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Efficiency of some new insecticides on physiological, histological and molecular level of cotton leafworm.

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ABSTRACT

Efficiency of indoxacarb, spinetoram and methoxyfenozide on cotton leaf worm (*Spodoptera littoralis*) was determined through exposure of second and fourth instar larvae to dipped castor bean leaves. LC_{50} estimates of second instar larvae ranged from 0.004 to 0.006 ppm of indoxacarb, 0.022 to 0.033ppm of spinetoram and 0.006 to 0.011ppm of methoxyfenozide. LC_{50} estimates of fourth instar larvae ranged from 0.36 to 0.54 ppm of indoxacarb, 1.78 to 2.64ppm of spinetoram and 1.36 to 1.69 ppm of methoxyfenozide. Also, the ovicidal activity of these compounds was studied. Three day old eggs are more affected than that of one or two days old in case of indoxacarb and spinetoram while the reverse was in the case of methoxyfenozide. In nutritional assays, all tested compounds reduce food intake and affected growth rate (GR) where indoxacarb caused the highest rate of efficiency and followed by methoxyfenozide and spinetoram against 4th larval instar fed for two days on castor leaves treated with LC_{50s} of the previous coumpounds. The histological examinations of 6th larval instar cuticle (after treatment of fourth larval instar by LC_{50} of methoxyfenozide) showed destruction in the cuticle layers, fissures in the endocuticle and irregular distribution of the hypodermal cells. While indoxacarb and spinetoram treatments showed slight effect in the cuticle layers as compared to methoxyfenozide. In addition, the deteriorative potentialities of aldehyde oxidase and α - glycerophosphate dehydrogenase pattern of *S. littoralis* were screened.

Keywords: Indoxacarb, spinetoram, methoxyfenozide, *Spodoptera littoralis*, ovicidal effect, food consumption, histopathology, cuticle, aldehyde, oxidase, α – glycerophosphate, dehydrogenase, pattern.

INTRODUCTION

The cotton leaf worm, *Spodoptera littoralis* (Boisd) is a well polyphagous herbivore and is regarded as one of the most important agricultural pest in the Middle East. It is a very harmful potential pest of many field crops and vegetables, causing important economic losses in both greenhouses and open field on a broad range of ornamental, industrial and vegetable crops. To overcome the losses and to increase the yield, chemical insecticide application is utmost important (Aslam *et al*., 2004). Currently new groups of chemical compounds are being tested against lepidopteran pests such as, indoxacarb (Steward 15% SC), spinetoram (Radiant 12% SC) and methoxyfenozide (Runner 24% SC).

Indoxacarb represents a new class of insecticides (the oxidiazines). The primary route of entry into target insects is through ingestion (Wise *et al*., 2006), although it also is absorbed through the cuticle. It has a novel mode of action that blocks the movement of sodium ions into certain nerve cell ion channels, resulting in paralysis and death of the pest species.

Spinetoram is a new generation of spinosyn group. It causes excitation of the insect nervous system by altering the function of nicotine and GABA-gated ion

channels. It dose not interact with the known binding sites of other classes of insecticides such as of neonicitinoids, fiproles or avermectins (Crouse and Sparks, 1998).

Methoxyfenocide is classified as a diacylhydrazine insecticide. It acts as ecdysone agonists with enormous potential for development as insect specific control agents with little or no effect on non target species (Dhadialla *et al*., 1998; Toya *et al*., 2002; Sawada *et al*., 2003). Also, it provides effective control of a wide range of lepidopteran insects. The chemical upon absorption into the haemolymph of the insect, binds to the ecdysone receptor which initiates the moulting process. As the normal process disrupted, the insects prevented from shedding its old cuticle. The larvae die of dehydration and starvation within 2-5 days (Kumar and Santharam, 2008).

Present studies were designed to evaluate some toxicological, physiological, histopathological and molecular effects of these compounds against *S. littoralis*.

MATERIALS AND METHODS

The cultural of *S. littoralis* used in this study originated from eggs obtained from a susceptible strain established in the cotton leaf worm Department, Plant Protection Research Institute, Dokki, Giza. This strain was reared in the laboratory under constant laboratory conditions of 27±2 ^{o}C and 65±5 % RH (EL- Defrawy *et al*., 1964).

