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Biochemical Studies of Chlorfluazuron and Diflubenzuron Effect on Chitinase and Phenol Oxidase and Biological Studies on the Black Cutworm *Agrotis ipsilon* (HUFN.) (Lepidoptera:Noctuidae).

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ABSTRACT

Chlorfluazuron and diflubenzuron effect were biologicaly and biochemicaly were studied by treated *Agrotis ipsilon* at early stage 2nd instar larvae with two concentrations to show the stages diformities and changes in chitinase and phenoloxidase activities at three time intervals. The chitinase activity showed increase at 48h compared with 24h and 72h. Else, significant differences between the three times of treatment were observed in treated and control larvae. For illustration the mean of chitinase activities of chlorfluazuron LC_{30} treatment were (333.3±7.0, 319.6±2.4 and 282.6±3.1ug NAGA/ min/g.b.wt. for 24, 48 and 72 h were noted. Phenoloxidase enzymatic activity after 72 hrs revealed significant decline at higher concentration. After 24 hrs of treatment, the highest phenoloxidase activity was with chlorfluazuron (170 ±4.58 O.D. units/ min/g.b.wt.) and followed by drastic decline at higher concentrations and exposure period (91.6±0.99 O.D. units/ min/g.b.wt.). The percentage of phenoloxidase activity compared with the control shows varied from each other according to larvae exposure period. The activity at the three different time intervals show increase at 48h in compared with 24h and 72h. The higher percentage comparing with control of phenoloxidase activity and the lowest were 83.58 to –5.4 %for chlorfluazuron at42 h, of LC_{30} and chlorfluazuron at 24h, of LC_{50}. The biological results showed that the two compounds induced morphological abnormalities of all life stages of *A.ipsilon,* increase larval and pupal duration and decrease fecundity and fertility.

INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Hufn.) (Lepidoptera:Noctuidae) is a major pest of cutworms permanently shakes Egyptian crops at three to four generations each year resembling different countries. Cutworm is nocturnal (night time) as it attacks the young seedling of the plants at night. Larvae can damage many cultivated and wild plant species as tobacco, potato, tomato, lettuce, cabbage, spinach, turnip, eggplant, broccoli and many ornamental plants (Watson 2016). The larva feed on the plants by cutting their stem either below or just above the ground level, hide and live inside the cracks and holes in the soil during the day Busching and Turpin (1977). The percent of plant damage varies from 20-37 % at optimum temperatures of 26 °C and 75% relative humidity, (Archer *et.al.* 1980), but in severe cases the damage occur as much as 80% depending on the severity of infestation (Fernandes *et.al.* 2013). All organisms generate a wide variety of hydrolytic enzymes that exhibit different substrate specificities useful for various functions. Chitin is a polysaccharide occurs in fungi, some algae and many invertebrates, including insects. Thus, chitin synthesis and degradation could represent specific targets for fungicides and insecticides. Chitinases catalyzes the hydrolysis of chitin, to produce N-acetyl-d-glucosamine and chitobiose or chitotriose, and can be classified as endochitinases or exochitinases, and currently classified into two different families of glycosyl hydrolases, namely family18 and 19, on the basis of amino acid sequence similarities (Hirose *et.al.* 2010). Phenoloxidase is responsible for the biosynthesis of melanin pigment in animals and plants and play important role in insect immunity system that showing both monophenol monooxygenase and diphenoloxidase activity. Melanization followed by tanning and Sclorotization that make insect cuticle is tanned and hardened, (Lu and Jiang 2007). Chitinase and phenoloxidase inhibitors have become subject of increasing interest very important to investigate and so are necessary to comprehend its biochemical properties. Insecticides with growth regulating properties (IGR); recently exist in markets affect insects by regulating or inhibiting specific biochemical pathways or processes essential for insect growth and development principally to death due to abnormal regulation of hormone-mediated cell or organ regulation (Gholami *et.al.* 2013). Chlorfluazuron ($C_{20}H_9Cl_3F_5N_3O_3$) and diflubenzuron ($C_{14}H_9ClF_2N_2O_2$) is stomach toxin act as insect growth regulator belonging to class benzophenylurea, inhibits chitin biosynthesis of insect cuticle, loses cuticle elasticity and firmness, results in abortive molting and give a good control of various pests and selective toxicity especially lepidoptera, even at low dose rate (10-50 g a.i. /ha), possess low environmental persistence, low mammal toxicity and rapid dissolution, (Perveen and Miyata 2000). This study aim to investigate the effect of the two insect growth regulators treatment on the biological fitness of the target pest and detect the changes that conferring biochemically by measuring two biomarkers is chitinase and phenoloxidase enzyme at three time intervals throughout the *A. ipsilon* 2nd instar larvae exposure period

