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Efficiency of Diple 2x ((*Bacillus thuringiensis* var. *kurstaki*) alone and its mixture with two insecticides against the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae).

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ABSTRACT

The Efficiency of the bioinsecticide, Diple 2x (*Bacillus thuringiensis*) alone and its mixtures with two insecticides on cotton leafworm *Spodoptera littoralis* (Boisd) was investigated. Data indicated that Lufenuron was the highest effective insecticides against both the 2nd and the 4th instar larvae of *S. littoralis* followed by Methomyl and B.t , respectively .All tested mixtures decreased the toxicity according to Co-Toxicity factor ,the tested mixture (B.t + Lufenuron) gave the high level of antagonism effect followed by the mixture (B.t + Methomyle). The field efficiency of tested insecticides (when used singular) can be arranged according to the general mean of reduction percentage during two seasons in a descending order as follows: Lufenuron, Methomyle and B.t. The addition of B.t. to Lufenuron gave raising the general mean of reduction percentage during two seasons in contrast the other tested insecticide gave a reduction but less than when applied without added to Bt. The same trend of effects were obtained when applied the two tested insecticides at the half recommended rate alone or mixed with the same dose of Bt on *S. littoralis* but with less level of reduction in case of (Bt+Lufenuron) mixture. Also the three toxicants affected the Chitinase ,prptease and acetycholinesterase enzymes.

INTRODUCTION

The entomopathogenic bacteria, *Bacillus thuringiensis* represents a good example for biological controlof insect pests. This bacterium, proved to be a highly successful for controlling some agricultural insect pests (Mohamed *et al*., 2005). Resistance to pesticides is probably the biggest challenge facing pesticides research today. Consequently, insecticides from different chemical groups with different mode of action and also some of their combination should be tested against *Spodoptera littoralis* to help developing a sound control program in the future (Ghoneim, 2002).

The combination of such bioactive agent with insecticides was investigated as attempt to increase their efficiency on *Spodoptera littoralis* and reduce the amounts of insecticides release in the environment which is appreciable from the environmental safety point of view (Aly and Eldahan,1987).

The aim of the work:-

The objective study was to evaluate the susceptibility of 2nd and 4th instars of *S.littoralis* to *Bacillus thuringiensis*, methomyle and chitin synthesis inhibitors Lufenuron. In addition assessment the joint action of the combinations of B.t when applied alone or mixed with the complete or half recommended rate of the same tested insecticides against *S. littoralis*. Also determine the effect of these insecticides on some enzymes activity.

MATERIAL AND METHODS

Tested Insecticides:

1- *Bacillus thuringiensis* subsp. *kurstaki* Berliner (diple2x) contain 32000 IU/mg as bioinsecticide, 300 gm /feddan.
2- Methomyle 90% SP ,300 gm / feddan.
3- Lufenuron , 160 ml /feddan .

Laboratory Experiment:

A laboratory strain of the cotton leafworm *S. littoralis* (Boisd.) was reared in the laboratory under constant conditions of 25°C±1 and 70 ± 5% RH and kept of any contamination with chemicals till the time of study as described by El-Defrawi *et al.* (1964).

A. Toxicity tests:

A series of concentrations (in water) for each insecticide was prepared on the active ingredient (a.i) based on ppm by diluting the commercial formulation. Castor-bean leaves were dipped for 30 seconds in each concentration then left to dry for one hour. The 2nd and 4th instars larvae of each tested strain were confined with treated leaves in glass jars covered with muslin for 24 hrs. Test also included a non treated control in which leaves were dipped in water (as a check). Treated leaves were then removed and fresh untreated leaves provided for four days. Three replicates (each of 20 larvae) were tested for each concentration. The average of mortality percentage was corrected using Abbott's formula (1925). The corrected mortality percentage of each compound was statistically computed according to Finney (1971). From which the corresponding concentration probit lines (LC-p lines) were estimated in addition to determine 25, 50 and 90% mortalities, slope values of tested compounds were also estimated. In addition, the efficiency of different compounds was measured by comparing the tested compound with the most effective compound by using the following equation:

Toxicity index = LC_{50} of the most effective compound / LC_{50} of the tested compound x 100 (Sun, 1950).

