ISOLATION AND IDENTIFICATION OF NOVEL LOCAL ISOLATES OF *Bacillus thuringiensis* ACTIVE AGAINST RED PALM WEEVIL (RPW)

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The gram-positive bacterium *B. thuringiensis* is well-known as sources of insecticidal proteins, most of which accumulate in crystalline inclusions during sporulation, these parasporal inclusions contain various types of insecticidal crystal protein (ICP). The crystal proteins from *B. thuringiensis* are among the most successful biological control agents for the suppression of agriculturally and medically important insect pests (Roh *et al.*, 2007), being toxic to the larvae of lepidopteron, dipteran, and coleopteran insects (Johnson *et al.*, 1998), as well as certain hymenoptera, homoptera, and mallophaga, in addition to many nematodes, flatworms, and Sarcomastigophora (Walters and English, 1995; Horak *et al.*, 1996).

At present, more than 130 *B. thuringiensis* crystal proteins have been described based on their gene sequences and amino acid homologies (Crickmore *et al.*, 1998). Recently, all crystal proteins show some relatedness is grouped into four main groups. *B. thuringiensis* isolates are distributed worldwide, and more than 60,000 have already been collected by various industries in an effort to obtain novel crystal proteins (Martin and Travers 1989; Li *et al.*, 2007). In addition, *B. thuringiensis* isolates showing different crystal protein gene patterns from the reference strains have also been reported (Li *et al.*, 2002).

The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier), a concealed tissue borer, is a lethal pest of palms and is reported to attack 17 palm species worldwide. Although the weevil was first reported on coconut (*Cocos nucifera*) in South Asia during the last two decades it has gained a foothold on date palm *Phoenix dactylifera* in several Middle Eastern countries from where it has moved to Africa and Europe, mainly due to the movement of infested planting material. In the Mediterranean region, RPW also severely damages *Phoenix canariensis.* Infested palms, if not early detected and treated, often declined and die. However, palms in the early stages of infestation usually respond to chemical insecticide treatments. RPW has been managed in several countries through an integrated pest management (IPM) pro-

gram (Faleiro, 2006). The search for effective natural enemies (biological control) for RPW still continues.

A novel *Bacillus thuringiensis* strain, NCIMB 40152, was isolated from dead *Tenebrio molitor L.* Larvae. The isolate is endowed with specific insecticide activity against the Colorado potato beetle (Leptinotarsa decemlineata (Say), Coleoptera, Crysomelidae), but has no effect against lepidopteran or dipteran insects (Cidaria *et al.,* 1991). *Bacillus thuringiensis* strains C-4, C-9, GM-7, and GM-10, with high toxicity against lepidopteran and coleopteran pests have been isolated from Northeast Mexico (Tamez-Guerra *et al.,* 2004).

Bacillus thuringiensis soil isolates A21, A51 and C17 isolated from different regions of the Cuban archipelago showed higher larvicide activity than Bactivec's isolated reference strain, against both *Aedes aegypti* and *Culex quinquefasciatus* (González *et al.,* 2011). Accordingly, This study describe the isolation and identification of novel isolates of *B. thuringiensis*, in order to study insecticidal activity of isolates produced proteins against Red Palm Weevil insect.

MATERIALS AND METHODS

Soil Samples and microbial strains

Soil samples were collected from different locations in the Governorate of Sharkia, Egypt. For the isolation and characterization of *B. thuringiensis* local isolates, bacterial reference strain; *B. thuringiensis var*. *kurstaki* was provided kindly from Microbial Biology Molecular Biotechnology Laboratory, Agricultural Genetic Engineering Research Institute, Giza, Egypt.

Bacterium isolation and morphological characterization

Soil suspensions were made by adding 5 g of soil to 50 ml sterile basic salt solution. Ten fold dilutions of these suspensions were plated on Luria-Bertani (LB) agar. Only colonies from the highest dilution of the soil suspensions were selected for the isolation and identification of bacteria. Standard physiological and biochemical identification analyses were carried out as described in Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The candidate isolates were examined for the distinct crystal morphology by using phase-contrast microscopy.

