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RESEARCH ARTICLE

Physiological and toxicological effects of dible 2X (Bacillus thuringiensis) on the Egyptian cotton leaf worm Spodopteralittoralis(Lepidoptera: Noctuidae).

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Bacillus thuringiensis delta-endotoxins are safe biological insecticidal proteins whose usefulness has long been recognized. The insecticidal effect of Diple 2x (*Bacillus thuringiensis* varkurstaki) was evaluated on 4th instar larvae of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). The data indicated that, the mortality percentage increased with increasing Dipel 2X concentration. The highest mortality (90%) was obtained by using 4 g/ L of Dipel 2x (5 days post treatment) while 5% mortality was recorded by using 1 and 0.5 g/L compared with the control which recorded no mortality (0%). Died larvae get shrinked and its color turned to dark red compared to the control while survival larvae exhibited several malformations. At the pupae stages changes ranged from dwarf pupa; larvae that fail to pupate (half larvae and half pupa) and the pupa that kept the last instars' molting skin remaining attached to its body were observed. Also some moth failed to emerge from the pupa cuticle was recorded. *B. thuringiensis* (Dible 2X) caused a remarkable decrease in amylase, invertase and trehalase enzyme activities compared with the control. The inhibition of carbohydrate hydrolyzing enzymes found in the present study might affect the molting process and subsequently may explain the reason of mortality occurred in *S. littoralis* larvae.

Introduction:

The cotton leaf worm, *Spodoptera littoralis* (Boisd) is one of the major insect pests that cause a considerable damage to many of the important vegetables and field crops in Egypt. The rising consumption of currently used insecticides in developing countries has led to a number of problems such as insect resistance, environmental pollution and the health hazards associated with pesticide residues (Abdel-Hafez *et al*., 2013).

Bacillus thuringiensis (Bt) preparations are prime candidates for use in Integrated Pest Management Programme (IPM). They have high pathogenicity for target pests. Safe for most non-target organisms, and have good integration with other pest control methods. It is known that most Bt formulations have a very short residual activity. Insecticidal Cry proteins, produced as protoxins (65–140 kDa). After crystal solubilization the protoxins released are activated by proteases found in the larval midgut, followed by the binding of toxins to the primary receptor cadherin (Vadlamudi *et al.,* 1995). Binding to cadherin induces the cleavage of helix α-1 located at the N-terminal end of the toxin, facilitating its oligomerization (Gomez *et al.,* 2002). The activated toxins (60–70 kDa) bind to the secondary receptor such as the enzymes aminopeptidase N (APN) (Knight *et al*., 1994; Bravo *et al.,* 2004; Gomez *et al*., 2006), and alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004), which are membrane

anchored by a glycosylphosphatidylinositol (GPI) group. Finally, the oligomeric toxin forms a pore in the membrane and inducing osmotic lysis of the epithelium that leads to cell death (Rausell *et al.,* 2004; Pardo-Lopez *et al*., 2006). Salama *et al*., (1999) reported that, when the clover plants was sprayed Dipel 2x at the rate of 500 g/f, the *S. littoralis* larval population decreased significantly after one week to an average 12.8 ±1.14 larvae/10 m^2. This decrease in larval population was more obvious when Dipet2x was used at 750 g/f, being6.3 ± 0.44 larvae/10 m^2.

Metabolism of carbohydrate hydrolyzing enzymes that play a principal role in digestion and utilization of carbohydrate by insect is controlled mainly by amylase, invertase and trehalase enzymes (Al-Shannaf *et al.,* 2012). Trehalose is one of the most important storage carbohydrates that is present in almost all forms of life except mammals. Trehalose is split into glucose units by trehalase enzyme. Amylase enzyme is required to digest carbohydrates (polysaccharides) into smaller units (disaccharides), and eventually into even smaller units (monosaccharides) such as glucose. Amylase enzyme plays a key role in plant defense toward pests and pathogens (Franco et al., 2000) which cause severe damage to field crops and stored grains (Franco et al., 2002). Invertase enzyme hydrolyzes sucrose, forming fructose and glucose.

The objective of this study is to evaluate the susceptibility of 4th instar of *S. littoralis* to the commercial formulation of *Bacillus thuringiensis* under laboratory condition and to determine the effect of this bio-insecticide on amylase, invertase and trehalase enzymes activities.