B. Determination of the joint action:

The tested insecticides were applied each at LC_{25} level when used singly. To test the joint action of the tested insecticides, equal volumes of the two insecticides were added together [Diple 2x and each insecticide 1:1 (w/w)]. Three replicates with twenty larvae each were used in each treatment. Mortality counts were recorded after five days of treatment. To determine the effect of applying pairs of insecticides, the expected LC_{25} concentrate of each insecticide in the paired combination was calculated from its corresponding LC-p lines, there for, the expected mortality for the mixture of two insecticides was the sum of the expected mortalities of each of the concentrate used in the combination. To evaluate the joined effect of the different pairs of used insecticides, the equation of the co-toxicity factor of Mansour *et al.,* (1966) was applied.

$$\text{Co-Toxicity factor} = \frac{\text{Observed \% mortality} - \text{Expected \% mortality}}{\text{Expected \% mortality}} \times 100$$

This factor was employed to differentiate the results into three categories. A positive factor of (+20) or more meant potentiation.a negative factor of (-20) or more meant antagonism, and any intermediate value (i.e. between -20 and +20) was considered only additive effect.

II. Field Experiment:

Experiments were conducted at kaha district, kalyobia Governorate during the two successive seasons 2010 and 2011 to evaluate the field efficiency of the bioinsecticide Diple 2x (*Bacillus thuringiensis* subsp. *kurstaki* Berliner) alone and mixed with the complete or half recommended rate of one insecticides (Methomyl) and one insect growth regulators (Lufenuron) on cotton leafworm *S. littoralis* (Boisd.). The fields were cultivated with Giza 86 cotton variety and the normal agricultural practices were applied. The experimental area was divided into plots of 42 m^2 each and the treatments were arranged in randomized complete blocks with four replicates each. Plots were isolated from each other by unplanted corridors (1 m width) that separated replicates. A motor sprayer was used to spray the chemical dilutions. The volume of spray solution was 40 litres /feddan. A pre-treatment count was measured for each treatment so as control. Post treatment count was recorded after 3,7 and10 days the initial effect was calculated at day 3 post treatment , the general mean residual effect was calculated as the mean reduction percentages of larvae observed at days 7 and 10 post treatment . After counting ,the appropriate amounts of each compound were extended with water and sprayed with ULV motor .Hand picking of egg masses was continued daily after spraying up to the end of the experiment and the collected egg masses were destroyed .Initial and reduction percentages in larvae counting were calculated according to Henderson and Tilton (1955).

III-Determination of biochemical aspects:-

A-Preparation of samples for biochemical studies:

The 4th instars larvae of both field and laboratory strain was fed on castor oil leaves treated with LC_{50} of tested compounds till the 6th instar. The collected larvae were placed in clean jars, and then starved for 4 hours. Samples of haemolymph were collected from the larvae by puncturing aproleg into tube and kept under freezing condition at-50°C.

B -Enzyme assays :

1- Determination of Chitinase activity:

Chitinase was assayed using 3,5-dinitrosalicylic acid reagent to determine the free aldehydic groups of hexoaminase liberated on chitin digestion according to the method described by Ishaaya and Casida (1974).

2-Determination of Protease activity:

The proteolytic activity was determined by the casein digestion method described by Ishaaya *et al.* (1971).

3-Determination of acetylcholine esterase activity:

The activity of acetylcholine esterase (AChE) was measured according to the method described by Simpson *et al.* (1964).

RESULTS AND DISCUSSION

I. Laboratory Experiment:

A. Toxicity tests:

Data presented in Table (1) showed that Lufenuron was the highest effective insecticides against the 2nd instar larvae of *S. littoralis* followed by methomyle and B.t, respectively. The toxicity index being, 0.004577 and 51.0986 % for Bt and methomyle based on LC_{50} of Lufenuron 100% .Concerning the efficiency of tested insecticides against the 4th instar larvae of *S. littoralis* (Table 2), also Lufenuron was the most effective insecticides followed by Methomyle and B.t, respectively. The toxicity index being 0.003426 and 50.798 % for Bt and Methomyle based on LC_{50} of Lufenuron 100%, respectively. The obtained results of lufenuron toxicity are in agreement with those obtained by Haga *et al.* (1984) who reported that Chlorfluazuron is very toxic to insects because it metabolizes slowly inside the insect body. The toxicity of lufenuron against *S. littoralis* larvae was some what similar to that of the Teflubezuron against *S. littoralis* (Aziza *et al.* 2011)