MATERIALS AND METHODS

Rearing Insects:

A big culture of *A. ipsilon* was reared in the CAPL for many years on castor-bean leaves (*Ricinus communis* L.) till the pupal stage under conditions 25 ± 2 °C, 65 ± 5% R.H. and light: dark period was 12 hours. Neonate Larvae reared in big glass containers with sawdust in the bottom covered with muslin cloth fastened with rubber bands and cleaned daily to avoid any type of infection. The 4th, 5th and 6th instar were reared individually in plastic vials to avoid cannibalism. Pupae were putted in ventilated cage and adults emerged were fed on piece of cotton soaked with a 10% sucrose solution and fresh leaves of *Nearium oleander* (L.) were used for oviposition. Laid eggs were collected and kept in glass container for hatching at the same rearing room. This culture was used for the

toxicological, biochemical bioassay and the growth parameter measurement. The process of culture rearing was repeated and maintained throughout the experimental studies.

Insecticides tested:

Insecticide formulations: chlorfluazuron 5% SC (Efcoron) also known as Atabron and diflubenzuron 48% SC (Newbenzuron) also known as Dimilin, were obtained from Central Agricultural Pesticide Laboratory under Kam Egyptian Agricultural Chemical Company as formulation supplier and Jiangyin Suli chemical Co.-China as supplier of the active ingredients.

1-Treatment procedure :

The initial bioassay was carried out by dipping castor bean leaves in serial insecticide concentration preparations based on ppm by diluting the commercial formulation in water. The treated leaves were allowed to dry at room temperature, introduced to 2nd instar larvae in 3 replicates petri dish and water only used for control. Ten larvae to each dish were provided. Then mortality percent was recorded after 96 h after treatment and corrected by Abbott formula (Abbott, 1925). Probit analysis for mortality data were completed using (Finney, 1971) and $LC_{30,\ 50}$ were calculated. Subsequently a quantity of 2nd larvae culture was exposed to castor bean leaves treated with LC_{30} and other quantity treated with LC_{50} values that achieved from the previously described bioassay. Every batch was divided into three exposure duration were 24, 48, and 72 h, and after each exposure time the survived larvae were collected and kept freezing for enzyme activities determination.

2-Determination of enzymes activities:

Determination of chitinase activity:

Colloidal chitin (substrate preparation) was prepared according to Bade and Stinson (1981). The reaction mixture was conducted according to Ishaaya and Casida (1974), and N-acetylglucose amine was determined by the sensitive method of Waterhouse *et.al.* (1961). The enzyme activity was expressed as µg N-acetylglucose amine (NAGA)×103/min/gm fresh weight.

Determination of Phenoloxidase activity:

Phenoloxidase activity was determined according to a modification of Ishaaya (I971), in a reaction mixture consisting of 0.5ml phosphate buffer (0.1 M, pH 7), 200µl enzyme solution and 200µl catechol solution (2%). Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25˚C). Enzyme reaction was initiated by adding catechol solution. Then after exactly 1 min, the optical density was determined. Zero adjustment was against sample blank at 405 nm.