Materials and Methods

Laboratory rearing of *S. littoralis* :

The cotton leaf worm, *S. littoralis* was reared under laboratory conditions for several generations at 25±2 $^{\circ}$C and 60 ±5% R.H. Larvae were fed on Egyptian clover leaves (*Trifolium alexandrinum* L .) in a wide glass jars until adult emergence. The newly emerged adults were mated inside glass jars and supplied with a piece of cotton wetted with 10% sugar solution as feeding source for the emerged moths and branches of Tafla (Nerium oleander L.) as an oviposition site. Egg masses were kept in plastic jars until hatching. The obtained 4th instar larvae were used for bioassay tests.

Bacterial insecticide:

The commercial formulation of *Bacillus thuringiensis* subsp. kurstaki Berliner (diple2x) that contains 32000 IU/mg as bio-insecticide, 200 gm /feddan (VALENT Biosciences Corporation -USA) was used in the present investigation.

Toxicity tests:

A series of concentrations (in water) from diple 2x was prepared based on the active ingredient by diluting the commercial formulation to reach the following concentration : 0.5, 1, 2, 3 and 4 g/L. Clover leaves were dipped for 30 seconds in each concentration then left to dry for one hour. Non treated leaves were dipped in water (as a control). The 4th instar larvae of S. littorals were confined with treated leaves in glass jars covered with muslin for 48 hrs. Treated leaves were then removed and fresh untreated leaves provided for four days. Five replicates (each of 20 larvae) were tested for each concentration. Samples collected after 2, 3 and 5 days. The mortality percentage was daily calculated.

Enzymes determination:

Preparation of larval enzymes solution:

The samples of larvae used in enzyme assays were obtained from those subjected and fed on the sub lethal concentration (LC50) of the experimental biopestside (Dible 2x).

The larval enzyme solution was prepared according to the method described by Ishaaya *et al.* (1971). The enzyme solutions were obtained by homogenizing 10 fourth-instar larvae, representing ca. 2 g larval weight, in 20 ml distilled water, using a chilled glass Teflon grinder. The homogenate was centrifuged at 8000 r.p.m. for 15 min at 5°C, the deposits were discarded and the supernatants were kept in deep freezer till use.

Determination of Digestive enzymes activities:

The determinations of invertase, amylase and trehalase activities were based on the digestion of sucrose, starch and trehalose, respectively, by spectrophotometric methods (Ishaaya and Swirski, 1970, 1976). Briefly, invertase, amylase and trehalase were assayed using 3, 5 dinitrosalicylic acid reagent for determining the free aldehyde groups of glucose caused significant decrease of total protein, lipids and carbohydrates in *Spodoptera littoralis* larva.

Statistical analysis:
All experiments contained 3-4 replicates (insects homogenates), and the results of biochemical determinations were pooled from triplicate determinations. The results were analyzed by one – way analysis of variance (ANOVA) using costat statistical software (cohort software, Berkeley). When the ANOVA statistics were significant ($P<0.01$), means were compared by the Duncan's multiple range test.

Results and Discussion

In the present study the effects of commercial formula of *Bacillus thuringiensis* known as diple 2X against 4th instars of *S. littoralis* was determined. Using gradual increase of concentration of diple 2X (0.5, 1, 2, 3 and 4 g/L), the mortality percentages were recorded after 2, 3, 4 and 5 days post treatment. The data presented in Figure (1) show that, the mortality percentage increased with increasing the Dipel 2x concentration. The highest mortality was obtained when the 4th instar larvae were treated with 4 g/ L ofDipel 2x (= 90% 5 days post treatment) while the concentration of 1 and 0.5 g/L recorded the lowest mortality percentage (5% 5days post treatment) compared with the control which recorded no mortality (0%).

The toxicity of *B. thuringiensis* was investigated by Abd El-Aziz (2000) who classified lepidopteran larvae into three types based on their susceptibility to:
Crystalline endotoxin which caused insects mortality by preparations of crystalline endotoxin alone. They also, found that spores of bacterium are not responsible crystalline for the increase of toxicity, in some cases, mid gut pH may be closer to neutrality, allowing germination or the action of endotoxin may cause a decrease in pH so that germination can occur. They also indicated that insects were susceptible to endotoxin but the effect was enhanced by the presence of spores. The mid gut pH of most susceptible larvae was too alkaline to allow spore germination but was suitable for dissolution and activation of protoxin. Aziza and El- Shikh (2012) recorded that *B. thuringiensis* are toxic to larvae of Lepidoptera upon ingestion.

The died larvae by feeding on treated Egyptian clover with Dipel 2X for 5 days show several morphological changes (Figure 1).The died larvae get shrinked and its color turned to dark red compared to the control.