The toxicity of *B. thuringiensis* is 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 δ- 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. They also indicated that spore-endotoxin mixtures only killed insects. The mid gut pH of most susceptible larvae was too alkaline to allow spore germination but was suitable for dissolution and activation of protoxin. Abd-El wahed *et al*., (2010) and Aziza *et al*. 2011 recorded that *B. thuringiensis* are toxic to larvae of Lepidoptera upon ingestion.

Table 1: Susceptibility of 2nd instar larvae of cotton leafworm, *Spodoptera littoralis* (Boisd.) to tested insecticides.

Tested Insecticides	LC_{25} (ppm) its limits at 95%	LC_{50} (ppm) its limits at 95%	LC_{90} (ppm) its limits at 95%	Slope	Toxicity index (%)
Bacillus thuringiensis	2931.21 (1261-7254)	20321 17532-41233.5	565720 76246-841E+5	0.76± 0.2	0.004577
Methomyle	0.91 1.004 -1.167	1.82 1.2211.412	2.304 1.77- 2.048	6.94± 0.8	51.0986
Lufenuron	0.30 0.095- 1.890	0.93 0.056- 0.212	1.96 3.821- 64.754	0.67±0 .2	100

Toxicity index = LC_{50} of the most effective insecticide / LC_{50} of the tested insecticide x 100.

Table 2: Susceptibility of 4th instar larvae of cotton leafworm, *Spodoptera littoralis* (Boisd.) to tested insecticides.

Tested Insecticides	LC_{25} (ppm) its limits at 95%	LC_{50} (ppm) its limits at 95%	LC_{90} (ppm) its limits at 95%	Slope	Toxicity index (%)
Bacillus thuringiensis	1526.21 (1861-1254)	35321 (27532-14233)	59657 (76246- 321E+5)	0.76± 0.2	0.003426
Methomyle	1.51 (2.01 -0.867)	2.382 (3.22-1.822)	3.304 1.77- 2.048	6.94± 0.8	50.798
Lufenuron	0.93 (1.92- 2.89)	1.21 (2.25- 0.82)	0.926 (1.821- 0.67)	0.53±0 .2	100

B- The joint action of Bt and both toxicants:-

The joint action data of the combinations of B.t with each other tested insecticides against 2nd instars larvae of *S. littoralis* at LC_{25} level are shown in Table (3). Data clearly indicated that the two tested mixtures decreased the toxicity of the 2nd instar according to Co-Toxicity factor. The tested mixture (B.t + lufenuron) gave the high level of antagonism effect – 52.55 compared with -29.98 for (B.t + methomyle) mixture. While (B.t + methomyl) and (B.t + lufenuron) gave -17.454 and -24.92 Co-Toxicity factor in case of 4th instars treatment respectively. Concerning the joint action of combination produced antagonism effect when tested on both instars larvae of *S. littoralis* except in case of B.t + methomyl mixture produced additive effect for 4th instar treatment. It could be concluded that both tested combinations didn't have positive effect on but in contrast it gave negative effects under laboratory condition. So, unfavorable the bioinsecticide (Diple 2x) mixed with tested insecticides Methomyle or Lufenuron .

Table 3: Joint action of the tested mixture of insecticides against 2nd instars larvae of *Spodoptera littoralis* (Boisd.) at LC_{25} level.

	2nd instars larvae			4th instars larvae		
	Expected (%) mortality	Observed (%) mortality	Co-Toxicity factor	Expected (%) mortality	Observed (%) mortality	Co-Toxicity factor
B.thuringiensis +Methomyle	47.31	36.54	- 29.98	40.22	33.2	- 17.454
B thuringiensis +Lufenuron	58.32	38.23	- 52.55	53.3	40.38	-24.92

C- Field experiments:-

Data in Table (4) showed that the field efficiency of the bioinsecticide, Diple 2x (*Bacillus thuringiensis*) alone and other complete dose of two insecticides on cotton leafworm *S. littoralis* (Boisd.) during 2010 and 2011 seasons .The initial effect (after three days from spraying), Lufenuron was the highest in activity gave 81.9 and 74.85% reduction in infestation followed by methomyl gave (51.7 and 41.2) whereas *Bacillus thuringiensis* gave the least effect (26.6 and 25 %) reduction during 2010and 2011 seasons, respectively.