3-The effect on biological stages estimation:

The biological effects were studied at the two concentrations against 2nd instar larvae of *A. ipsilon*. One hundred larvae were introduced to a big container covered with muslin and filled with a potato leaves treated with LC_{30} and LC_{50} concentration that estimated from the initial bioassay and three replicate were prepared. Control was treated with water only. After 24h feeding on treated leaves, untreated leaves were introduced. Each instar developmental periods, body weight, percentage of pupation and adult emergence were recorded, also count of egg to every female survived were recorded and hatchability were calculated.

Statistical analysis:

Data were subjected to the probit regression analysis using the Ehab-Soft program according to Finney 1971 methods and Abbott 1925 was included and the LC_{50}, and LC_{90} values and their 95% confidence limits were calculated for the baseline toxicity. The differences between the two compounds, exposure time and the concentration were analysis using the SPSS-19 statistical programme 2016. Means were separated using the least significant different at 5% level.

RESULTS AND DISCUSSION

Lethal and Sublethal Effects on 2nd instar larvae of *A.ipsilon*:

1-Toxicity of chlorfluazuron and diflubenzuron:

Table 1, show the lethal and sublethal concentrations of both IGR compound tested (LC_{30} and LC_{50}) that determined against *A.ipsilon*. Both goodness-of-fit tests indicated that the regression analysis fitted well to the chlorfluazuron ($p = 0.93$) and diflubenzuron data points ($p = 0.98$). Significant differences were observed between the IGR slope lines ($p < 0.001$). The LC_{30} and LC_{50} values in ppm that achieved were (134.38, 266.9 and 2002, 3744.5 for chlorfluazuron and diflubenzuron respectively. Nearby the toxicity effect result of both compounds that represent the phenylurease chemical group some searches stated that those compounds exerted excellent control for lepidopteran pests, identically, the phenylthiourea insecticide members differ between each other in their potency and toxicity, however (Li, *et.al.* 2013), discovered the insecticidal mechanism of NK-17 that was could bind to sulfonylurea receptor (SUR) of *Blatella germanica* to inhibit the chitin synthesis with stronger affinity comparing to diflubenzuron and glibenclamide. Da Silva and Mendes (2007) reported that diflubenzuron residual effects caused great *Aedes aegypti* (L) larval mortality. The diflubenzuron WP brought about 100% inhibition of adult housefly emergence (Tilak *et.al.* 2010), and inhibited 80 % of *Culex* adult emergence for 7-21 days in abandoned wells (Sadanandane *et.al.* 2012). Peters and Fitzgerald (2003) proved that the mound-building of subterranean termite *Coptotermes acinaciformis* (Froggatt) and Colony was declined till 12 week after bait application of chlorfluazuron.

Table 1. Toxicity of chlorfluazuron and diflubenzuron on 2nd instar larvae of *A.ipsilon*.

Insecticide	Calculated Responses			
	LC_{30}(F.L.) ppm	LC_{50} (F.L.) ppm	Slope ±SE	χ^2
Chlorfluazuron	134.38 (81.28-222.15)	266.91 (186.1-359.3)	1.72 ±0.289	0.43
Diflubenzuron	2002 (1225.7-3269.3)	3744.5 (2864.8-4882.5)	2.06 ±0.305	0.153

2-Effect on Chitinase and phenoloxidase activity:

In this study result of the *A.ipsilon* larvae treatment by benzophenylurease insecticides is found to

be induces differential in chitinase and phenoloxidase activity as compared to untreated. Chitinase activity resulted were mentioned in Table 2. Comparing between all the means, treated larvae had greater chitinase activity of both compounds than untreated controls. A significant differences between chitinase activity of all treatments of both compounds were found (F=1.34, df1=5, df2=39 and P= 0.267). With increase in exposure time the level of chitinase were increased slightly from 24h to 48h and began to slightly decrease in 72h exposure time. Otherwise, significant differences between 24, 48 and 72 h of treatment were observed in enzyme activity of treated and control larvae (F= 0.279, df=1 and P=0.60). For instance the mean of activities of chlorfluazuron LC_{30} treatment were ranged between (333.3±7.0 to 282.6±3.1ug NAGA/ min/g.b.wt. for 24, 48 and 72 h noted. The percentage of activity compared with the control also was found in Table 2 and shows that the results were varied from each other according to larvae exposure period. The activity at the three different time intervals show increase at 48h in compared with 24h and 72h. The higher value and the lowest were 23.3 to -1.77 % for chlorfluazuron at 24 h, of LC_{30} and diflubenzuron at 72h, of LC_{50}. Similar results were found to be a better review for the effect of IGRs compound on chitinase activity likewise Abd El-Mageed and Shalaby(2011), Found that mixing IGR compounds with some pyrethroids or organophosphates insecticide caused an increase in chitinase activity ranging between 17.14% and 53.34% in *Spodoptera littoralis* (Boisd.) more than the control. Also Sabry and Khedr (2014), observed elevation in phenoloxidase in 4th instar larvae of *S. littoralis* treated with tebufenozide, flufenoxuron and teflubenzuron and reduction in chitinase activity. Also molting hormone agonist methoxyfenozide and tebufenozide decreased phenoloxidase activity. Either Al-Mokhlef *et.al.* (2012), recorded that, inhibition in chitin and protein synthesis was calculated to be 88.9% and 61.85%, respectively in 5th instar *Schistocerca gregaria* nymphs, treated with LC_{75} of the chitin synthesis inhibitor teflubenzuron, and cuticle chitin and total soluble protein dry weight were 8.5 and 7.85 mg/nymph less than control, respectively.

Table 2. Chitinase activities at three time intervals of treated 2nd instar larvae of *A.ipsilon*.

Insecticide	Conc.	Chitinase (ug NAGA/ min/g.b.wt.)					
		24h±SE	% control	48h±SE	% control	72h±SE	% control
Chlorfluazuron	LC_{30}	333.3±7.0	23.3	319.6±2.41	20.9	282.6±3.1	0.462
	LC_{50}	297±1.27	9.87	305.3±2.16	15.5	264.6±2.16	-5.93
Diflubenzuron	LC_{30}	272±1.27	0.628	318±2.99	20.3	357±2.92	26.9
	LC_{50}	284.6±1.21	5.29	337±3.36	27.5	276.3±2.46	-1.77
Control	------	270.3±5.0	------	264.3±2.27	------	281.3±1.7	-----

a:% of control = (Test – Control)/Control × 100; Letters mean the significant differences between treatments

The result of phenoloxidase activities were cited in Table (3). The percentage of activity compared with the control also was found in Table (3) and shows that the results were varied from each other according to larvae exposure period. The results shows that after 24 hrs of chlorfluazuron treatment of *A.ipsilon* 2nd instar larvae, only the LC_{50} concentrations resulted in significant increase in phenoloxidase activity as compared to control. The analysis of phenoloxidase enzymatic activity after 72 hrs revealed significant decline at higher concentration. After 24 hrs of treatment, the highest phenoloxidase activity was to chlorfluazuron (170 ±4.58 O.D. units/ min/g.b.wt.) and followed by severe decline at higher concentrations and exposure period (91.6±0.99 O.D. units/ min/g.b.wt.). The percentage of the control was found in table 3. The activity at the three different time intervals show increase at 48h in compared to 24h and 72h. The higher value and the lowest were 83.58 to -5.4 % for LC_{30} and $LC_{50}$of chlorflua zuron at42 h.

