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Biochemical and Toxic Characterization of Some Insect Growth Regulators to the Pink Bollworm, *Pectinophora gossypiella* (Saunders)

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ABSTRACT

Toxicity of three insect growth regulators; pyriproxyfen (juvenile hormone mimic), methoxyfenozide (an ecdysone agonist) and lufenuron (chitin synthesis inhibitors) were assayed against the first instar larvae of pink bollworm under laboratory condition. Data showed that lufenuron was the most toxic insecticides followed by methoxyfenozide and pyriproxyfen. The LC_{50} was 20.6, 47.4 and 50.8 ppm, respectively. The biological aspects (larval duration, pupal period, adult longevity, sex ratios, number of laid eggs and hatchability) were affected also by the tested insecticides. Lufenuron was the most effective on biological aspects than other insecticides. The life span of this insect was prolonged to 62.6 days compared to 49.7, 56.7 and 53 days with methoxyfenozide, pyriproxyfen and control, respectively. The number of laid eggs per female and hatchability were affected in lufenuron followed by pyriproxyfen and methoxyfenozide. Biochemical characterization showed that the activities of chitinase and protease were decreased compared to control, while amylase activity increased. These results confirmed that chitinase, protease and amylase play an important role in metabolism and degradation of insect growth regulators and thus play a major role in insect growth regulators resistance.

KEY WORDS
pink bollworm, lufenuron, methoxyfenozide, pyriproxyfen, biochemical characterization

INTRODUCTION

Cotton growing and production in Egypt have been faced with several infestations, especially lepidopteran insects; most of them being the pink bollworm, *Pectinophora gossypiella*, which causes an enormous damage in cotton yield [12]. The larvae of the pink bollworm attack plants at the beginning of the fruiting stage causing huge losses to the cotton green bolls, fibers and seeds and accordingly great reduction in the cotton yield [22].

Molting and metamorphosis are two critical physiological events in the life of insects. All insects molt periodically in order to grow, and all but a very few go through either gradual or complete metamorphosis to become an adult. These two events are regulated by the steroid 20-hydroxyecdysone, and the sesquiterpenoid juvenile hormone [26]. Due to harmful effect of the residual toxicity of conventional pesticides on human and environment, control agents with comparative less toxic were recommended. The systemic synthetic mimics of the insect hormones, which are best known as Insect Growth Regulators (IGRs) have been reported to be potent control agents against a number of pests of agriculture [14]. Insect growth regulators need not necessarily be toxic to its target, but may instead lead to an abnormality which impairs the survival of the insect [32]. However, it is important to note that those IGRs which have found practical uses cause the relatively rapid death of the insect through failure in the operation of a key process such as emergence of adults from pupae. Insect growth regulators have a good effect on certain physiological regulatory processes essential to the normal

development of insects or their progeny. They are quite selective in their mode of action and potentially act only on target species.

Pyriproxyfen is a juvenile hormone mimic with low toxicity to mammals. Pyriproxyfen inhibits metamorphosis and embryogenesis in several insects [10]. It is a fenoxycarb derivative in which a part of the aliphatic chain replaced by pyridyl oxyethylene. It is a potent juvenile hormone mimic affecting the hormonal balance in insects resulting thereby in strong suppression of embryogenesis, metamorphosis, and adult formation [23].

Methoxyfenozide belonging to a group of insecticide known as insect growth regulators (IGR's) is one of the most effective molt-accelerating compounds to control the lepidopteran insect pests of different crops [33]. It mimics the insect growth hormones thus causing premature molting of larvae.

Lufenuron (a chitin-synthesis inhibitor) has remarkable effects on the development and reproduction of *Drosophila melanogaster* [36]. The authors also found that the eggs from *D. melanogaster* adults exposed to 10 ppm of lufenuron failed to hatch and examined the embryos. Lufenuron disrupted the molting process, prevented egg hatch, and eradicated the German cockroach, *Blattella germanica* after 12 months of spraying in a commercial freight containers that had been specially modified to serve as a simulated domestic environment [25].

