Evaluation of Some Environmentally Safe Cemicals Against *Spodoptera littoralis*

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ABSTRACT

Toxicity effect of Dipel-2X, and three insect growth regulators IGRs: Diflubenzuron; Spiromesifen and Pyriproxyfen, were determined against 2nd larval instar of *Spodoptera littoralis*. The results showed that Diflubenzuron was the most potent toxicity followed by Spiromesifen; Pyriproxyfen, and Dipel-2X. The effect of LC_{50} of the tested IGRs on the *in vivo* inhibition of chitinase from *Spodoptera littoralis* was assayed. The interaction effect of Dipel-2X with IGRs was investigated. Results proved that pretreated of Dipel-2X with IGRs caused more toxicity effect than single treatment. The sensitivity of chitinase activity to the three tested IGRs was measured by the I_{50} values. The I_{50} values of Diflubenzuron; Spiromesifen and Pyriproxyfen on Lab strain larval chitinase are 0.54, 0.60 and 0.73µM respectively. While the I_{50} values are 0.60, 0.72 and 0.81µM respectively against Field strain. The results proved that chitinase was sensitive to the IGRs. Generally, Dipel-2X pretreated with IGRs will produce a new trend so as increase toxicity of the bioinsecticide, enhance the role of beneficial insects. The results of the present study may add some forward steps to use bioinsecticide as alternative to conventional insecticides especially against this insect. So, the tested compounds can be involved in important steps necessary for successful IPM programmes applied against *S. littoralis*.

INTRODUCTION

The Egyption cotton leafworm, *Spodoptera littoralis* is one of the major pests in the middle east. It has quickly developed resistance to chemical pesticides (Chung and Cote, 1992 & Amin, *et al*., 2001). Therefore, the cotton leafworm in Egypt exhibits multiple resistance to nearly all insecticides used (Keddis, *et al*., 1988 & Ishaaya and Kleen, 1990).Owing to the endless and various problems that have been arisen by using insecticides (eg., the development of pest resistance, rapid resurgence of target species and outbreaks of secondary pests), the need to develop novel alternatives or functional combinations of pest control techniques is emphatically a product of this decade. Attention was therefore paid to control insects using different non traditional insecticides, e.g., insect growth regulators (IGRs) and *Bacillus thuringiensis* (BT). (Klein, *et al*., 1996; Abdel-Halim, 1997 & Rizk, *et al*., 1999).

IGRs show good effect against scale insects on cotton. Their effects have been observed in development, on behavior and several forms of diapauses. Also IGRs compounds which are considered nowadays one of the mainly component of IPM program term IGRs describe a new class of bio-rational compounds. (Mesbah, *et al*., 1982; Abdel-Naby, *et al*., 1990; Palma and Meola, 1993; Pawar, *et al*., 1995 & Shiotsuki, *et al*., 1999).

In recent years, much attention has been paid to the use of the *Bacillus thuringiensis* (B.t.) against *Spodoptera littoralis* (Lecadet and Martouret, 1987; Entwistle, *et al*., 1993; Klein, *et al*., 1996 & Abdel-Halim, 1997). *Bacillus thuringiensis* subsp. *Kurstaki* active vs. lepidopterous insects (Lereclus, *et al*., 1989). The mixtures of Bt. with some insecticides have been evaluated against various insects. (Pree and Daly, 1996 & Abou-Taleb, 2000).

The present investigation aimed to study the efficiency of bioinsecticide (Diple-2X) either alone or in their combination with some IGRs (Diflubenzuron; Spiromesifen and Pyriproxyfen) on *Spodoptera* larvae. Also the study was directed to throw the light on the effect of these tested IGRs on the activity of chitinase.