This data reveal a significant differences between phenoloxidase activity of all treatments of both compounds were found (F=4.5, df1=5, df2=39 and P= 0.002) and significant differences between time of exposure 24h to 48h, 72h and control (F= 0.08, df=1 and P=0.779). These results were similar to that of Rahimi, *et.al.* 2013, who they found no statistical differences in phenoloxidase activity of *Ephestia kuehniella* larvae treated by pyriproxyfen, but hexaflumuron after 1-3 hours of post-injection. The phenylthiourea is the well-known and widely used inhibitor of phenoloxidase, the inhibitor function is the control of the activity in insects and prevent melanization and sclerotization reaction that might be regulating the activity during infection, wounding and metamorphosis (Sugumaran and Nellaiappan 2000& Ryazanova *et.al.* 2012). Phenol oxidase activity always affected by some stressor as pesticides and others like the proceeding reviews, Bai *et.al.* (2014), said that the phenoloxidase activity were affected by kojic acid treatment in larval of oriental fruit fly, *Bactrocera dorsalis*, and developmental periods were prolonged. While in rosaceous branch borer, *Ospheranteria coerulescens* phenoloxidase activities were lost 50% after a60 min incubation when treated with kojic acid (Gholami *et.al.* 2013). Assar, *et.al.* (2012), found decrease in phenoloxidase in 4th larval instar of culex treated with cyromazine. Ibrahim *et.al.* 2015, mentioned that phenoloxidase activity decreased in the *Heterorhabditis zealandica* infected *A. ipsilon* larvae compared with the control. Mirhaghparast, *et.al.* (2013), reported highest activity of phenoloxidase obtained at 6-12 hours post-injection of 5th larval instars of *S. littoralis* by spor of *Beauveria bassiana* and *Metarizium anisopliae*. And a significant increase in phenoloxidase level were found in 3 and 4th instar larvae *S. litura* treated by *B. bassiana* at 4.0×106 spores/ml after 24 hrs of infection (Bali and Kaur 2013). The fluctuation in decrease and increase in phenoloxidase activity may affected by some factors as stated by, Cornet *et.al.* (2013), found *Culex* phenoloxidase activity was higher in larvae than adults, in females than males, and in resistance males than their susceptible, but activity declines with adult age.

Table 3. Phenol oxidase activities at three time intervals of treated 2nd instar *A.ipsilon*.

Insecticide	Conc.	Phenol oxidase (O.D. units/ min/g.b.wt.)					
		24h±SE	% control	48h±SE	% control	72h±SE	% control
Chlorfluazuron	LC_{30}	170±4.58	83.58	119.6±3.63	33.48	91.6±0.99	11.3
	LC_{50}	87.6±1.46	-5.4	96.6±1.46	7.8	90.0±1.27	9.3
Diflubenzuron	LC_{30}	96.6±0.73	4.32	112.6±2.37	25.6	128.0±2.1	55.5
	LC_{50}	95.3±1.21	2.91	143.0±1.68	59.6	102.3±1.68	24.3
Control ± SE	------	92.6±1.46	------	89.6±0.99	------	82.3±1.54	------

% of control = (Test – Control)/Control × 100; Letters mean the significant differences between treatments

3-The effects on biological stages:

The effect of the growth regulation effect on biological stages of *A.ipsilon treated by* chlorfluazuron and diflubenzuron at the 2nd instar by LC_{30} and LC_{50} (134.38, 266.91 and 2002, 3744.5 ppm respectively and the control were in Table 4. Most larvae died during the molting process and a few individuals developed to the 6th instar and then died, while most larvae continue to live. The obtained results also clearly were revealed that the major effect were prolongation of the developmental times in all stages, decrease in body weight for larvae and pupae, deformation of all stages and survived longer before death as compared with the control. Table 4 also shows the effect on fecundity and fertility of each survived female at both concentrations of the two compounds. There was a significant differences between both insecticide in fertility that mean the number of eggs that hatched to every survived adult (F= 12.78, df= 15, P= 0.075) and in adult longevity (F= 1.88,df=6,P=0.171) and adult survival (F=0.55, df= 16, P=0.824). However there is no significant different were found towards fecundity or the number of eggs that deposited from every survived female. There is a significant differences between both insecticide about larval mortality or larval weight (F= 0.55, df=3,P=0.728) and (F=0.861, df=6,P=0.525) respectively. The results clearly show decrease in number of posited eggs and decrease in hatchability of both compounds that spot on the effect of emberyocidal effect against larvicidal effect of both compounds. The Benzoylphenylureas have several effects on larvae lead to diformation of the cuticle in addition to larvicidal and emberyocidal effect. Perveen and Miyata 2000 stated that sublethal exposure of chlorfluazuron topically applied on 5th instars of *S. litura* (F.) affect the fecundity and fertility through significant reduction of ovarian weight and number of mature eggs in pupae and adults. The protein content of ovaries was reduced but the carbohydrate and lipid contents were not affected. Apparently IGRs compound employed malformation effect beside larvicidal and emberyocidal effect in comparison with conventional insecticides. Rumpf *et.al*. (1998) found that the *Micromus tasmaniae* Walker treated by diflubenzuron, female Longevity was reduced, total, daily number of eggs was reduced and pre-oviposition period were extended, that in contrast with azinphos-methyl. The effect of the tested compounds on biological deformities here were similar to other IGR insecticide in *A.ipsilon* like Khatter (2014) who found that , Flufenoxuron induced morphological abnormalities of all life stages of *A.ipsilon,* and form incomplete adult wings, increase larval and pupal duration and decrease fecundity and fertility.

Table 4. Development of *A. ipsilon* larvae, pupae and adult under two treatment doses of chlorfluazuron and diflubenzuron.

Estimated parameters	Chlorfluazuron		Diflubenzuron		Control
	LC_{30}±SE	LC_{50}±SE	LC_{30}±SE	LC_{50}±SE	
Larval Mortality %	30±0.48	50±0.48	30.33±1.11	50.33±0.27	4.66±1.8
Larvae Weight (gm)	1.05±0.024	0.93±0.07	1.24±0.02	0.96±0.01	1.32±0.05
Larvae diformation %	28.66±1.94	40±2.1	35±2.9	49.3±1.8	2±0.48
Pupation %	58.66±4.86	45.3±1.9	59±1.92	36.3±0.73	95.66±2.3
Pupae Weight (gm)	0.49±0.0048	0.41±0.0096	0.5±0.005	0.39±0.0028	0.44±0.015
Pupae diformation %	19.33±4.2	27.67±0.73	26.7±1.99	47±2.54	2±0.48
Adult emergence %	62.7±2.42	47±2.67	51±3.6	33±1.27	94±1.27
Adult diformation %	41.3±3.9	45.67±5.46	44.3±2.46	51.6±0.73	1.33±0.27
Longivity (days)	7±0.48	6.66±0.73	8.66±0.73	7.66±0.55	10.33±0.27
Adult survival %	52±2.2	39.67±1.94	58.3±0.99	44±2.2	89.33±1.21
Fecundity	146.7±19.4	88±9.16	153.3±18.66	76.66±5.13	348.66±8.2
Fertility	54.3±1.99	39±4.1	60±3.36	36±1.92	91.66±4.46

Means followed by the same letter at the same column are not significantly different at p= 0.05.

For explain how insect affected: firstly: the insect dies within the old cuticle and the new cuticle formed within partial moult inhibition, along the ecdysidalline to where the old cuticle remains attached at one point or other. The effect of larval treatment can be displayed at the pupal moult (diformities). Secondly the failure to feed because of displaced mandibles.