Amylase was found to be the most important enzyme that plays the major role in the digestion and metabolism of carbohydrates in insects [37]. This enzyme has received a great deal of attention in concern with digestion and utilization of carbohydrates in insect. However, little is known about their physiological and biochemical contribution to insecticidal toxicity [16].

This work aims to evaluate toxicity of some insect growth regulators to the pink bollworm larvae, their efficacy to biological aspects and some enzymes activities

MATERIALS AND METHODS

Insect:

The pink bollworm adults were obtained from Bollworm Division, Plant Protection Research Institute, Agriculture Research Centre. These adults were laid eggs which incubated at 25 ± 1C^{o} and 70 ± 5% RH in the laboratory. The eggs were hatched after five days and the first instar larvae were used in this work. The newly hatched larvae were reared and treated on semi-artificial diet according to [28].

Insecticides:

Three insect growth regulators in different groups were used:

Lufenuron:

(Match 5% EC), produced by Syngenta, Swaziland. This insecticide acts as a chitin synthesis inhibitors and thereby prevents Lepidoptera larvae from molting from one stage to another. It acts by preventing the formation of the new cuticle. Field rate is 200 ml per feddan (4200 m^2). Three concentrations were used (the field rate (25 ppm and other two lower concentrations 12.5 and 6.25 ppm).

Methoxyfenozide:

(Runner 24% SC), produced by Dow AgroSciences. This insecticide acts as ecdysone agonist (antimoulting). When insects are exposed to methoxyfenozide it is rapidly absorbed into the insect's circulatory system and binds with the ecdysone receptor. This disruption of the normal molt cycle prevents the larvae from completely shedding its old cuticle resulting in starvation, dehydration, and death within a few days. Field rate is 200 ml per feddan. Three concentrations were used (the field rate 60 ppm and other two lower concentrations 30 and 15 ppm).

Pyriproxyfen:

(Admiral 10% EC), produced by Sumitomo Chemical Company. This insecticide acts as juvenile hormone mimic and a potent inhibitor of embryogenesis, metamorphosis and adult formation. Field rate is 100 ml per feddan. Three concentrations were used (the field rate, 25 ppm and other two lower concentrations 12.5 and 6.25 ppm).

Toxic effect of the tested insecticides to the first instar larvae of the pink bollworm, P. gosssypiella:

The first instar larvae were starved for about 6 hours and treated with the tested insecticides. Three concentrations (the field rate and other two lower concentrations) from every tested pesticide were used. About 1ml of each concentration was added to 50 g of freshly prepared diet and mixed very well. Diets were divided into three replicates and each one poured into a convenient Petri dish (12 cm diameter). Twenty healthy newly hatched larvae were gently transferred to the surface of the diet on each Petri dish by using a soft

brush. Similar numbers of larvae were transferred to untreated diet as a control treatment. The dishes were covered and maintained in an incubator at the temperature of 27±1 °C and 65–75 R.H. with complete darkness during all the daytime. To simulate the nature, after ca. 1h, by exposing the first instar larvae to the treated and untreated diet, the healthy larvae were transferred individually into clean and sterile glass tubes (2x7 cm), each one containing 5g of untreated diet and each tube contained one alive larva.

All tubes were inspected after one, two and five days for estimating the mortality percentages. The LC_{50} values were calculated according to [13].

Efficacy of the tested insecticides on some biological aspects:

It was known that the insect growth regulators have effects on some developmental processes. So, the survived larvae were taken to determine the efficacy of the tested pesticides on some biological aspects. These aspects include the larval duration, pupal stage, adult longevity, sex ratio, number of eggs lay per female and hatchability in comparison with the untreated larvae (control).

Biochemical experiment:

The survived larvae fed on semiartifiacal diet contamenated by the first concentration (field rate) untile reached at forth instar larvae (full grown larvae) were taken to detarmine the activity of chitinase, amylase and protease enzymes.

