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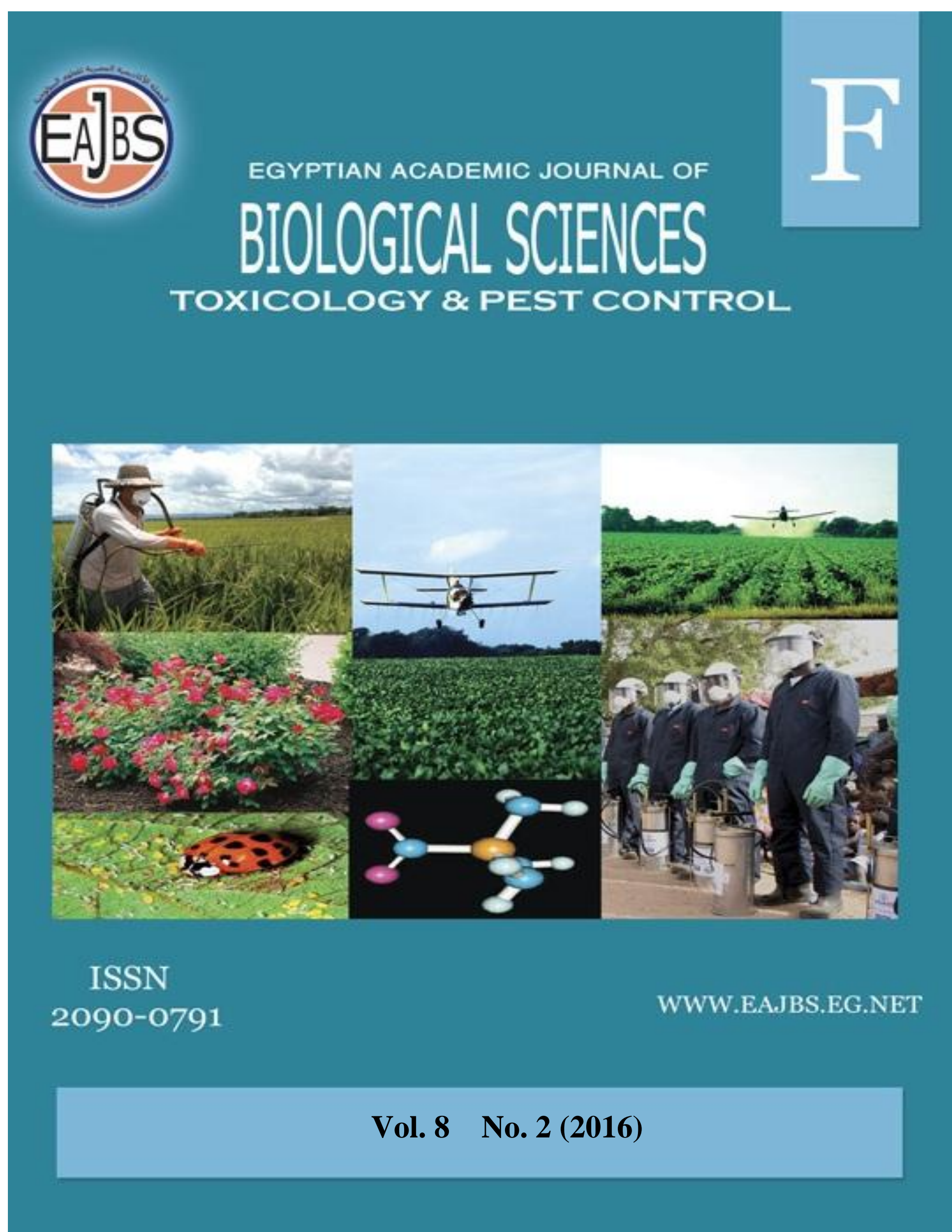

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Citation : *Egypt. Acad. J. Biolog. Sci. (F. Toxicology & Pest control) Vol.8(2)pp.145-153(2016)*