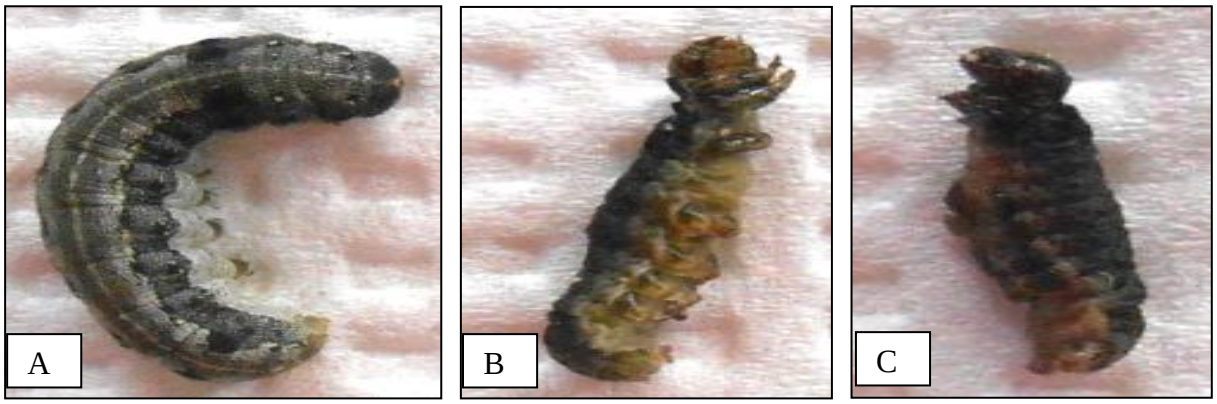

Fig. 1: Morphological changes in *S. littoralis* larvae treated with Dipel 2X compared to the control, A: control , B and C: dead larvae

The survival larvae of S. littoralis that have been fed on clover leaves treated by 4g / L of Dipel 2X shows several malformation. The data presented in Fig.2 show the changes in pupa stage, in comparison with the control pupa (Fig. 2-a) dwarf pupa (Fig. 2-b) was observed; the larvae fail pupate (half larvae and half pupa - Fig. 2-C), the pupa that kept the last instars' molting skin remaining attached to its body (Fig. 2-D) . On the other hand some moth failed to emerge from the pupa cuticle was evident (Fig. 3).

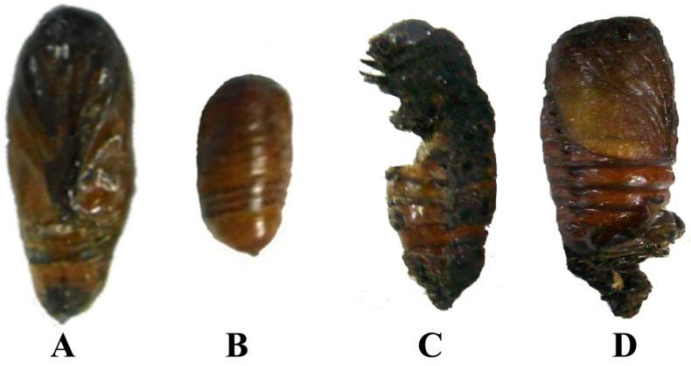

Fig. 2 : The effect of Dipel 2X on pupa stage, A: represent control, B: dwarf pupa and C: malformation