Protein and DNA extraction

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of (Li *et al.,* 2002).The DNA was extracted from the bacterial cells by boiling the bacterial growth in a water bath for 5 min to lyses the cells and then the tubes were spun briefly to collect the condensate cells (Carozzi *et al.,* 1991).

Amplification of 16S rRNA gene

16SrRNA analysis was carried out using specific universal primers for 16SrRNA, forward primer 5'-

CAGGCCTAACACATGCAAGTC-3' and reverse primer 5'-GGGCGGTGTGTACAAGGC-3' (Yoon *et al.*, 1998). Isolated DNA from pure bacterial culture was used as template. PCR was performed with a 50 µl reaction mixture containing 16S gene primer, DNA template buffer, $MgCl_2$, dNTPs and *Taq* polymerase. PCR program was carried out in Bio-RAD i-cycler which comprises of three steps; 1) Denaturation at 94ºC for 40 seconds; 2) Annealing at 51ºC for 50 seconds; 3) Extension at 72ºC for 1.5 minute all running for 35 cycle, with initial denaturation 94ºC for 4 minute and final extension 72ºC for 7 minute. PCR Products were analyzed by electrophoresis in 1.5% agarose gel then one of them is gel-purified using (Promega Wizard SV Gel and PCR Clean Up-system Kit cat. # A9282). After cloning PCR product into pGEM-easy vector (Promega, Madison, USA), 4 µL of the purified DNA containing cloned PCR product was sequenced using ABI PRISM (310 Genetic Analyzer). Obtained sequence was edited to exclude the PCR primer binding sites and manually corrected with Chromas 2.3 (Chromas version 2.3; www.technelysium.com.au. chromas.html).

Analysis of 16SrRNA gene sequence

16SrRNA gene sequence compared with already submitted in database using the standard nucleotide-nucleotide BLAST algorithm (Altschul *et al.*, 1990). Further, most similar sequences were aligned by ClustalX2 version 2.0.8 software (Thompson *et al.*,1997), using the same software phylogenetic tree was constructed to analyze evolutionary relationships among sequence of isolated microorganism and closest relatives using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence. The identities of the relatives were determined on the basis of the highest GenBank accession numbers score. Phylogenetic tree was visualized using Dendroscope 2.6 software (Huson *et al.*, 2007).

Preparation of crystal proteins for insect bioassay

To evaluate crystal protein production, wild-type isolate and reference strain of *B. thuringiensis* were typically grown in T3 sporulation medium (Donovan *et al.*, 1988), at 25-30°C for 3-4 days, cultures that fully sporulated and lysed were harvested by centrifugation, washed once or twice in an equal volume of wash buffer (10 mM Tris-HCl [pH 7.5]- 0.005% Triton X-100 or 0.005% Triton X-100 alone), and suspended at 1/10 of the original volume in the washing buffer. This 10X spore-crystal concentrate was used directly in bioassays. Sucrose step gradients (7.5 ml each of 79, 72, 68 and 55% sucrose in wash buffer) were prepared in 25- by 89-mm Ultra-Clear centrifuge tubes (Beckman Instruments, Inc.). Five ml of Spore-crystal suspensions were layered on top of the gradients. The gradients were centrifuged at 18,000 rpm at 4ºC in an L8-70 M ultracentrifuge (Beckman

Instruments, Inc.) for overnight period. The protein crystals of EG11529 separated into two distinct bands, one at the 68-72% interface, and the other at the 72-79% interface. The bands were recovered from the gradients by use of a pipette and the protein crystals were concentrated by centrifugation. The protein crystals were washed twice in buffer. Crystal proteins were quantified by SDS-PAGE and densitometry by using bovine serum albumin as a standard.

Determination of insecticide activity

Bioassay screenings were performed *via* surface inoculation of an artificial diet (Marrone *et al.*, 1985), Spore-crystal suspensions or gradient-purified protein crystals were diluted in an aqueous solution of 0.005% Triton X-100 and applied to the surface of the diet. After the solution had dried, first-instar larvae were placed on the diet and incubated at 28ºC. At least five concentrations and five replicated per dose were performed. Bioassays were replicated on at least three different times. Ten larvae were tested per concentration. Mortality was scored after 1-3 days by using the diluent-only treatment as the untreated check assay. All the tests were conducted at 27ºC in 60-70% humidity with a light 16 h: dark 8 h cycle and repeated three times. The number of surviving larvae per concentration as well as control was recorded at 24, 48 and 72 hours from the beginning of the experiment to estimate. The mortility percentages were plotted on Log-probit regression line. The median lethal concentrations (LC_{50}), standard error (SE) and the slop were determined from the regression line. The LC_{50} were expressed in mg/l. A strain *B. thuringiensis var*. *kurstaki* was used as reference for comparing the results of bioassays.