Egypt. Acad. J. Biolog. Sci., 8(2): 145-153 (2016)

Egyptian Academic Journal of Biological Sciences
F. Toxicology & Pest control
ISSN: 2090 - 0791
www.eajbs.eg.net

Estimation of the Efficiency of Bacterial Isolate Compared to Commercial Formulated Bioinsecticides Against *Spodoptera littoralis* (Boisduval) in Vitro

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ARTICLE INFO

Article History
Received: 5/9/2016
Accepted: 1/11/2016

Key words:
cotton leafworm
Spodoptera littoralis
Bacillus thuringiensis kurstaki
commercial formulations
biological aspects
non-specific esterases
ALP
ACP

ABSTRACT

The present study was performed in order to evaluate the biological and biochemical effects of commercially formulated *Bacillus thuringiensis kurstaki* (BTK) and a locally isolated BTK against the cotton leafworm, *Spodoptera littoralis*, under laboratory conditions. Two commercial bioinsecticides; Protecto® and Dipel DF®, and the local isolated *B. thuringiensis kurstaki* (BTK) were tested against 2nd and 4th instar larvae of *S. littoralis*. The results showed a decrease in both mean larval and pupal duration of the larvae, which were treated as 2nd and 4th instar larvae. The results also showed a decrease in the rate pupation and the adult emergence percentage compared to the control. The results also showed influenced by the mean adult longevity for both male and female moths as a result of treatment with studied compounds. In addition, results showed that the treatment with tested bioagents led to a negative impact on the activity of certain enzymes.

INTRODUCTION

In Egypt, the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is destructive phytophagous lepidopterous pest causing various ravages not only for cotton plants (Ahmad, 1988 and Hatem *et al*., 2009) but also for other field crops, vegetables (Hosny *et al*., 1986), ornamentals and orchard trees (Domínguez, 1993 and Belda *et al*., 1994) all over the year in Egypt (Hamouda and Dahi, 2008). Development of an effective control method against this pest is urgently needed since it does serious damage to many important agricultural crops in Egypt. There is a serious interest in the use of microbial insecticides for biological control of the cotton leafworm, as alternatives to chemical control, since they neither leave toxic chemical residues in the environment nor do they develop resistance in their insect hosts. And hence, the public awareness and concern for environmental quality, has led to more focused attention on research aiming at developing biological agents. A promising strategy with good potential to control insect pests and, at the same time, to minimize the adverse effects of chemical insecticides is the use of microbial agents (Ahmed and El-Katatny, 2007). For the purpose of evaluating the different biological and biochemical effects of commercially formulated *Bacillus thuringiensis kurstaki* (BTK) and a locally isolated BTK, the present study was conducted under laboratory conditions.

MATERIALS AND METHODS

Insects used

A laboratory susceptible strain of the cotton leaf worm *Spodopter littoralis* (Boisd.) (Lepidoptera: Noctuidae), reared for more than 10 generations was obtained from the Research Division of the cotton leaf worm, Plant Protection Research Institute. Insects were reared under controlled conditions in an incubator at 26 ± 2° C, of 65 ± 10% R. H., and 8:16 L: D photoperiod at the Plant Protection Research Institute, Dokki-Giza, Egypt (El-Sawaf, 1971).

Bacterial isolation:

Soil samples from maize field at El-Dakhaly village, the western side of Rashid branch, Kom Hamada Center, Beheira Governorate were collected in glass flasks. About 1g of soil was cultured in 10mL of Nutrient Broth for 24h at 30°C with orbital shaking at 150rpm. From the culture, 2mL was taken and heat-shocked in water bath at 60°C for 1h. Then the samples were spread on Nutrient Agar plates (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar), incubated at 30°C for 24h and checked for the presence characteristic colonies of *B. thuringiensis* phenotype (matte white colour, flat, dry and uneven borders). Each sporulated culture was then heat fixed and stained (0,133 % Coomassie Blue stain in 50 % acetic acid) in a glass microscope slide (Ammons *et al*., 2002). The Bt-like cells identification was performed by microscopic examination using a Leyca DM 750 bright field microscope (Leyca Microsystems).