MATERIALS AND METHODS

Insect:

Laboratory strain of cotton leafworm, *Spodoptera littoralis* was chosen for bioassays and biochemical assessment. This strain start as field strain reared under laboratory condition for several years in centeral lab. of pesticides, Agricultural Research Center (ARC) Cairo, Egypt. Field strain of *Spodoptera littoralis* egg masses were collected from cotton fields at Abeis area, the 2nd larval instar used for assessments.

Chemical:

Three IGRs insecticides: Diflubenzuron, 25% (WP) Novartis Co. (Syngenta). Spiromesifen, 24% (SC); was obtained from MyTrade Co., and Pyriproxyfen, 10% (EC) was obtained from Sumitomo Chemical Co.

Bioinsecticide *Bacillus thuringiensis* subsp. *Kurstaki*; Diple-2X 6.4% (WP) (32,000 International Units/mg). The product was produced by Abbott Laboratories. Chemical and Agricultural Products

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Division, North Chicago, USA, and Provided by Bayer Company.

Bioassay tests:

1-Toxicity of Bt.:

Second instar larvae were starved for 6hrs before exposed test. The selected larvae were bioassayed against commercial strain (Diple-2X). Using three replicates for each concentration with ten larvae in each replicate.

Disc dipping technique was used since it has been proved to be the most common procedure for assessing toxicity to commercial formulation of Bt. (Tabashnik and Cushing, 1987). Each castor leaves disc ($2Cm^2$) was dipped into the suspension of tested formulation for 10s. Tested concentration were prepared in glass distilled water (GDW) (Toni and Fred, 1996) discs were held vertically to allow excess solution to drip off and placed on a rack to dry for at least 2hr. Treated discs were offered to starved larvae (on disc per cup) and left under constant conditions (27±2 ^{o}C). There after survivors were transferred with fresh castor oil plant leaves to clean cups and kept under the same conditions. Control larvae were allowed to fed on castor oil leave discs treated with distilled water. Mortality was percentage calculated for each concentration daily for 24; 48, and 72hrs and corrected according to Abbott (1925) and subjected to probit analysis using the computer program (Finney, 1971).

2-Toxicity of the Tested IGRs Against *S. littoralis*:

Diflubenzuron; Spiromesifen and Pyriproxyfen, were bioassayed against the 2nd larval instar *S. littoralis*. The castor leaves were dipped in different concentrations of the tested IGRs. All insecticides concentrations were prepared in distilled water. The treated leaves were placed in clean glass container at the laboratory conditions of (27±2°C) and 65-70%RH. Ten larvae (Lab and Field strains) were used for each test with three replicate. Mortality was recorded after 24; 48 and 72hr and subjected to probit analysis.

3-Toxicity of Tested Diple-2X in Presence of IGRs:

S. littoralis 2nd instar (Lab and Field strains) were treated with solution of Diflubenzuron; Spiromesifen and Pyriproxyfen at LC_{50} values concentrations before 24; 48 and 72hr of feeding on discs of castor oil leaves discs treated with LC_{50} of Diple-2X, joint action experiments have tow controls. Larvae of the first control were allowed to fed castor oil leaf discs treated with concentration equivalent LC_{50} of Diple-2X alone, while larvae of the second control were fed with untreated discs. Mortality counted and recorded daily for 3days. Percentage of mortality were calculated according to Abbott (1925) and subjected to probit analysis (Finney, 1971).

Enzyme Preparation and Activity Assay:

Chitinase was prepared from *S. littoralis*. 2nd instar larvae (Lab and Field strains) according to the method of Deul, *et al*., (1978). Homogenate was prepared in 10^{-3}M Cleland's reagent (dithiotheritol, DTT) (v/w=2). The homogenate was centrifuged for 15min at 12,000g. An equal volume of saturated ammonium sulfate solution was slowly added to the supernatant. After stirring for 1hr, the suspension was centrifuged for 10min at 10,000g. The precipitate was washed with half-saturated ammonium sulfate solution and was recentrifuged, after which it was suspended in a small volume of water, followed by dialysis 20hr. Any occasional precipitate was removed by centrifugation and was discarded as it proved to be enzymatically inactive. After dialysis, water was added to the original ratio (v/w=2). All manipulations were carried out at 0-2?C.