Preparation of homogenate samples for biochemical analysis:

The collected larvae were homogenized in distilled water at 500 rpm using a Teflon homogenizer - (Mechianika Precyzyjna Warszawa type MPN-309-Poland) - surrounded with a jacket of crushed ice for 3 minutes. Homogenates were collected in cold tubes (on ice) previously coated with crystals of phenylthiourea to prevent melanization. centrifuged at 6000 rpm for 10 min at 5°C using (BECKMAN GS-6R Centrifuge). After centrifugation, the supernatant fluid was divided into small aliquots (0.5 ml) and stored at –20 °C until analysis. Three replicates were carried out for each biochemical determination.

Biochemical characterization:

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 [18]

Determination of amylase activity:

The method was based on the digestion of starch amylase according to the method described by [17]. The free aldhydic group of glucose formed after starch digestion were determined using 3,5 dinitrosalicylic acid reagent.

Determination of Protease activity:

The proteolytic activity was determined by the casein digestion method described by [19].

Statistical analysis:

Data were analyzed by the analysis of variance (one ways classification ANOVA) followed by a least significant difference, LSD at 5% [8].

RESULTS AND DISCUSSION

Toxic effects of the tested insecticides on the of pink bollworm larvae:

Data in (Table 1) show that the field rate (first concentration) of lufenuron is the most toxic insecticides against the pink bollworm larvae compared with the other insecticides. The percent of mortalities are 55.7, 36.7, 50.7 and 8.3% for lufenuron, methoxyfenozide, pyriproxyfen and control, respectively. The lethal concentrations for 50% of insect population (LC_{50}, s) are 20.6, 47.4 and 50.8 ppm, respectively. The slope value in lufenuron treatment is 1.6 compared with 1.3 and 1.5 in methoxyfenozide and pyriproxyfen, respectively. The statistical analysis shows that there are significant differences between all treatments with the first concentration (field rate). On the other hand, there is no difference between lufenuron and pyriproxyfen treatment in second and third concentrations. This means that lufenuron not only developmental insecticides (acts on development processes) but also has a larvicidal effects. This result was consistent with [30]. The authors cleared that lufenuron had a larvicidal effect on *Lobesia botrana* larvae, resulting in similar LC_{50} values for different instars: 0.07 ppm for first instars, 0.08 ppm for third instars, and 0.11 ppm for fifth instars. Lufenuron is more active than tebufenozide (act as ecdysone agonist and belong to the same group of methoxyfenozide) on *Spodoptera littoralis* larvae [15]. Methoxyfenozide (ecdysone agonist) has a low toxic compared than other insecticides. The percent of mortality with the first concentration (field rate) was 36.7% compared with 55.7 and 50.7% in

lufenuron and pyriproxyfen, respectively. The same result was found by [38]. The authors found that larval mortality of the fall armyworm, *Spodoptera frugiperda* reached to 8% and 26% in the low and high concentration of methoxyfenozide. A leaf-disk bioassay to seven concentrations of pyriproxyfen ranging from 0 to 30 ppm on fifth-instar of obliquebanded leafroller, *Choristoneura rosaceana* [31]. Male and female larvae were assessed separately for mortality as well as other parameters of growth and development. The LC_{50} values for males and females were 2.4 and 4.8 ppm, respectively.

Table 1: The lethal effect of lufenuron, methoxyfenozide and pyriproxyfen to the first instar larvae of the pink bollworm larvae, *P. gossypiella*