Molecular characterization of *Bacillus thuringeinsis kurstaki*:

The locally isolated *B. thuringiensis* strains were grown for 24 h on LB broth medium at 30°C as follows: 1) Pellets of 5×10^6 cells were grown in suspension. This was done by spinning at 1200xg in a centrifuge tube. The supernatant was discarded. Then the cells was washed once with PBS and resuspended with 200 µl cold (4°C) PBS; 2) 25 µl of OB Protease (D3496) or Proteinase K (D3495) was added at 20 mg/ml solution; it was vortexed to mix well, and incubated at 65 °C for 5 min to complete lysis; 3) 220 µl Buffer BL was added and vortexed to mix well. It was incubated at 70 °C for 10 min. 4) The column was placed in a second 2 ml tube and washed by pipetting 750 µl of wash Buffer diluted with ethanol; it was centrifuged at 8,000xg for 1 min, and collection tube and flow-through liquid were disposed; 5) A new collection tube was used, the column was washed with a second 750 µl of wash Buffer and centrifuged as above. Discard flow through; 6) the same collection tube was used, centrifuged at highest speed (10,000 x g) for 2 min, to dry the column. 7) The column was placed in a sterile 1.5 ml microfuge tube and 200 µl of preheated (70°C) Elution Buffer was added. Tubes were allowed to sit for 3 min, at room temperature. Incubating 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.

Polymerase chain reaction (PCR)

PCR amplification of the CryI gene of the local *B. thuringiensis* strains was conducted using according to the procedure previously published by Ahmed *et al*. (2015). All molecular identification techniques were performed at the Central Laboratory, Faculty of Science, Ain Shams University.

Tested compounds

Two commercial bioinsecticides; Protecto® WP 9.4% (32×10^6 IU/mg of *Bacillus thuringeinsis kurstaki*) and Dipel DF® WP 6.4% (32×10^3 IU/mg of *Bacillus thuringeinsis kurstaki*), and the local isolated *B. thuringiensis kurstaki*

(BTK) were tested against 2nd and 4th instar larvae of *S. littoralis*. The tested bioagents were obtained from Plant Protection Research Institute Biopesticide Unit Production and applied at half recommended dose.

Biological tests

The half of the recommended concentration of the product was obtained according to the recommendation of the Egyptian Ministry of Agriculture. 0.125g of each commercial product was dissolved in a 100ml of distilled water. The isolated *B. thuringeinsis* was used at concentration 65×10^8 spores/ml. Newly ecdysed 2nd and 4th instar *S. littoralis* larvae (12–24 h old) were offered castor bean oil leaves treated with Protecto and Dipel DF at 0.125 gm/100 ml distilled water, and isolated *B. thuringeinsis* at concentration 65×10^8 spores/ml for 48h. After treatment time, larvae were offered untreated leaves. Treatment of larvae was conducted by the leaf dipping technique (Tabashnik *et al*., 1991). Two sets of five replicates each contain 10 newly molted 2nd and 4th instar larvae for each concentration of each tested product. The same numbers of larvae were considered as a control, these larvae were offered castor oil leaves immersed in distilled water. The following parameters were recorded:

Larval instars duration, from the initial treated instar up to pupation.

Percentage of pupation and mean pupal duration.

Percentage adult emergence and mean adult longevity.

