santos *et al.*, 2009).** The analysis of toxic genes cloned from different strains in the same subspecies *kurstaki* by **(Kronstad *et al.*, 1983).** And **Whiteley *et al.*, (1984)** showed the existence of different toxin genes located on different plasmid species in a single subspecies strains. Also, **Thorne *et al.*, (1986)** pointed out that the considerable DNA homology in the similarity among Cry1 and Cry1V genes. Similar to our result **Ceron *et al.* (1995)** identified the most common Cry1 (lepidopteran-active) and Cry111 (coleopteran-active) gene profile. They described a PCR screening method to determine which genes are present in a particular strain. Four general PCR primers, which amplify DNA fragments from the known Cry1 or CryIII genes, were selected from conserved regions. Once a strain was identified as an organism containing a particular type of Cry gene, it could be easily characterized by performing additional PCR with specific Cry1 and Cry111 primers selected from variable regions.

2- Electron Microscope: The present study showed that the crystal shape and size of these isolates were different according to the strain; the isolated strain have only one shape of crystal (cuboidal) but in case of *Bacillus thuringiensis* var. *Kurstaki* HD-1, have also one type of crystals (bipyramidal) these results are nearly similar to **(Ohba and Aizawa, 1986)** he found that total of 189 isolates of Bt producing parasporal inclusions, isolated from soil of Japan, were examined for their oral toxicity against larvae of silk worm and the mosquito and adults of a chrysomlid Coleopteran. Also these results are nearly similar to **(Whiteley and Schnepf, 1986)** demonstrated that identification of novel crystal protein genes toxic to lepidopteran insects, 52 *B. thuringiensis* isolates, which were isolated from Korean soil samples, were selected.

Toxicity of the isolated bacterial strains against *S. littoralis.* Our results demonstrated that the production of protein crystals toxic, *Bacillus thuringiensis* Alex-13 compared to *Bacillus thuringiensis* HD-1, against worms sagacious. The results showed that *Bacillus thuringiensis* Alex-13 gave a death rate of 69% after 5 days concentration of 5000 ppm. While the proportion of Commercial powder showed 100% mortality after (2 days). On the other hand, the standard strain of *Bacillus thuringiensis* HD-1, showed the death rate of 86% after 5 days. Using an isolation protocol involving the use of a selective medium, over 150 Bt strains were recovered from the sample material. Isolates causing over 80% mortality in the screening assays against second instar partellus larvae were then bioassayed **(Sneh and Schuster, 1981)**. Near similar to our result, **Walgenbach *et al.* (1991)** studied the persistence of some insecticides on tomato foliage and implications for control of tomato fruit-worm, *Helicoverpa zea*, and found that *B. thuringiensis* was toxic for very short periods (<48 h) after application. Our result demonstrate that the most toxic effect was found to Cry1 insecticidal crystal protein, similar toxic effect of *B. thuringiensis* isolates was also observed on the *S. littoralis* and the most toxic strain was found to be 85PPb. In addition to 8 Cry1 gene subgroups, presence of Cry1C in the 85PPb. These results is in agreement with the results of **Escriche *et al.* (1998)** they showed that some of the Cry1 insecticidal crystal proteins, especially Cry1Ca, was active against *S. littoralis*, made brush border membrane vesicles permeable to KCI. Moreover,

Chenot and Raffa, (1995) demonstrate that the larval stage which combined dosage that affect of different toxins was also found to play determinative role **(Chilcott and Tabashnik, 1997).** The LC_{50} values strongly favour the view that more than one protoxin is present and they are efficiently processed within the gut to provide sufficient active toxin molecules in HD1 dipel compared to remaining three isolates. Other studies have also shown similar variability in toxicity within Btk serovars **(Saitoh *et al.*, 1996).**

Similar to our result there are twenty-seven isolates killed 100% of tested larvae of *S. frugiperda, A. gemmatalis* and *P. xylostella* after 5 days in selective bioassay and were submitted to dose response bioassay. Of the isolates, 19 exhibited LC_{50} values that were higher than that of the standard strain Btk. HD-1, against the entire insect tested at 95% confidence **(Rose *et al.*, 2007).**

Conclusion

The present data demonstrated the isolated strain belonging to the genus *Bacillus thuringiensis* produced insecticidal crystal proteins, which are effective against Egyptian cotton leaf worm (*Spodoptera littollaris*), that is have biological economic importance, many of chemical compounds are used for agriculture treatment which major problem in the ecosystem Therefore, it is very important to isolate this strain in large scale to reduce the using of chemical substances in agriculture field.