Chitinase activity was determined according to the method of Reissig, *et al*., (1955) which modified by Andrew, *et al*., (1982), using sodium acetate buffer instead of tris-HCl buffer and wave-leangth 416nm was used instead of 544nm. 25µl of chitin (20mg/ml), 100µl of enzyme prep and 225µl of sodium acetate buffer, (pH 4.5) in total volume 350µl. The enzyme substrate mixture was incubated at 35?C for 60min, then the reaction was stopped by adding 100µ1 of 0.8M borate buffer (pH10.0) followed by determination of N-acetylglucoseamine by the method of Reissig, *et al.,* (1955). By adding 1.5ml of p-dimethyl amino benzaldhyde (DMAB, reagent). The samples were incubated in a shaker water bath at 35?C for 20min, the samples were measured spectrophotometerically at λ416nm.

The protein content of *S. littoralis* 2nd instar larvae homogenates was assayed spectrophotometrically by the method of Lowery, *et al*., (1951) at λ750nm using bovine serum albumin as a standard protein.

Inhibition of Chitinase Activity:

The inhibition of chitinase was determined in 2nd instar larvae *S. littoralis* using the LC_{50} values of each of the three tested IGRs. The method of Dixon and Webb, (1964) was adopted to draw the Dixon-plots by plotting 1/V versus concentrations of the inhibitor at two concentrations of the substrate. Chitine (the substrate of chitinase) concentrations were 3.0 and 5.0mM. Estimation of I_{50} value (the concentration of the inhibitor which inhibits 50% of the enzyme activity) was carried out by pre incubating the enzyme with the inhibitor for 30 min.

RESULTS AND DISCUSSIONS

Toxicity of Dipel-2X and Three IGRs:

The results of the toxicity of the Dipel-2X and IGRs in terms of LC_{50} are given in Table (1) for 2nd instar larvae of *S. littoralis*. LC_{50} values after 24hr were 7.31, 3.62, 4.25 and 5.38ppm for Dipel-2X, Diflubenzuron, Spiromesifen and Pyriproxyfen, respectively against *Spodoptera* Lab strain. For Field strain, LC_{50} values were 9.44, 4.39, 5.66 and 6.17ppm respectively. While LC_{50} values after 48hr were 5.40, 1.22, 3.41 and 4.00ppm respectively against Lab strain. For Field strain, the LC_{50} were 7.36, 2.16, 3.02 and 4.40ppm respectively. LC_{50} values after 72hr were 1.23, 0.23, 0.44 and 0.74ppm respectively against Lab strain. For Field strain, the LC_{50} were 2.13, 0.45, 0.68 and 0.91ppm respectively. According to the LC_{50} values, it is quite clear that the susceptibility of *Spodoptera* larvae to Dipel-2X, and the Lab strain of *Spodoptera* larvae is more susceptible to Dipel-2X in comparison to the Field strain. Also IGRs may act as growth distruptor, it interferes with moulting by softening the larval endocuticle through reduction in its chitin content and by hardening of the exocuticle as result of enhanced phenoloxidase activity. These results are in agreement with many investigators, Dulmage, 1971; Ibrahim, 1974; Ascher and Nemny, 1979; Dimetry, *et al*., 1979; Grosscurt and Anderson, 1980; Radwan, *et al*., 1980; El-Sayed, 1981; El-Nockrashy, *et al*., 1986; Lecadet and Martouret, 1987; Marguerre-M and Daniel, 1987; Chilcott and Ellar, 1988; Chung and Cote, 1992; Fisk and Wright, 1992; Tabashnik, 1992; Chandler, 1993; Palma and Meola, 1993; Forrester, 1994; El-Kordy, *et al*., 1995; Pawar, *et al*., 1995; Smagghe, *et al*., 1997; Barker, 1998; Said, 1998; Abd-Allah, 2000; Abou-Taleb, 2000; Ali, 2001 & El-Aw, 2006.