Biochemical tests

Larvae at 6th instar larvae that survived treatment with tested bioagents at 4t instar larvae were selected. The larvae of each instar were anaesthetized and rinsed with 5 ml acetone to remove surface residues, the larvae were weighed then homogenized in phosphate buffer (pH7) using a Teflon tissue homogenizer surrounded by crushed ice. The homogenates were centrifuged at 8000 rpm for 20 min at 4° C and the supernatant was used directly for the determination of the following:

Enzymes assay

The following enzymes activity was determined:

Non-specific α- and β- esterase activity was measured as described by Van Asperen (1962) using α-naphthyl acetate and β-naphthyl acetate, respectively, as substrates.

Acid and alkaline phosphatase activity was measured from the larval heamolymph as described by Laufer and Schin (1971).

Statistical analysis:

Data were statistically analyzed by ANOVA procedure (Snedecor and Cochran, 1980) at $P<0.05$ using SPSS statistics 17.0 release 17.0.0 software. When the ANOVA statistics were significant at ($P \leq 0.01$), means were compared by the Duncan's multiple range test using SPSS 17.0 software.

RESULTS AND DISCUSSION

Bacterial identification

Microscope examinations of the *B. thuringiensis* strain indicated that the bacteria were gram positive, rod-shape cells containing only one endospore. The polymerase chain reaction products for *B. thuringiensis* subsp. *Kurstaki* were 521 bp for Cry1A (Fig. 1).

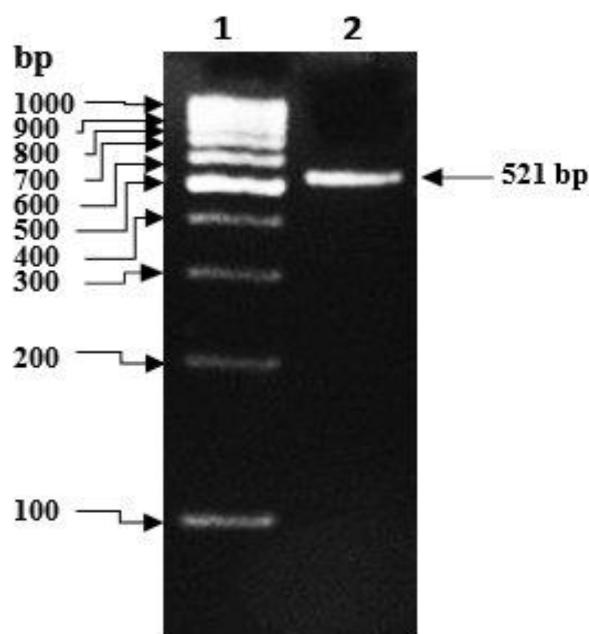

Fig. 1: Agrose gel electrophoresis of PCR amplified product of Cry1A gene from local isolate of *B. thuringeinsis kurstaki*; Lane (1): DNA ladder (1000 bp), Lane (2): Amplified product of the isolated *B. thuringeinsis kurstaki* at 521 bp.

Biological studies:

Larvae that treated as 2nd and 4th instar larvae, and survived treatment with half recommended concentrations of Protecto® and Dipel DF® (0.125g/100ml), and isolated *B. thuringeinsis* (at concentration 65×10^{8} spores/ml) showed that the duration of those instars and their subsequent instars up to pupation was affected (Table 1).

Obtained results showed that the mean larval durations of treated 2nd and 4th instar larvae with tested agents were decreased compared to control. Results also showed that there was a low significant difference obtained in case of Protecto® compared to control, while no significant difference obtained when both Dipel DF® and isolated BTK. Results also revealed that the pupation percentage of 2nd and 4th instar larvae survived treatment with tested bioagents decreased to approximately half. Obtained results showed that treatment with tested bioagents shortened the mean pupal duration of survived pupae that treated as 2nd and 4th instar larvae. Low significant difference was obtained in case of Protecto® and Dipel DF®. No significant difference was observed in case of treatment with BTK in survived pupae that treated as 2nd and 4th instar larvae.