RESULTS AND DISCUSSION

Isolation and identification of local isolates

Twenty soil samples collected from different sites in Sharkia Governorate were used for the isolation of *B. thuringiensis*. Morphological comparison was employed between obtained bacterial colonies from soil suspensions and reference isolate colony in order to determinate candidate *B. thuringiensis* colony.

Candidate *B. thuringiensis* colonies were tested for the presence of crystals and only those with crystals were considered for further examination. The type of crystal observed were rhomboidal and cuboidal but don't show bi-pyramidal, triangular and irregular shapes. Candidate *B. thuringiensis* was designated *BTRW1* and *BTRW2* which was found to produce rhomboidal parasporal inclusions, while candidate *BTRW3*, *BTRW4* and *BTRW5* were found to produce cuboidal parasporal inclusions (Fig. 1), and significant differences were found in the shape and size of the vegetative cells, spores, and parasporal inclusions between BTRW isolates and reference strain *B. thuringiensis subsp. var. kurstaki*, which produces the bi- pyramidal crystal shape.

Isolation and sequence analysis of 16S rRNA gene

Amplification of new isolate 16SrRNA gene showed a candidate band about (1350 bp) in Fig. (2). The purified candidate 16SrRNA fragment was ligated into pGEM-T easy vector and transformed into *E. coli* DH5α competent cells. Plasmids were isolated from candidate colonies obtained from transformation. Isolated fragment was sequenced using ABI PRISM (310 Genetic Analyzer). The sequence data (Fig. 3) was utilized to run a homology search using blast tool provided by NCBI, with specifying blast to the microbial 16SrRNA data. The results of the homology search revealed that the isolated fragment was designated as 16SrRNA of *B. thuringiensis*, Sequence showed high similarly to *B. thuringiensis* strain IAM 12077 16S ribosomal RNA and accession NR_043403. Blast also was performed with the sequence specifying only *B. thuringiensis* data, the sequence showed similarly to many *B. thuringiensis* accessions. Phylogenetic tree (Fig. 4) was drawn with both new isolate and its closest relative (Table 4) which determinate on the basis of highest score obtained in the blast result, tree constructed for the determination of evolutionary relatedness. New isolate witch designated as BTAGERI was found to be close to accessions JN205290, GQ342294 and JF496321. The use of 16S rRNA gene sequences as one of the housekeeping genetic marker to study bacterial phylogeny and taxonomy has been carried out for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel., 2001). An interesting example of using 16S rRNA gene sequencing, Strains of *B. globisporus* and *B. psychrophilus* which share >99.5% sequence similarity with regard to their 16S rRNA gene, and yet at the DNA level exhibit only 23-50% relatedness in reciprocal hybridization reactions (Fox *et al.*, 1992).

SDS-PAGE analysis of vegetative and sporulated cells

Protein SDS-PAGE analysis of vegetative and sporulated cells of the five isolates and reference strains revealed that the five isolates were different from each other in their banding pattern as shown in (Figs. 5 and 6). The protein contents of the sporulated cells (Fig. 5) showing two major bands (≈ 34-40 kDa). The higher molecular weight band of ≈ 130-140 KDa for reference strain *B. thuringiensis subsp. var. kurstaki* representing the bi-pyramidal crystal (lepidopteran toxin) and the lower band of ≈ 34-40 kDa for Bt isolates *BTRWs* representing the rhomboidal crystal for isolates *BTRW1* and *BTRW2*, and cuboidal crystal for isolates *BTRW3*, *BTRW4* and *BTRW5* (Coleopteran toxin).