References

1. Abbott, W. S. (1925): A method of computing the effectiveness of an insecticide. J. Econ. Entomol.18: 265-267.
2. Al-Momani, F.; Obeidat, M.; Saadoun, I. & Meqdam, M. (2004): Serotyping of *Bacillus thuringiensis* isolates, their distribution in different Jourdanian habitats and pathogenicity in Drosophila melanogaster. World J. Microbiol. and Biotech., 20: 749-753.

3. Aronson, A. I. (1994): *Bacillus thuringiensis* and it's use as a biological insecticide. Plant Breed. Rev., 12: 19-45.
4. Ceron, J.; Ortiz, A.; Quintero, R.; Guereca, L. & Bravo, A. (1995): Specific PCR primers directed to identify *cryI* and *cryHI* genes within a *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol., 61: 3826-3831.
5. Chenot, A. B. and Raffa, K. F. (1995): Effect of parasitoid strains and host instar on the interaction of *Bacillus thuriengensis* subsp. *kurstaki* with the gypsy mouth (Lepidoptera: Lymantriidae) larvae parasitoid *Cotesia melanoscela* (Hymenoptera: braconidae) Environ. Entimol., 27: 137-147.
6. Chilcott, C. F. and Tabashnik, B. K. (1997): Independent and combined effects of *Bacillus thuringiensis* and the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) on susceptible and resistant Diamondback mouth (Lepidoptera; plutellidae) J. Econ. Entomol., 90: 397-403.
7. De Barjac, H. (1981): Identification of H-serotype of *Bacillus thuringiensis.* In "Microbial Control of Pests and Plant Disease" (Ed. Burges), Academic Press, NY., 35-43.
8. De Barjac, H.; and Franchon, E.; (1990): Classification of *Bacillus thuringiensis* strains. Entomophaga. 35: 233–240.
9. De Lucca, A. J.; Simonson, J. G. & Larson, A. D. (1981): *Bacillus thuringiensis* distribution in soils of the United States. Can. J. Microbiol., 27: 865-870.
10. dos Santos, K. B.; Neves, P.; Meneguim, A. M.; dos Santos, R. B.; dos Santos, W. J.; Boas, G. V.; Dumas, V.; Martins, E.; Praça, l. B.; Queiroz, P.; Berry, C. & Monnerat, R. (2009): Selection and characterization of the *Bacillus thuringiensis* strains toxic to *Spodoptera eridania* (Cramer), *Spodoptera cosmioides* (Walker) and *Spodoptera frugiperda* (Smith) (Lepidoptera:Noctuidae). Biological Control 50: 157-163.
11. Escriche, B.; Decker, D.; Van rie, J.; Jansens, S. & Van Kerhove, E. (1998): Changes in permeability of brush border membrane vesicles from *Spodoptera littoralis* midgut induced by insecticidal crystal proteins from *Bacillus thuringiensis*. Appl. and Environ. Microbiol., 64: 1563-1565.
12. Finney, D. J. (1971): Probit analysis (Third edition). Cambridge Univ. Press, London., 333.
13. Gao, M.; Li, R.; Dai, S.; Wu, Y. & Yi, D. (2008): Diversity of *Bacillus thuringiensis* strains from soil in China and their pesticidal activities. Biological Control., 44: 380-388
14. Han, C. S. H.; Xie, G.; Challacombe, J. F.; Altherr, M. R.; Bhotika, S. S.; Bruce, D.; Campbell, C. S.; Campbell, M. L.; Chen, J.; Chertkov, O. & Gilnal, P. (2006): Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. J. Bacteriol., 188: 3382–3390.
15. Hofte, H. and Whiteley, H. R. (1989): Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbial. Rev., 53: 242–255.
16. Hongyu, Z.; Ziniu, Y. & Wangxi, D. (2000): Composition and ecological distribution of Cry proteins and their genotypes of *Bacillus thuringiensis* isolates from warehouses in China. J. of Invertebr. Pathol., 76: 191-197.
17. Jaquet, F.; Hutter, R.; & Luthy, P. (1987): Specificity of *Bacillus thuringiensis* delta endotoxin. Appl. Environ. Micobiolo., 53: 500-504.
18. Kronstad, J. W.; Schnepf, H. E. & Whitetey, H. R. (1983): Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol., 154: 419-428.
19. Lecadet, M. M.; Franchon, E.; Cosmao Dumanoir, C.; Ripoteau, H.; Hamon, S.; Laurent, P. & Thilery, I. (1999): Updating the H-antigen classification of *Bacillus thutingiensis*. J. Appl. Microbiol., 86: 660–672.