Table 1: Effect of Protecto®, Dipel DF®, and isolated *Bucillus thuringiensis kurstaki* (BTK) on mean larval duration, mean pupal duration, and pupation percentage of *Spodoptera littoralis* treated as 2nd and 4th instar larvae

Treatment	Larval duration (days) Mean ±S.E.		Pupation %		Pupal duration(days) Mean ± S. E.	
	2nd	**4th**	**2nd**	**4th**	**2nd**	**4th**
Protecto®	13.6±0.24b	12.6±0.24a	65.9	49.8	11.3±1.5b	9.3±1.30c
Dipel DF®	14.5±0.12a	12.01±1.70a	52.0	50.0	12.0±0.55b	11.2±0.37b
BTK	14.3±0.10a	12.11±0.43a	49.33	48.65	12.9±0.67a	13.0±1.40a
Control	15.4±0.24a	10.4±0.24b	100	100	13.6±0.5a	14.0±1.70a

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

Data presented in Table (2) showed the latent effect of tested bioinsecticides on adult emergence rate and mean adult longevity for adults treated as 2nd and 4th larval instars. Results showed that Treatment of 2nd and 4th instar larvae with tested bioagents reduced the adult emergence rate. In addition, results showed that the smallest adult emergence percentage was observed when the BTK isolate was applied. Furthermore, obtained data revealed shortening in mean male adult longevity for all treatments, although no significant difference was detected. On the other hand, the mean female adult longevity was increased and no significant difference was observed.

Table 2: Effect of Protecto®, Dipel DF®, and isolated *Bucillus thuringiensis kurstaki* (BTK) on adult emergence percentage and mean adult longevity of *Spodoptera littoralis* treated as 2nd and 4th instar larvae

Treatment	% Adult emergence		Adult longevity (days) Mean ± S.E.			
	2nd	**4th**	**2nd**		**4th**	
			♀	♂	♀	♂
Protecto®	94.40	94.20	15.3±0.57[a]	11.30±0.10[b]	16.3±0.57[a]	11.30±0.10[b]
Dipel DF®	93.70	94.10	15.3±0.57[a]	11.54±1.00[b]	14.3±0.57[b]	11.54±1.00[b]
BTK	81.25	89.20	15.7±1.15[a]	12.87±0.87[a]	14.7±1.15[b]	12.87±0.87[a]
Control	100.0	100.0	14.6±0.58[a]	13.60±1.15[a]	14.9±0.58[b]	12.90±0.98[a]

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

These results agreed with previously published researches by Dutton *et al*. (2003); Gamil (2004); Abdel-Aziz (2007); and Abdel-Salam *et al*. (2013) found that the development time of larvae and pupae were extended as well as adult emergence after treatment with bacterial agents. Results also agreed with Hegab and Zaki (2012) who found the same effects when treated both the pink bollworm, *Pectinophora gossypiella* and the spiny bollworm, *Earias insulana* larvae, with biocide *Btk* (Dipel 2×). Abedi *et al*. (2014) reported similar delay in mean larval duration, mean pupal duration, and mean adult longevity.

Biochemical studies:

Effect of tested bioinsecticides on α- and β- esterases activity:

Data presented in Table (3) showed the effect of treatment of 4th instar larvae with tested bioagents on the nonspecific esterases (α- and β- esterases) in late 6th instar larvae. Results showed that treatment with Protecto® and Dipel DF® decreased the β-esterase activity compared to control. However, treatment with BTK isolate increased the β-esterase activity.

Results also showed that the α-esterase activity was highly increased due to treatment with all tested bioagents compared to control. BTK treatment has the highest enzyme activity.