Table 4: Field efficiency of Diple 2x (*Bacillus thuringiensis*), Methomyle and Lufenuron on cotton leafworm during 2010 and 2011 seasons.

Treatments	Rate of application / feddan	Season	No. befor spraying	Reduction %					
				Initial effect After 3 days	Residual effect (days) After 7days	Residual effect (days) After 10days	Mean of % Residual effect	General mean of % Reduction	General mean of two seasons
B.t	200gm	2011	95	26.8	43.5	56.4	49.95	38.37	40.26
		2012	120	25	41	44.5	33.9	58.9	
Lufenuron	300 gm	2011	85	81.9	85	92.3	88.65	85.3	77.37
		2012	100	74.85	75.4	77.4	76.4	71.2	
Methomyle	160 mll	2011	90	51.7	60.5	68.8	64.4	58.1	54.17
		2012	122	41.2	45.9	72.6	59.25	50.23	
Control	-----	2011	92	90	-	-			
		2012	112	110					
Bt+ Lufenuron	200gm+300 gm (complete dose)	2011	90	86.4	89.8	94.3	92.4	89.4	85.3
		2012	110	76.8	83.3	87.9	85.6	81.2	
Bt+Methomyle	200gm+160 ml (compete dose)	2011	96	41.6	49	66	57.5	49.55	41.138
		2012	135	24.4	38.8	43.3	41.05	32.725	
Bt+ Lufenuron	200gm+150 gm (half dose)	2011	102	75	80	85	82.5	78.5	70.35
		2012	112	63.8	71	82.3	67.65	65.7	
Bt+Methomyle	200gm+80 ml (half dose)	2011	95	30.2	84.5	67.8	56.15	46.8	47.69
		2012	115	37.4	54.8	64.78	59.79	48.59	

Regarding the mean of residual effect percentage for tested IGR, Lufenuron showed the highest residual effect gave 88.65 and 67.4% followed by Methomyl and B.t with values (64.4 and 59.2%) and (49.9 and 33.9 %) during two seasons, respectively. The efficiency of tested insecticides (when used singular) can be arranged according to the general mean of reduction percentage of two seasons in a descending order as follows: Lufenurone , Methomyle and BT they were 77.37,54.17 and 40.26 respectively .

Concerning the field efficiency of B.t when mixed with other complete dose of either IGR or insecticide on cotton leafworm *S. littoralis* (Boisd.), data indicate the addition of B.t to Lufenuron gave raising the general mean of reduction percentage during two seasons with value 85.41% reduction, in contrast the other tested insecticide gave a reduction but less than when applied without added to Bt (41.138%).

The same trend of effects were obtained when applied the two tested insecticides at the half recommended rate alone or mixed with the same dose of Bt on *S. littoralis* but with less level of reduction in case of Bt+Lufenuron mixture (Table4).

Based on the LC_{50} values, the highest larvicidal activity of the selected insecticides alone was exhibited by chlorpyrifos, fenpropathrin and thuringiensin (Radwan *et al*. 1993). Mohamed and Ahmed (1990) reported that, in leaf-dip bioassays using castor leaves, sethoxydim (at 1 and 0.1%) the activity of *B. thuringiensis* against 2nd instar larvae of the noctuid *S. littoralis*, whereas fluazifop at the same conc. was antagonistic. Al-Zubadi et al., (1988) investigated the compatibility of Bactospeine (a commercial preparation of *B. thuringiensis* subsp. thuringiensis) with several chemical insecticides in Iraq . Effective control of *S. littoralis* was achieved with mixtures of Bactospeine (at half the recommended dosage) with Decis 2.5% EC [deltamethrin], Sevin 85% WP [carbaryl] or Sumithion 50% EC [fenitrothion]. El-Hamaky *et al.* (1990) applied Cyfluthrin, triazophos and thiodicarb alone or as mixtures with *B. thuringiensis* (as Bactospeine) to cotton fields in Egypt, and the initial and residual mortality to 2nd and 4th instar larvae of the noctuid *S. littoralis* on treated leaves were determined in the laboratory. All treatments caused 95% initial mortality, but the microbial pesticide did not increase knockdown performance. Positive correlations were found between residual mortality and insecticide concentrations, as well as for feeding periods on treated leaves. Negative correlations were found for time after treatment. *B. thuringiensis* did not potentiate residual performance of the chemical insecticides.