Treatments	Percents of mortalities			Slope values ± SE	LC_{50} and confidence limits/ ppm
	C_1 (mean±SE)	C_2 (mean±SE)	C_3 (mean±SE)		
Lufenuron	55.7 ±4.1[a]	35 ± 3.6[a]	20.7 ± 2.5[a]	1.6±0.3	20.6 (16.4 – 30.4)
Methoxyfenozide	36.7 ±3.1[c]	22 ± 3[b]	14.3 ± 1.2[b]	1.3±0.3	47.4 (29.1 - 95.7)
Pyriproxyfen	50.7 ± 7.3[b]	30.3 ± 4.6[a]	20 ± 2[a]	1.5±0.3	50.8 (38.3 – 89.6)
Control	8.3 ± 2.9[d]	6.7 ± 2.9[c]	5 ± 0.0[c]	-------	-------
F- values	133.4***	35.9***	54.1***	-------	-------
LSD (5%)	5.8	6.8	3.2	-------	-------

C_1 – first concentration C_2 – second concentration C_3 - third concentration

On the other hand, methoxyfenozide, lufenuron and pyriproxyfen caused 96.6, 89.9 and 90.6% mortality, respectively, to the canola aphids [2].

Effect of the tested insecticides on some biological aspects of the pink bollworm, P. gossypiella:

The survived larvae are kept to determine the latent effect of the tested insecticides on some biological aspects. These biological aspects include larval duration, pupal period, adult longevity, sex ratio, number of lay eggs and hatchability.

Data in (Table 2) show that the larval duration is prolonged in lufenuron treatment compared with control. The larval duration is 29.7 days compared to 25.3 days in control. The same data also, found with pyriproxyfen treatment. The larval duration is increased to 26.7 days. On the other hand, the larval duration is decreased to 23.7 days in methoxyfenozide treatment compared to 25.3 days in control. Statistical analysis shows that there is no difference among methoxyfenozide, pyriproxyfen and control treatments, but there is a great difference between these treatments (methoxyfenozide, pyriproxyfen and control treatments) and lufenuron treatment.

The larval duration in lufenuron treatment was increased sharply compared with control by more than 4.4 days. On the other hand, the larval duration in methoxyfenozide treatment was decreased to 1.6 days compared to control. This result was consistent with [20]. The authors found that the larval duration of the raisin moth, *Ephestia figulilella* was increased by lufenuron treatment to more than 12 days. Treatment of halofenozide (belong to ecdysone agonist) to the mosquito larvae caused a significant reduction in the length of the larval and pupal stage [5]. The incorporation of methoxyfenozide into the diet had a significant effect on the timing of larval development of fall armyworm, *Spodoptera frugiperda* [27]. Studies of the sublethal effects of ecdysone agonists in several important pests have been widely documented, in both larvae and adults. These effects include delayed or accelerated developmental time, loss of weight in both larvae and pupae, mortality and deformations in pupae, wing deformities in adults, disturbed diapause, and impaired reproductive parameters [11]. Pupal period also is affected in lufenuron treatment compared with other treatments and control. The pupal periods are 16.7, 10.7, 12.7 and 12 days in lufenuron, methoxyfenozide, pyriproxyfen and control, respectively. The statistical analysis confirmed that no significant difference among methoxyfenozide, pyriproxyfen and control (Table 2). While, there is a significant difference between these treatments and lufenuron.

The pupal duration was increased to 4.7 days compared to the control. [1] found that larval and pupal duration of *plutella xylostella* and oviposition period were increased in treated groups compared with the control (treated with distilled water). The pupal period was reduced in methoxyfenozide treatment compared other treatments. Methoxyfenozide reduced pupal stage duration of Mediterranean flour moth, *Ephestia Kuehniella* Zeller and inhibited adult exuviations with an LD50 of 0.01µg/pupa and LD90 of 0.37µg/pupa [34].

Adult longevity is increased slightly in lufenuron (16 days) and pyriproxyfen (16.3 days) treatments compared with control (15.7 days). On the other hand, it's decreased in methoxyfenozid to 15.3 days. The statistical analysis shows that there are no significant differences among all treatments. Data also show that the percent of males is more than female in all treatments. Table 2 shows that the life span is increased in lufenuron and pyriproxyfen to 62.6 and 56.7 days, respectively, compared with 53 days in control. The life span in methoxyfenozid is decreased to 49.7 days.