Toxicity of Dipel-2X Alone or Pretreated with the LC_{50} Values of IGRs Against *S. littoralis* Larvae:

Data in Table (2) show the LC_{50} values of Dipel-2X are 7.31, 5.40 and 1.23ppm after 24; 48 and 72hr against Lab *Spodoptera* strain respectively, while the LC_{50} values are 9.44, 7.36 and 2.13ppm against Field *Spodoptera* strain respectively. The interaction of IGRs with Dipel-2X against Lab and Field strains of *Spodoptera* larvae were studied. Larvae were allowed to feed on castor oil leave discs treated with LC_{50} of the different IGRs.

The LC_{50} values, of Dipel-2X pretreated with the LC_{50} values of Diflubenzuron; Spiromesifen and Pyriproxyfen on Lab and Field strains of *Spodoptera* larvae are presented in Table (2). The LC_{50} values of Dipel-2X when pretreated with IGRs was lower than LC_{50} of Dipel-2X alone in Lab or Field *Spodoptera* strains. The enhancement of toxicity is calculated as a Potentiation factor (P.f.) Table (2). Potentiation factor (P.f.) values for Diflubenzuron; Spiromesifen and Pyriproxyfen are 13.54, 11.97 and 9.14 respectively, after 24hr for Lab strain, while the P.f. values of three IGRs are 13.88, 12.26 and 10.04 respectively, after 24hr treatment, for Field strain. The P.f. values of three IGRs are 16.36, 13.17 and 8.57 respectively, after 48hr for Lab strain, while the P.f. values for Field strain are 13.63, 12.07 and 10.51 for three IGRs respectively. While the P.f. values of three IGRs are 9.68, 6.87 and 5.33 respectively, for Lab strain after 72hr treatment, while the P.f. values for Field strain are 13.33, 9.52 and 7.41 for three IGRs respectively. It is clear that the LC_{50} values concentrations of IGRs enhancement the toxicity of the Dipel-2X on *S. littoralis* larvae. The mixtures of Diflubenzuron+Dipel-2X were the most toxic treatments than Spiromesifen+Dipel-2X and Pyriproxyfen+Dipel-2X respectively.

In general, the susceptibility of *Spodoptera* larvae to Dipel-2X increases when treatment after IGRs. The IGRs+Dipel-2X caused more toxic effect than single treatment with Dipel-2X, it could be concluded that IGRs enhanced the toxicity effect of Dipel-2X. Based on P.f. values, the Lab strain of *Spodoptera* larvae is more susceptible to Dipel-2X in comparison to the Field strain. Generally, efficacy of IGRs have a very good additive toxicity for Dipel-2X either in Lab or Field *Spodoptera* strains. These results are agreement with finding (Salama, *et al*., 1992; David and Joanne, 1996; Klein, *et al*., 1996; Pree and Daly, 1996; Liburd, *et al*., 2000 & Mona, *et al*., 2004) whom found that when certain pairs of drugs or insecticides are administered

Table 1. LC_{50} values of Dipel-2X and three IGRs to 2nd instar *S. littoralis* larvae

Compounds	LC_{50}(ppm)					
	24hr		48hr		72hr	
	Lab strain	Field strain	Lab strain	Field strain	Lab strain	Field strain
Dipel-2X	7.31	9.44	5.40	7.36	1.23	2.13
Diflubenzuron	3.62	4.39	1.22	2.16	0.23	0.45
Spiromesifen	4.25	5.66	3.41	3.02	0.44	0.68
Pyriproxyfen	5.38	6.17	4.00	4.40	0.74	0.91

Table 2. Comparative toxicities of Dipel-2X alone or pretreated with three IGRs on *Spodoptera* larvae