Evaluation of Insecticidal activity

The insecticidal activities of the crystal proteins produced by five local isolates *BTRW* were evaluated against red palm weevil and cotton leaf worm (*Spodoptera littoralis*) larvae compared with reference strains *B. thuringiensis subsp. Var. kurstaki*. The bioassay data for the local isolates against Red palm weevil and cotton leaf worm were recorded after 24 hr, 48 hr and 72 hr. First, the mean percent mortality of treated fifty red palm weevils instars larvae against each one from five local isolates. The highest mortality was recorded at 24 hr due to applying isolate, *BTRW2* crystal protein result in 76% mortality, While reference strain *B. thuringiensis subsp. var. kurstaki* crystal protein result in 20% mortality. At 48 hr, cumulative mean percent mortality of instars larvae ranged from 76% to 84% and no change was occurred in cumulative mean percentage mortality value for applying reference strain *B. thuringiensis subsp. var. kurstaki* crystal protien. At 72 hr, the all local isolates crystal protein resulted in 100% larva mortality (Table 1). Secondly, local isolates crystal protein applied to cotton leaf worm (Table 2) at 24 hr period, the mean percent mortality of treated fifty instars larva against each one from five local isolates ranged from 20% to 32.5%. The highest mortality was recorded due to treatment with reference strains *B. thuringiensis* subsp. var. kurstaki crystal protein 60%. At 48 hr, cumulative mean percent mortality of instars larva ranged from 30.0% and 49.5%. Treatment with reference strain *B. thuringiensis subsp. var. kurstaki* crystal protein showed 86.66 % mortality. At 72 hr, the reference *B. thuringiensis subsp. var. kurstaki* crystal protein resulted in 100 percent larval mortality. LC_{50} value was determinate in (Table 3) and slopes were drawn (Fig. 7). The highest LC_{50} value was for local isolate *BTRW2* crystal protein (0.252) compared to the rest of the isolates. *BTRW2* crystal protein showed a high toxicity towards *R. ferrugineus* larvae and recorded a high slope, whereas *B. thuringiensis subsp. var. kurstaki* was no significant toxic to *R. ferrugineus* larva.

In conclusion, we showed evidence of the potential of Egyptian *Bt* strains for insect pest control. Despite the concerns on the use of *BTRW* strains as a bioinsecticide, the capacity of these strains to control coleopteran pests is clear. We are also reporting a new strain with potential use for the control of coleopteran pests. These strains can be grown using inexpensive ingredients and similar fermentation technology for *Bt* commercial production at pilot scale. However, commercializing their use as bioinsecticides in field may be delay, in spite they have been shown to be an alternative for chemical insecticides in Egypt.

SUMMARY

Bacillus thuringiensis, a gram positive bacterium known for it's widely usage to control insect pests represent a threat for agriculture. Five local isolates of *Bacillus thuringiensis* were found in soil

samples collected from different areas in Sharkia governorate, Egypt. Morphological, Biochemical analysis and molecular biology methods were employed to identify these isolates. When crystal morphology of bacterial isolates was examined, isolates were found to produce distinct crystal proteins which were very similar to crystal proteins produced normally by *Bacillus thuringiensis.* Moreover, Identification was performed using 16S rRNA gene sequencing which revealed that the isolates were *Bacillus thuringiensis*. Protein SDS-PAGE analysis of vegetative and sporulated cells of the five isolates revealed that the protein banding pattern of the five isolates was different from each other. Insecticidal activity of the novel isolates have been tested against the red palm weevil (RPW), *Rhynchophorus ferrugineus*. The LC_{50s} of the local isolates BTRW1, BTRW2, BTRW3, BTRW4 and BTRW5 against RPW have been determined and were 0.062, 0.252, 0.178, 0.060 and 0.061 µg/ml, respectively. In the same time, no insecticidal activities have been detected for these isolates against cotton leaf worm.

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Table (1): Efficiency of local *B. thuringiensis* isolates and reference strain *B. thuringiensis subsp. var. kurstaki* crystal protein toxin against Red palm weevil larva.