20. Martin, P. A. W. and Travers, R. S. (1989): Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbial., 55: 2437–2442.
21. Masson, L.; Erlandson, M.; Puzstai-Carey, M.; Brousseau, R.; Juarez-Perez, V. & Frutos, R. (1998): A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains Appl. Environ. Microbiol., 64(12): 4782-4788
22. Misuki, E.; Ohba, M.; Akao, T.; Yamashita, S.; Saitoh, H. & Park, Y. S. (1999): Unique activity associated with non insecticidal *Bacillus thuringiensis* inclusions: in vitro cell-killing action on human cancer cells. J. Appl. Microbiol., 86: 92-96.
23. Ohba, M. and Aizawa, K. (1986): Distribution of *Bacillus thuringiensis* in soils of Japan. J. Inverteb. Pathol., 47: 277 282.
24. Rose, G. M.; Andera, C. B.; Patricia, T.; Erica, S. M.; Viviance, M. M.; Lilian, B. P.; Vinicius, F. D. (2007): Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis* Biological Control., 41: 291-295
25. Saitoh, H.; Higuchi, K.; Mizuki, E. & Ohba, M. (1996): Larvicidal activity of *Bacillus thuringiensis* natural isolates, indigenous to Japan, against two nematoceran insect pests occurring in urban sewage environments. Microbiol. Res., 151: 263-271.
26. Santiago-Alvarez, C. and Quesada-Moraga, E. (2001): Empleo de *Bacillus thuringiensis* en los sistemas agrcolas. In: Caballero, P., Ferr!e J. (Eds.), Desarrollo y aplicaciones de *Bacillus thuringiensis* en el Control Integrado de Plagas. Phytoma Press, Valencia., 191-217.
27. Schnepf, E. and Crickmore, N. (1998): *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev., 62: 775-806.
28. Seleena, P.; Lee, H. L. & Lecadet, M. M. (1995): A new serovar of *Bacillus thuringiensis* possessing 28a28c flagellar antigenic structure: *Bacillus thuringiensis* serovar jegathesan, selectively toxic against mosquito larvae. J. Nm. Mosq. Control Assoc., 11:471-473.
29. Sneh, B. and Schuster, S. (1981): Recovery of *Bacillus thuringiensis* and other bacteria from larvae of *Spodoptera littoralis* previously fed *Bacillus thuringiensis* treated larvaes. J. Inverteb. Patho., 37: 295-303.
30. Thorne, L.; Garduno, F.; Thompson, T.; Decker, D.; Zounes, M.; Wild, M.; Walfield, A. M. & Pollock, T. J. (1986): Strucural similarity between the Lepidoptera and Diptera specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* and *israelensis* J. Bacteriol., 166: 801-811.
31. Walgenbach, J. F.; Leidy, R. B. & Sheets, T. J. (1991): Persistence of insecticides on tomato foliage and implications for control of tomato fruitworm (Lepidoptera: *Noctuidae*). J. Economic Entomology., 84(3): 978-986.
32. Whiteley, H. R. and Schnpf, H. E. (1986): The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Annu. Rev. Microbiol., 40: 549-576.
32. Whiteley, H. R.; Schnepf, H. E.; Kronstad, J. W. & Wong, H. C. (1984): Structural and regulatory analysis of a cloned *Bacillus thuringiensis* crystal protein gene. In Genetics and Biotechnology of Bacilli, Ganesan, A. T. and Hoch, J. A. (eds). Academic Press, Inc., Orlando, Fla. 375-386.

ISSN 1857-8179 (paper). ISSN 1857-8187 (online). http://www.anglisticum.mk
Proceedings of the 1st International Conference on New Horizons in Basic and Applied Science, Hurghada – Egypt, Vol 1(1), 2013.

Session V

Geological studies and future development