The adult longevity was reduced in methoxyfenozide treatment compared the other treatments and control. This result was consistent with [24]. The authors found that methoxyfenozide significantly reduced adult male longevity of beet armyworm, *Spodoptera exigua* compared with females by 1.1 and 1.5 d at 75 and 150 mg (AI)/liter, respectively. Methoxyfenozide was reduced adult longevity of *Spodoptera littoralis* by 2.3 days at the

higher concentrations [27]. The pyriproxyfen (PPF) has no discernable effects on adult longevity of *Aedes aegypti* [9].

Sex ratio was not affected by all tested insecticides compared with control. The number of emerged females and males (sex ratios) of diamondback moth, *Plutella xylostella* was close to 1:1 in all treatments, and there were no differences among all treatments [1]. No significant effects were observed on pupal sex ratio in beet armyworm, *Spodoptera exigua* [29].

The number of eggs lay per female decreased sharply in all treatments especially lufenuron. It was 85.3, 163.7, 137.0 egg/female in lufenuron, methoxyfenozide and pyriproxyfen, respectively, compared to 206 in control. The same results are found with hatchability. The percent of hatchability is decreased sharply with lufenuron treatment. It is 33% compared with 81.3% in control. The percents of hatchability are 61.3 and 53% in methoxyfenozide and pyriproxyfen, respectively.

Table 2: Effect of the tested pesticides on some biological aspects of the pink bollworm, *P. gossypiella*

Insecticides	LC_{50} and confidence limit	Larval duration	Pupal period	Adult longevity	Sex Ratio %		No. of laid eggs/female	Hatchability %
					male	female		
Lufenuron	20.6 (16.4 – 30.4)	29.7 ± 1.5^{a}	16.7 ± 2.1^{a}	16 ± 1.7^{a}	55	45	85.3 ± 10.7^{c}	33 ± 9.2^{c}
Methoxyfenozide	47.4 (29.1 - 95.7)	23.7 ± 1.2^{c}	10.7 ± 1.2^{b}	15.3 ± 1.5^{a}	60	40	163.7 ± 22.2^{b}	61.3 ± 5.5^{b}
Pyriproxyfen	50.8 (38.3 – 89.6)	26.7 ± 0.6^{b}	12.7 ± 1.2^{b}	16.3 ± 0.6^{a}	60	40	137.0 ± 15.9^{b}	53 ± 4.4^{b}
Control	-----	25.3 ± 0.6^{bc}	12.0 ± 1.0^{b}	15.7 ± 0.6^{a}	55	45	206.0 ± 9.6^{a}	81.3 ± 3.2^{a}
"F" values	------	17.8^{***}	10.1^{**}	0.37^{ns}	------	-----	32.19^{***}	34.60^{***}
L .S. D.	------	1.96	2.66	2.30	-----	-----	29.1	11.7

Statistical analysis shows that there are significant differences between lufenuron and other treatments. The egg hatch decreased in adults of *Ceratitis capitata, Bactrocera dorsalis, B. cucurbitae,* and *B. latifrons* reared on lufenuron treated diets [6]. Lufenuron treated media did not influence fertility after one gender was reared on experimental and the other on control media before mating. The adults of beet armyworm, *Spodoptera exigua* from the methoxyfenozide treatment did not show reduced fecundity, but fertility as measured by the percentage of eggs hatched (fertility) was significantly reduced compared with untreated control insects [29].

On the other hand, *Spodoptera frugiperda* adults that resulted from fifth instars treated with methoxyfenozide were not affected in their mean cumulative number of eggs laid per female (fecundity), nor percentages of eggs hatched (fertility), or the sex ratio [38].