Isolate	Percent total mortality		
	24 hr	48 hr	72 hr
BTRW1	72	84	100
BTRW2	76	84	100
BTRW3	60	76	100
BTRW4	48	76	100
BTRW5	68	80	100
var. kurstaki	20	20	20

Table (2): Efficiency of local *B. thuringiensis* isolates and reference strain *B. thuringiensis subsp. var. kurstaki* crystal protein toxin against cotton leaf worm.

Isolate	Percent total mortality		
	24 hr	48 hr	72 hr
BTRW1	25	32.5	47.5
BTRW2	32.5	37.5	45
BTRW3	32.5	42.5	52.5
BTRW4	30	49.5	67.5
BTRW5	20	30	32.5
var. kurstaki	60	86.66	100

Table (3): LC_{50} and slope values for *B. thuringiensis* local isolates against red palm weevil larva, *R. ferrugineus*

Sample	LC_{50}	Slope
BTRW1	0.062	0.875
BTRW2	0.252	2.059
BTRW3	0.178	1.48
BTRW4	0.060	1.053
BTRW5	0.061	1.050

Table (4): Local isolate BTRW1 closest relatives were used in constructing phylogenetic tree.

Organism	Definition	Accession
Bacillus thuringiensis	isolate PSA102	JN205290
	strain 2110	JF947357
	strain A1-1 16S	JF496321
	strain X5	HM585283
	strain DSS7	HM217124
	BMB171	CP001903
	strain CG-T1	GQ342294
	serovar asturiensis strain IEBC-T53 001	FJ358616
	Zhou-1	FJ210466
	strain: NBRC 13866	AB363741
	serovar tolworthi strain IEBC-T09 001	EF210288
	serotype H69	DQ286356
	serotype H27	DQ286336
	serotype H16	DQ286326

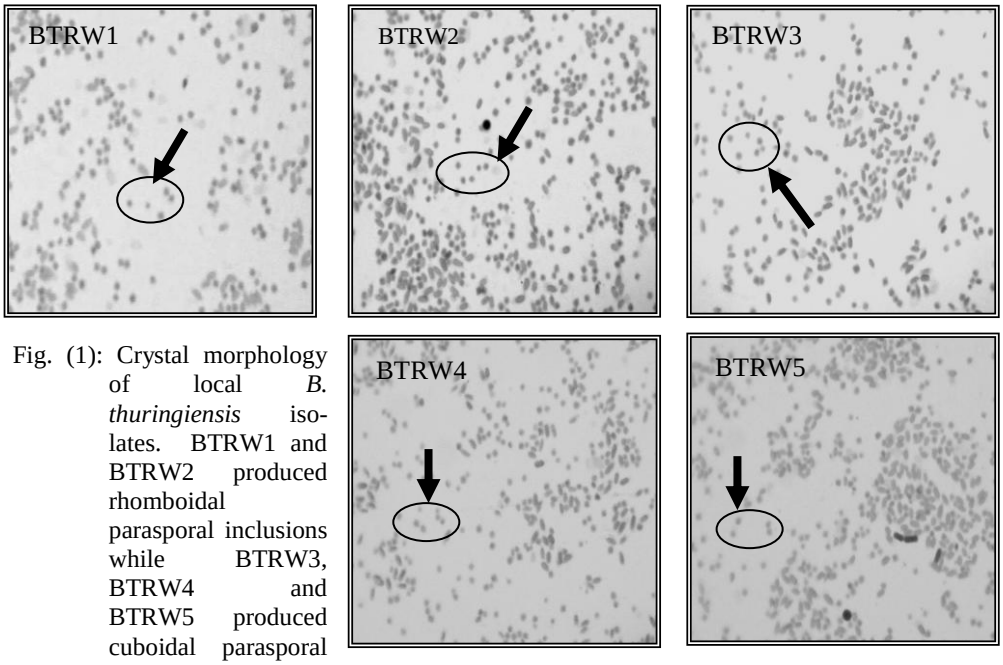

Fig. (1): Crystal morphology of local *B. thuringiensis* isolates. BTRW1 and BTRW2 produced rhomboidal parasporal inclusions while BTRW3, BTRW4 and BTRW5 produced cuboidal parasporal inclusions.