Compounds	LC_{50}(ppm)											
	24hr				48hr				72hr			
	Lab strain	P.f.	Field strain	P.f.	Lab strain	P.f.	Field strain	P.f.	Lab strain	P.f.	Field strain	P.f.
Dipel-2X	7.31		9.44		5.40		7.36		1.23		2.13	
Diflubenzuron +Dipel-2X	0.54	13.54	0.68	13.88	0.33	16.36	0.54	13.63	0.22	9.68	0.30	13.33
Spiromesifen +Dipel-2X	0.61	11.97	0.77	12.26	0.41	13.17	0.61	12.07	0.31	6.87	0.42	9.52
Pyriproxyfen +Dipel-2X	0.80	9.14	0.94	10.04	0.63	8.57	0.70	10.51	0.40	5.33	0.54	7.41

*Potentiation factor (P.f.) = LC_{50} Dipel-2X alone / LC_{50} IGRs + Dipel-2X

together, the effects may be greater or less than might be expected from the sum of the activities of the components when administered separately. The phenomena involved, included under the term "synergism" "potentiation" and "antagonism", are becoming increasingly important in, for example, practical insect control and mammalian toxicology.

The observation that Dipel-2X had the lowest effect when applied alone but it was the best when mixed with IGRs. These findings may be resulted insect cuticle easily penetration which caused by IGRs in the mixture, and these results show that IGRs are act in similar manner in reducing chitin incorporation in the cuticle of *S. Littoralis*. So these mixture are a good control of Lepidopterous larvae.

Generally, it could be concluded that the use of insect growth regulators (IGRs) and their mixtures with biological insecticides (Dipel-2X) instead of conventional hazardous insecticides; and these my reduce the environmental pollution and hazard effects on human health. Dipel-2X may play an important role in future insect pest management programs especially when mixed with IGRs.

***In Vivo* Inhibition of *S. littoralis* Chitinase Activity:**

The *in vivo* inhibition effect of the LC_{50} values three IGRs against to the *Spodoptera* 2nd instar Lab and Field strains larval chitinase is shown in the data given in Table (3). The data declared that Diflubenzuron; Spiromesifen and Pyriproxyfen exhibited the high percentages of reduction of chitinase activity as values were 87.2, 80.5 and 75.4% respectively, for Lab strain, while values were 80.6, 76.9 and 70.5% respectively, for Field strain. Results indicated that Diflubenzuron in more effect on the chitinase activity than the Spiromesifen and Pyriproxyfen on 2nd instar. It is quite clear that the IGRs at LC_{50} concentration acts as potential inhibitors for *Spodoptera* larvae chitinase activity.

Table 3. *In vivo* inhibition of *Spodoptera* larvae 2nd instar Chitinase activity by LC_{50} of three IGRs

IGRs	%Inhibition	
	Lab strain	Field strain
Diflubenzuron	87.2	80.6
Spiromesifen	80.5	76.9
Pyriproxyfen	75.4	70.5

***In Vitro* Inhibition of *S. littoralis* Chitinase Activity:**

Table (4) show the *in vitro* inhibition of IGRs on chitinase activity of *S. littoralis* 2nd instar. The I_{50} values of Diflubenzuron; Spiromesifen and Pyriproxyfen for Lab strain larval chitinase are 0.54, 0.60 and 0.73µM respectively. While the I_{50} values are 0.60, 0.72 and 0.81µM respectively against Field strain. To characterize more details about the *in vitro* inhibition of chitinase by the inhibitor, the I_{50} and Ki values of each inhibitor were estimated from the graphical method of Dixon and Webb, (1964) Table (4). The obtained data proved that IGRs competitive inhibition of chitinase activity and Ki values were 40.0 and 15.0µM for Lab and Field strains respectively, in the case of Diflubenzuron. While these values were 52.0 and 18.0µM for Lab and Field strains respectively, in the case of Spiromesifen. On the other hand, Ki values were 61.0 and 56.0µM for Lab and Field strains respectively, in case of Pyriproxyfen.