Hatchability also was tested. The percent of hatchability decreased sharply in all treatments and especially in lufenuron treatments compared that control. This result means that these tested pesticides have more effectiveness on insect fertility. This result was consistent with [3]. The authors evaluated that the Adult fecundity and egg hatchability until the 34 d after the adult of *Diabrotica speciosa* emergence. The mean number of eggs laid/female (177.5) and the egg hatchability (19.8 %) were lower when insects were fed on lufenuron-treated leaves compared to 375.4 eggs and 68.7 % of hatchability when insects were fed on untreated leaves, respectively. Found that egg number and percent of hatchability (27.6±7.5 and 9.77±4.89% respectively) of sun pest, *Eurygaster integriceps* were significantly lower than the treatment of both males as well as females with pyriproxyfen (53.5±4.8 and 33.18±2.7%, respectively)

Biochemical characterization of the survived larvae of the pink bollworm:

The activities of chitinase, protease and amylase are determined in survived larvae (4^{th} instar larvae). Data in (Table 3) showed that the activity of chitinase is decreased in all treatments compared the control. The enzyme activity is decreased sharply in lufenuron treatment. Data in Table 1 showed that lufenuron is the most toxic compound than other compounds followed by pyriproxyfen and methoxyfenozide (with the first concentration). This result means that decreasing of chitinase activity has a relationship with increasing toxicity of lufenuron and also its resistance. These results were consistent with [4]. The authors cleared that the potency of the chitinase was generally, reduced when tests were conducted using the 5^{th} instar larvae of *Spodoptera littorals*. Reduction in values for such efficacy ranged between 1.3 to 2.5 -fold. In a descending order of the tested compounds according to chitinase activity was teflubenzuron, chlorfluazuron and flufenoxuron, respectively. The same authors found that the enzyme activity was not much affected as a result of flufenoxuron treatment compared to that of the control. The reduction in such activity was only 3.1%. On the contrary, chlorfluazuron detrimentally affected the enzyme activity by 32.6%, whereas teflubenzuron caused 26.8% reduction in activity. The reason behind the reduction in the enzyme of activity may be due to blockage and inhibition of the enzyme.

Table 3 also, showed that the activity of protease is decreased in all treatments compared with control especially in pyriproxyfen treatments. Significant reduction in the activities of protease in *Callosobruchus maculatus* larvae treated by insect growth regulators [21]. This decreasing of enzyme activity may be due to

decrease of feeding larvae. The enzyme production is clearly related to the feeding behavior (amount of food that passes through the alimentary canal) [7]. In addition, the obtained changes in the enzyme activities may due to the variation in the protein synthesis as a response to the different treatments.

Table 3: Determination of some enzyme activity of the pink bollworm, *Pectinophora gossypiella* larvae treated with some insect growth regulators

Tested insecticides	Enzymatic activity ± S.E.		
	Chitinase ug NAGA /min /g	Protease OD*1000/ min /g	Amylase ug glucose / min /g
Lufenuron	257.37 ± 3.77	469.51 ± 4.02	611.11 ± 5.02
Methoxyfenozide	294.10 ± 3.91	450.52 ± 5.62	644.84 ± 6.6
Pyriproxyfen	265.53 ± 4.35	400.66 ± 3.74	519.24 ± 4.39
Control	344.28 ± 8.24	488.79 ± 4.72	453.50 ± 4.42

Amylase activity is changed also, in treated compared to the untreated. The activity of amylase was increased in tested pesticides compared the control especially in methoxyfenozide treatment. The activity of amylase in *Osmia bicornis* was increased when it treated with methoprene (insect growth regulators) [35]. The authors stated that this increasing of activity is probably connected with the start metabolic activity of imago and mobilization of carbohydrates which are the main fuel during flight in bees.

Finally, these results confirmed that lufenuron as chitin synthesis inhibitor was the most effective pesticides compared the other pesticides. Lufenuron also, was the most effective pesticides on biological aspects (larval duration, pupal period, adult longevity, number of laid eggs and hatchability. So, the results confirmed that lufenuron not only developmental insecticide but also toxic pesticide. The results showed that chitinase, protease and amylase have great role in pesticides metabolism and detoxification.

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