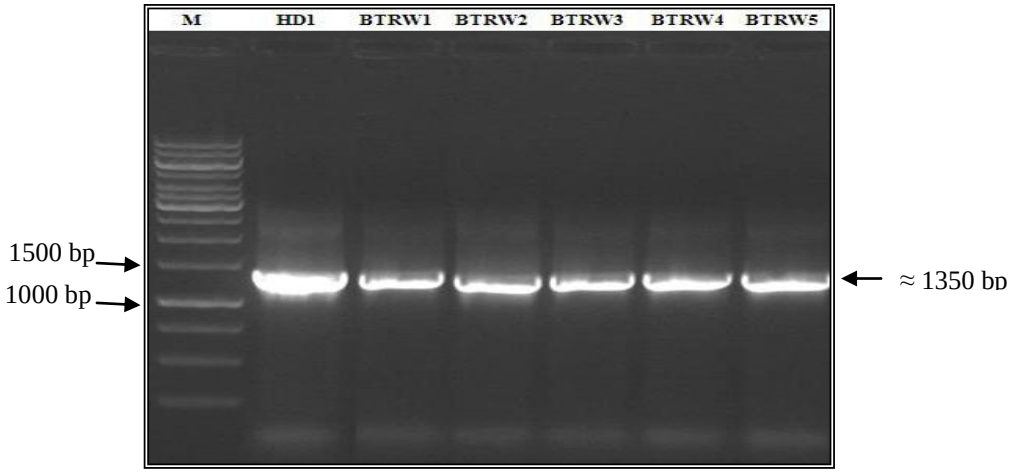

Fig. (2): Amplification of 16SrRNA gene from different Egyptian *Bacillus thuringiensis* local isolates. M: 1 kb DNA ladder GeneRuler™ 1 kb Fermentas, catg.# SM0311, laneHD1: amplification of 16SrRNA gene from positive control *B. thuringensis subsp.* var. kurstaki and lanes (BTRW1 to BTRW5): 16SrRNA gene amplification form *Bacillus thuringiensis* local isolates *BTRW1: BTRW5* (about 1350 bp*).*

> BtAGERI
GAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCT
AATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGG
CTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGT
CGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAG
GTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT
GGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCG
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GAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCT
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TTCCCGG

Fig. (3): Nucleotide sequence of 16SrRNA gene amplified from BTRW1 local isolate.

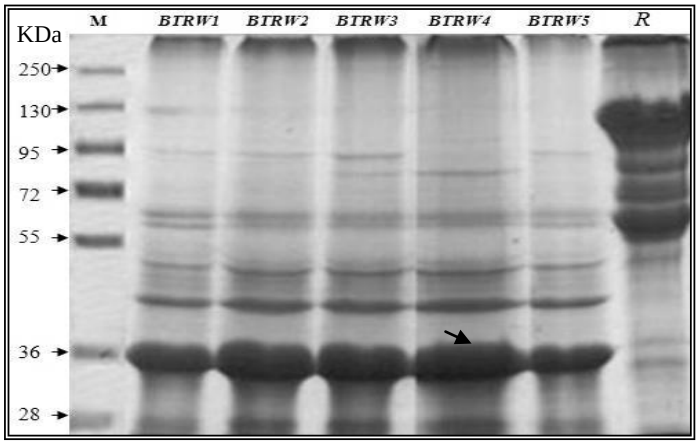


Fig. (5): SDS-polyacrylamide gel electrophoresis of Bt local isolates soluble crystals protein. Lane M: PageRuler™ Plus prestained protein ladder Fermentas (Catg. # SM1811) Lanes (1:5) represent Bt local isolates, Lanes (BTRW1:BTRW5) represent Bt local isolates vegatitave proteins, Lane R*: B. thuringensis* subsp. var. kurstaki vegatitave protein.