The penzoylphenylurease group structures show that the pyridyloxy group is responsible for the transport process of the compound. Substitution of the hydrophobic group on the pyridine ring that lead to increase the hydrophobicity (the intrinsic factor) of the substituents on the aniline and benzoyl ring is that the controlling factor of the insecticidal activity and embryocidal activity Verloop and Ferrell (1977). Additionally the oxidative or degradative metabolism (stability) of the insecticide in the insect body is also reflecting the insecticidal activity Casida and Hajjar, (1979). Those are two points, compensate for each other

Chitin synthesis in insects begins by the formation of oligosaccharides synthesized in the epidermal cell, transport to the cuticle and polymeriz to

form discrete chitin microfibrils and are covalently bound to proteins. Structural integrity is essential for the polymerization of the oligosaccharides, the second polymerization step is the inhibition site by benzoylphenyl ureas. the inhibitory effects is through inhibits the enzyme that catalysing the polymerisation of UDP-N-acetyl glucosamine to chitin or inhibits the transport of UDP-N-acetylglucosamine across biomembranes, inhibits a protease that activates the chitin synthase zymogen and inhibits ecdysone metabolizing enzymes resulting in accumulation of ecdysone. This stimulates chitinase production which results in moult disruption. Additionally the active metabolite of the benzoylphenyl urea is responsible for blocks conversion of glucose to fructose-6-phosphate resulting in inhibition of chitin synthesis (Retnakaran, and Grant (1985).

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التـاثير البيوكيمـاوي للمبيـدين كلورفلوازيـورون والـدايفلوبنزورون علـي مـستويات انزيمـي الكيتينيـز والفينـول اوكسيداز والبيولوجي علي المراحل العمريه للدوده القارضه اجروتيس ابسيلون

حنان صلاح الدين طه دياب و مني قطب الحادق

المعمل المركزي للمبيدات – مركز البحوث الزراعيه- الدقي- جيزه

تم اختبار تاثير المبيدين كلورفلوازيورون والدايفلوبنزورون بيوكيماويا علي انزيم الكيتيناز والفينول اوكسيداز علي مدي ثلاث فترات من تعرض الافه للمبيد وبيولوجيا علي المراحل العمريه للسلاله المعمليه لحشره الدوده القارضه وذلك بمعامله الطور اليرقي الثاني بجرعتين من كلا المبيدين والتي تم تحديدها مسبقا باجراء التقييم المعملي الحيوي علي الحشره وحساب التركيزات المميته النصفيه وغيرها . اظهرت النتائج ارتفاع ملحوظ في نشاط انزيم الكيتيناز في الفترات الثلاثه للقياس وخاصه في الفتره ٤٨ ساعه وكانت القياسات البيوكيماويه لنشاط الانزيم كالتالي :ug NAGA/ min/g.b.w . ٣٣٣.٣ ، ٣١٩، ٢٨٢ علي التوالي . اظهرت النتائج ان اعلي نشاط لانزيم الفينول اوكسيديز سجل بعد ٢٤ ساعه من تعرض اليرقات لمبيد الكلورفلوازيورون وكان النشاط كالتالي O.D. ١٧٠unit/min/g.b.w. ثم انخفض الي O.D. units/g.b.w.٩١.٦ وذلك بعد ٢٤ ساعه اخري من تعرض اليرقات للمبيد. ومقارنه بالكنترول فان النسبه المئويه لنشاط انزيم الفينول اوكسيداز التي تم قياسها اختلفت فيما بينها اعتمادا علي مده تعرض اليرقات للمبيد وكانت تتراوح بين ٨٣.٥٪ الي ٥.٤٠ ٪ وذلك لمبيد الكلورفلوازيورون .بالاضافه الي ذلك فان الاختبارات البيولوجيه لليرقات المعامله بكلا التركيزين اظهرت تاثير المبيدين بيولوجيا علي اطوار الافه باحداث تغيرا ملحوظا تتلخص في زياده مده الطور اليرقي للافه وكذا احداث تشوهات في طور اليرقه والعذراء والفراشه ووجد ايضا تاثيرا ملحوظا علي كميه البيض الموضوع من الفراشه الناجيه وكذا نسبه الفاقس منه.