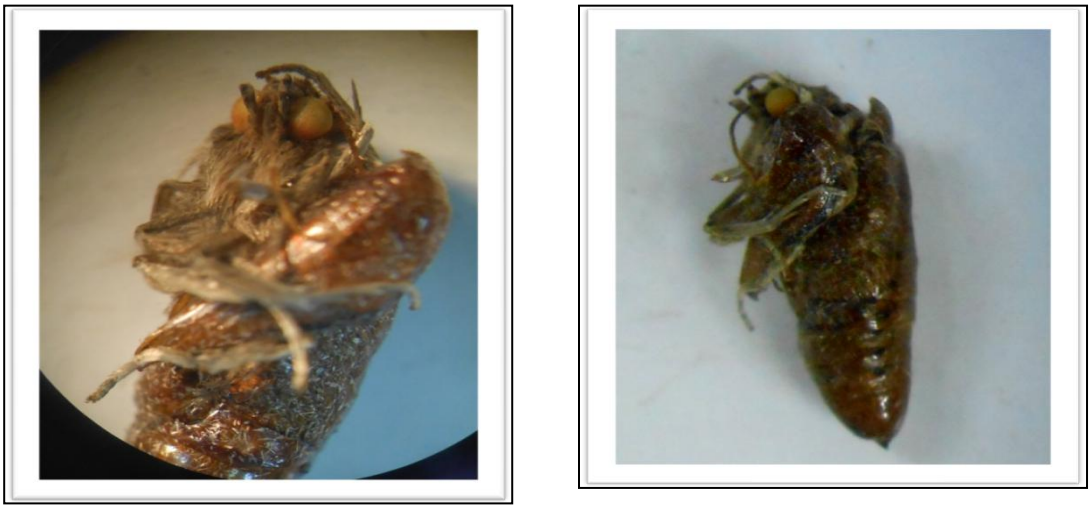

Fig. 3The effect of feeding larvae with treated clover on adult stages

The effects of *B. thuringiensis* on carbohydrates hydrolyzing enzymes of4th instar larvae of *Spodoptera littoralis* were studied. The amylase enzyme reduced to 70% after 72 hrs post treatment (Fig.4). The results in the present study are similar to the data reported by El-Ghar *et al*. (1995) in which *B. thuringiensis* caused a remarkable decrease in amylase activity. According to their results the maximum inhibition, about 77% was recorded 3 days after treatment which agreed with our data.

The results agreed also with Al-Shannaf *et al*., (2012) who found that the level of amylase activity in the supernatant of the homogenated larvae was lower than that obtained with the untreated larvae at all inspected times. The data disagree with El- Sheikh (2012) who showed that, the treatment with *B. thuringiensis* insignificantly decrease amylase activity as compared to the untreated one.

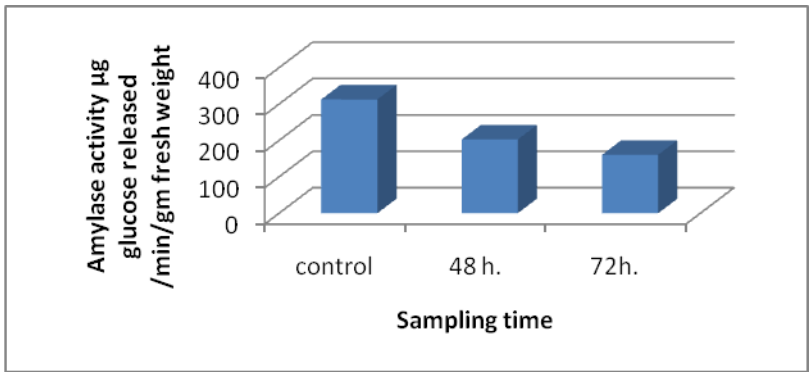

Fig. 4: the effect of *the B. thuringiensis* on amylase activity

The larval invertase activity remarkably decreased after three days of feeding on Egyptian clover leaves treated with Dible 2X compared to the control (Fig.5). The results in the present study were agree with the results that obtained by El- Sheikh (2012)who showed that the tested compound have a remarked effect on invertase activity. *Bacillus thuringiensis* significantly decreased the invertase activity compared to the untreated larvae as follow (27.7%, 27.5% and 32.9 %, respectively, compared to untreated one. The data also similar to data obtained by El-Ghar *et al*. (1995) who found that *B. thuringiensis*, caused a pronounced decrease in digestive enzyme activity especially invertase. Al Shannaf *et al*., (2012) reported that, regarding to invertase enzyme, there were decrease in the activity in *H. armigera* resulted from all treatments during all tested periods as compared to control.