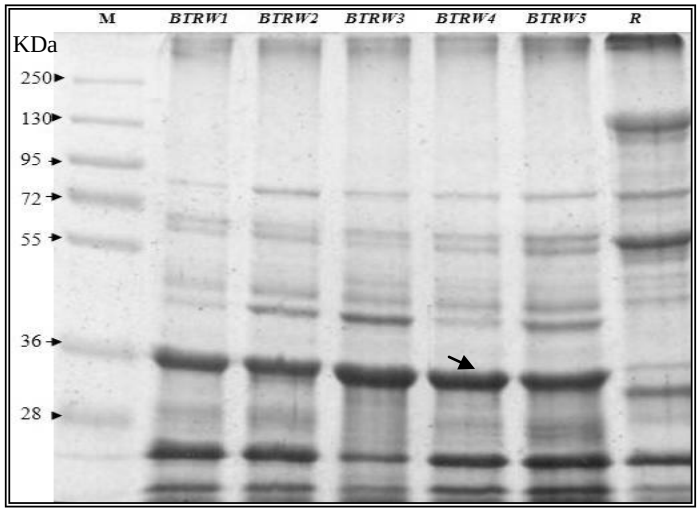
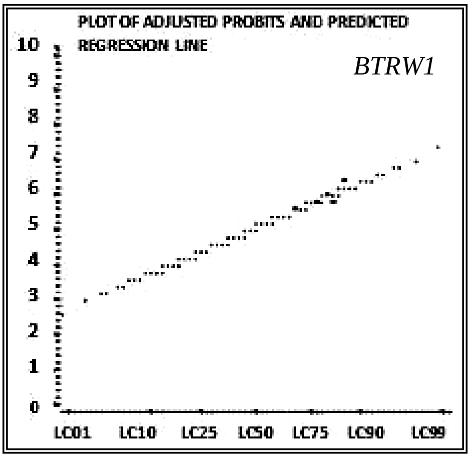
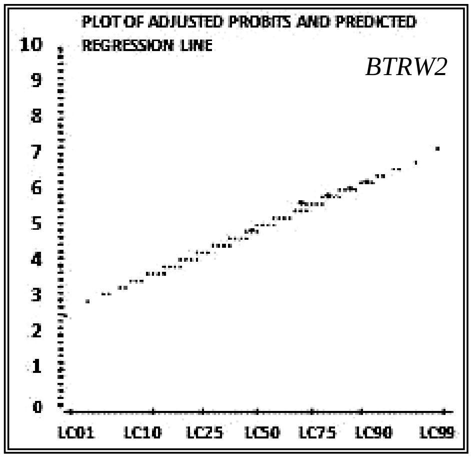
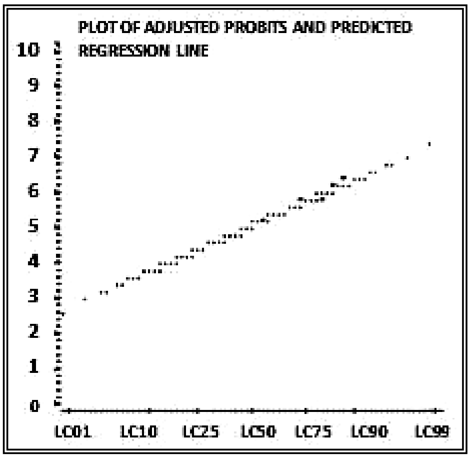


Fig. (6): SDS-polyacrylamide gel electrophoresis of *Bt* local isolates vegatitave proteins. Lane M: PageRuler™ Plus prestained protein ladder Fermentas (Catg. # SM1811), Lanes (BTRW1:BTRW5) represent Bt local isolates vegatitave proteins, Lane R*: B. thuringensis* subsp. var. kurstaki vegatitave protein.







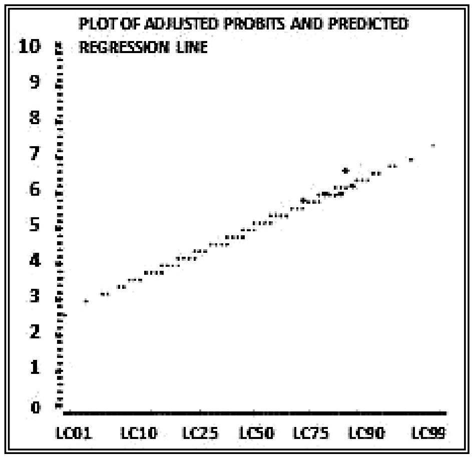


Fig. (7): Slopes of the LC_{50} (µg/ml) values of *Bt* local isolate soluble purified crystal which showed a different degrees of toxicity towards *R. ferrugineus* larvae.