In conclusion, Chitinase plays an essential role during ecdysis chitin. This enzyme is vital to moulting in insects, and may also affect gut physiology through their involvement in peritrophic membrane turnover. The exoskeleton of insects might constitute a useful target site for insecticidal chemicals.

Table 4. *In vitro* inhibition of *Spodoptera* larvae Chitinase activity by some IGRs

IGRs	I_{50}(µM)		Ki(µM)	
	Lab strain	Field strain	Lab strain	Field strain
Diflubenzuron	0.54	0.60	24	30
Spiromesifen	0.60	0.72	33	44
Pyriproxyfen	0.73	0.81	42	56

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الملخص العربي

تقييم بعض المركبات الآمنة بيئياً ضد دودة ورق القطن

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الهدف من البحث هو تقيم التأثير الأبادى للمبيد الحيوى دايبل-٢أكس مع ثلاث تجهيزات من منظمات النمو الحشرية هى الدايفلوبيتزيرون؛ سبيروميسيفين وبيريبروكسيفين ومخاليطهما وذلك على يرقات العمر الثانى لدودة ورق القطن للسلالة المعملية والحقلية بهدف تلاشى تأثير المبيدات التقليدية الضار على البيئة. وقد تم تسجيل قيم التركيزات النصف مميتة (LC_{50}) لكلا" من الدايبل-٢أكس ومنظمات النمو الحشرية تحت الدراسة بصورة فردية. ثم تم معاملة اليرقات العمر الثانى لدودة ورق القطن بتركيزات مختلفة (LC_{50}) من منظمات النمو الحشرية تحت الدراسة ثم معاملة هذه اليرقات بتركيز (LC_{50}) من الدايبل-٢أكس بعد ٢٤ و ٤٨ و٧٢ساعة من المعاملة بمنظمات النمو الحشرية تحت الدراسة، فأوضحت النتائج أن قيم الـLC_{50} بعد المعاملة أنخفضت بدرجة ملحوظة ويتضح ذلك من قيم معامل التنشيط (P.f.) الذى تم حسابها. وكذلك تم دراسة المقدرة التنشيطية لمنظمات النمو الحشرية المختبرة على النشاط الأنزيمى لأنزيم هام وحيوى بالنسبة للحشرة وهو أنزيم الكيتينيز ولقد أوضحت النتائج أن فى حالة الدايفلوبيتزيرون كانت النسبة المئوية للتنشيط هى ٨٧,٢ و ٨٠,٦% للسلالة المعملية والحقلية على الترتيب، بينما فى حالة السبيروميسيفين كانت النسبة المئوية للتنشيط هى ٨٠,٥ و ٧٦,٩% لكلا" من السلالة المعملية والحقلية على التوالى، ولقد كانت النسبة المئوية للتنشيط بواسطة البيريبروكسيفين هى ٧٥,٤ و ٧٠,٥% للسلالة المعملية والحقلية على الترتيب. وكذلك تم دراسة تأثير منظمات النمو الحشرية المختبرة على قيم I_{50} أوضحت النتائج حدوث زيادة فى الفعل الأبادى للدايبل-٢أكس على يرقات العمر الثانى للدودة ورق القطن المعاملة من قبل بمنظمات النمو الحشرية. ومن هذه النتائج نجد أن الخلائط أعطت تأثير أكبر من الدايبل-٢أكس ومنظمات النمو الحشرية عند تطبيقهم بصورة فردية مما يوضح أن منظمات النمو الحشرية تنشط عمل الدايبل-٢أكس ولذلك تعتبر هذه الدراسة خطوة فى إتجاه إستخدام هذه المخاليط كأحد عناصر المكافحة المتكاملة لدودة ورق القطن حيث إنها أكثر أمانا" للأنسان والبيئة.