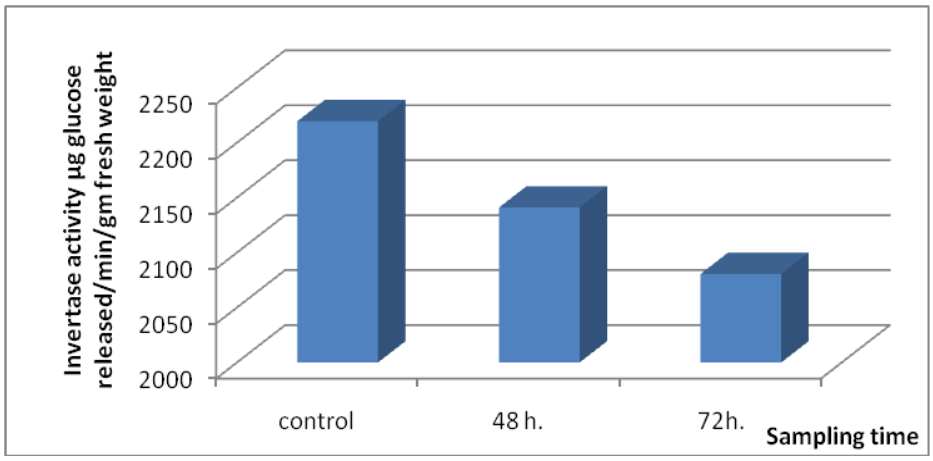

Fig. 5: the effect of the *B. thuringiensis* on invertase activity

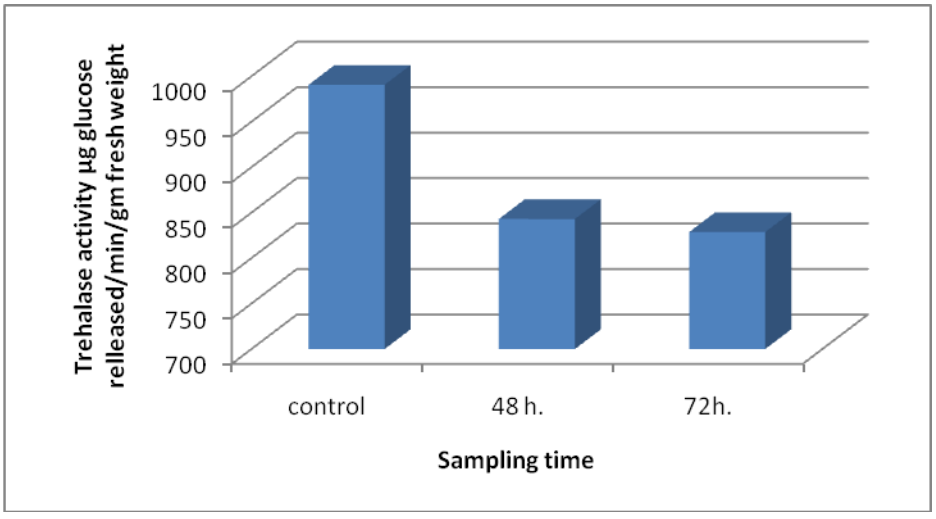

Fig. 6: the effect of the *B. thuringiensis* on trehalase activity

The data presented in Figure (6) indicate that feeding of *Spodoptera littoralis* larvae on the treated leaves with the bio-insecticide (dipel 2x) after 48 and 72 h results in significant reduction in trehalase activity compared to the control. It is well known that in insects, trehalase degrades trehalose to glucose for internal energy supply and generation of glucose needed for chitin build-up (during moulting), so the inhibition of trehalase observed in the present work might affect chitin build-up. Trehalase has the important function for liberating glucose for energy, and

is activated during moulting to generate glucose for chitin build up (Meisner *et al.*, 1978). In addition, trehalase played a significant role in the supply of energy to the insect and the activity of trehalase might serve as an indicator of energy reserves resulting from availability of carbohydrate nutrients (Wyatt 1967). The results reported by El-Sheikh (2012) are not similar to our data in which, the changes in trehalase activity insignificant decrease in enzyme activity was recorded after treatment with *B. thuringiensis*. The decrease in trehalase activity was similar with El-Ghar *et al*. (1995) who stated that *B. thuringiensis* at concentration of 200 ppm reduced trehalase activity by 53% after 2 days of treatment. Data presented by Al Shannaf *et al*., (2012), indicate that the activity of trehalase enzyme in the larvae of *H. armigera* was generally decreased in Dipel DF compared to other treatments in both times. It was 73.6 and 83.7% after 3 and 7 days, respectively.

The inhibition of carbohydrate hydrolyzing enzymes recorded in the present study might affect the molting process and subsequently may explained the reason of mortality occurred in *S. littoralis* larvae as illustrated previously in the toxicological experiments. These results are in agreement with previous research who observed pronounced decrease in the carbohydrate hydrolyzing enzymes especially amylase and invertase was observed after treated 5th instar larvae of cotton leaf worm, *S. littoralis* (Lepidoptera: Noctuidae) with sub-lethal concentrations of thuringeinsin (beta-exotoxin of *B. thuringiensis*) (El- Ghar *et al.* 1995). Zibaee *et al*., (2010) reported that when larvae fed on leaves treated by Bt, activity of all digestive enzymes was decreased

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