Biochemical studies:-

Activity of Chitinase and protease: Results in table (5) indicated that both chitinase and prptease activity increased significantly in case of Lufenuron treatment while there is no significant difference for both Methomyl and Bt. Chitinolytic enzymes are usually produced from cells in epidermis, gut, salivary glands or fat body of insects (Kramer and Koga, 1986). Chitinolytic enzymes have been demonstrated in the moulting fluid which appears in the space between the old and the new cuticles during ecdysis and secreted by the hypodermis (Kimura, 1976), and in the integument (Koga *et al*., 1989).Both enzymes Chitinase and protease are essential for digestion of old endocuticle in moulting process. So any change in this enzyme activity may attributed to the inference of the IGRs with this process.

Table 5: Enzymatic Activity *Spodoptera littoralis* Larval haemolymph Treated with LC_{50} of some Insect Growth Regulators.

IGRS	Enzymatic activity		
	Chitinase µgNAGA/min/ml	Mean AChE AChBr/min./ml± S.E.	Protease OD X 100/ hr./ml
Bacillus thuringiensis	3.21±.0.23b	17.9 ± 1.3b	89.3±0.042 b
Methomyle	3.92±0.30b	13.52 ± 2.3c	90±0.056 c
Lufenuron	6.35±0.042a	29.14 ±1.3a	132±0.042 a
Control	3.80±0.05b	18.21 ± 0.29b	85.3±1.5 b

Activity of Acetyl cholinesterase: The data in Table (5) indicated that the Acetyl cholinesterase activity decreased significantly for Methomyl treatment but insignificantly for Bt treatment .On the other hand Lufenuron increase the Acetyl cholinesterase activity significantly. Methomyl act as carbamate insecticide through excitation of the insect nervous system, which in turn cause alteration in the function of nicotinic and GABA-gated ion channels which leads to involuntary muscle contractions and tremors (Salgado *et al*., 1998). According to such an activity, it was expected that such insecticide may produce cytotoxic action either in neurons or non-target cells.

The cytotoxic action in neurons may alter the neurotransmitter mechanisms through interfering processes of methomyl with the production of acetylcholine in the synaptic region which affect in turn the activity of the acetycholinesterase to be in form of false inhibition.

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ARABIC SUMMARY

تقدير مدى فاعلية المركب الحيوى دايبل 2x بمفردة او مختلطا بمبيدين على دودة ورق القطن سبودوبترا ليتوراليس حرشفية الاجنحة نوكتويدى.

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تقدير مدى فاعلية المركب الحيوى دايبل 2x بمفردة او مختلطا بمبيدين على دودة ورق القطن سبودوبترا ليتوراليس أوضحت النتائج ان مركب اللوفينورون اكثر المركبات تأثيرا على كلا العمرين الثانى والرابع لدودة ورق القطن يلية مركب الميثوميل ثم المركب البكتيرى .أدت المعاملة بكلا المخلوطيين الى نقص السمية تبعا لمعامل السمية حيث ادى الخلط بمركب اللوفينورون الى اكبر تأثير مضاد يلية الخلط بالميثوميل. تقديرالمركبات المستخدمة فى التجربة الحقلية منفرده رتبت تبعا للمتوسط العام للخفض تنازليا كما يلى ليفنورون ،ميثوميل والمركب البكتيرى فى كلا الموسميين 2010و2011 .أدت المعاملة بخلط المركب البكتيرى لكلا المركبيين الاخريين الى نقص فى الاصابة ولكن اقل من الليفينورون بمفردة سواء الاضافة للجرعة الموصى بها كاملة او نصفها .كما ادت المعاملة بكل المركبات الى تغيير في نشاط انزيمات الكيتيينيز ،البرتييز والاستيتيل كولينيستيراز.