Table 3: Effect of Protecto®, Dipel DF®, and isolated *Bucillus thuringiensis kurstaki* (BTK) on α-esterase and β-esterase activity in the late 6th instar larvae of *Spodoptera littoralis* treated as 4th instar larvae

Treatment	β-esterase activity (µg β-naphthol/min./g.b.wt) (Mean ±S.E.)	α-esterase activity (µg α-naphthol/min./g.b.wt) (Mean ±S.E.)
Protecto®	1188.00±7.81[a]	618.33±5.49[b]
Dipel DF®	1096.66±10.52[b]	667.33±4.33[a]
BTK	1214.66±20.62[a]	684.33±8.09[a]
Control	1132.66±18.70[a]	424.33±8.69[c]

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

Effect of tested bioinsecticides on Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) activity:

Effect of treatment of the 4th larval instar with tested bioagents on the detoxification enzymes, Acid phosphatase (ACP) and Alkaline phosphatase (ALP), in the late 6th instar larvae of *S. littoralis* is shown in Table (4). ACP activity decreased significantly in all treated groups comparing to the control. In addition, treatment with tested bioagents decreased the ALP activity compared to untreated larvae. Generally speaking, increase of activity of detoxification enzymes is the most universal resistant mechanism in insects. As a result of infection with *B. thuringiensis kurstaki* the epithelial cells undergo an excessive oxidation due to bacterial endotoxin, viral replication and their combined action. This may be caused a stress on the lysosome, leading to their rupture, and finally the release of the acid phosphatase enzyme. It has been reported that substances and bioagents can increase the number of lysosomes inside the cell as defensive mechanism against the attack of these substances (**Assar** ***et*** *al*., 2010; Gaikwad *et al*., 2010; and Mohamed and Mohamady, 2010). The levels of acid phosphatase can be used as a marker on the cell death (Mohamed and Mohamady, 2010).

Table 4: Effect of Protecto®, Dipel DF®, and isolated *Bucillus thuringiensis kurstaki* (BTK) on Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) activity in the late 6th instar larvae of *S. littoralis* treated as 4th instar larvae

Treatment	ACP activity ($U \times 10^3$ ml/gm per body weight) (Mean± S.E.)	ALP activity ($U \times 10^3$ ml/gm per body weight) (Mean± S.E.)
Protecto®	34.00 ± 0.76^b	293.00 ± 3.60^b
Dipel DF®	36.90 ± 0.66^b	311.33 ± 3.48^b
BTK	29.70 ± 0.62^c	276.00 ± 7.37^c
Control	42.26 ± 5.42^a	512.66 ± 9.82^a

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test).

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ARABIC SUMMERY

تقدير كفاءة البكتريا المعزولة ومقارنتها بالمركبات التجارية من حيث الفاعلية فى مكافحة دودة ورق القطن معملياً

سارة محمد إبراهيم عبد الكريم- محمد فتحي عبد العزيز
قسم بحوث دودة ورق القطن- معهد بحوث وقاية النباتات

هدفت الدراسة الحالية الي تقييم بعض الآثار البيولوجية والبيوكيميائية لدودة ورق القطن عند معاملتها ببعض المستحضرات التجارية لبكتريا الباسيليس ثورينجينسيز ومقارنتها بالعزلة المحلية لنفس البكتريا. تم استخدام التركيز الموصى به لكلاً من مستحضري البروتكتو والدايبل دى اف والعزلة المحلية BTK على كلاً من العمرين الثاني والرابع ليرقات دودة ورق القطن تحت الظروف المعملية. وقد أظهرت النتائج انخفاض فى كلاً من متوسط العمر اليرقي ومتوسط العمر العذارى لليرقات التى تمت معاملتها فى كلا العمرين الثاني والرابع. كما أوضحت النتائج انخفاضاً فى كلاً من نسبة التعذير ونسبة خروج الفراشات مقارنة بالكنترول. وقد أوضحت النتائج أيضا تأثر متوسط عمر الحشرات الكاملة من الذكور والإناث نتيجة المعاملة بالمركبات محل الدراسة. أيضاً، أظهرت النتائج أن المعاملة بالمركبات محل الدراسة أدت إلى التأثير سلباً على نشاط بعض الإنزيمات.