Experimental Insecticides

Three new chemical insecticides, indoxacarb (Steward 15% SC, DuPont), spinetoram (Radiant 12% SC, Dow Agro sciences) and methoxyfenozide (Runner 24% SC, Dow Agro sciences) were obtained from their respective manufacturers and used in the present studies.

Susceptibility Test

To assess the insecticidal activity of the tested compounds, different concentrations were prepared ranged from 8 to $3x10^{-3}$ ppm. The larvae used in the experiments were freshly moulted 2nd and 4th instar larvae within 6 hrs after ecdysis. The leaf dipping technique was adopted, where freshly castor oil beans leaves were dipped for 30 second in one of the prepared concentrations. The treated leaves were left to dry for approximately 15 minutes at room temperature before being offer to *S. littoralis* larvae. Three replicates contained 20 larvae / jar were used for each treatment and also for the control experiments. The mortality percentages of treated larvae were corrected against those of the control by using Abbotts formula, (Abbott, 1925). Probit analysis was determined to calculate LC_{50} and slope values of the tested compounds (Finney, 1971), through software computer program. Statistical analysis of results according to (SAS, 1996).

Treatment of Egg Masses

Different ages of *S. littoralis* egg masses (0-24; 24- 48 and 48-72 hrs old) which deposited on *Nerium oleander leaves* were dipped in different concentrations of the tested compounds for 15 seconds, left to dry, placed in petri-dishes and incubated at 27± 2^{o}C and 70± 5 RH. The control one was dipped in water and left to dry. Hatchability was recorded after 2 to 4 days.

Food Consumption and Utilization

Three replicates contained 20 second and/or fourth instar larvae / jar were left to feed on treated leaves with LC_{50} of the tested compounds for 48 hrs, then the survived larvae were transferred to untreated leaves in clean jars and left to feed until

death or pupation. The fresh weight of larvae, faeces and castor bean leaves in each rearing jar were recorded daily. Fresh leaves were kept in a similar rearing jar under the same condition to estimate the actual loss of moisture, which was used for calculating the corrected weight of consumed fresh leaves. Food consumption and utilization were calculated according to the equations given by (Waldbauer, 1968) and (Slansky and Scriber, 1982) as follows:

$$\text{Food ingested (I)} = [1- a/2] [w- (L+bL)]$$

Where : a = the ratio of loss of water to the initial weight of leaf.
b = the ratio of loss of water to the final weight of leaf.
w = weight of food introduced.
L = weight of uneaten food.

$$\text{Consumption index (CI)} = \frac{I}{TA}$$

Where: A = mean body weight of larvae during the feeding period (mg).
T = feeding period (days).

$$\text{Relative growth rate (RGR)} = \frac{W}{TA}$$

Where: W = weight gain of larvae (mg).

Approximate weight of digested food (mg) (D) = I – F

Where: F = weight of fasces (mg).

$$\text{Approximate digestibility (AD)} = \frac{\text{Approximate weight of digested food}}{\text{Amount of food ingested}} X100$$

Efficiency of conversion of ingested food into body matter (ECI) =

$$\frac{\text{Weight gain of larva (mg)}}{\text{Amount of food ingested}} X\ 100$$

Efficiency of conversion of digested food into body matter (ECD) =

$$\frac{\text{Weight gain of larva (mg)}}{\text{Amount of food digested}} X\ 100$$

Histopathological Studies

The tested compound were selected for a comparative study to clarify their effects on the histopathology of the cuticle of 6th larval instar surviving after treatment of the 4th larval instar with the LC_{50} values of the tested compounds. These larvae were dissected in 0.85% Saline solution, and the cuticle was taken and fixed in carnoy's fixative for half an hour, then two changes (for about 15 minute) in absolute ethyl alcohol (100%) for dehydration. The dehydrated organs were cleared in methyl benzoate for about 24 hrs., washed in toluene for three-five minutes, then transferred to liquid Paraffin (melting point 58^{o}C) for two-three hrs. changes. The different organs were embedded in liquid paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin for microscopic examination. Control sections of non- treated larvae were also studied.

Molecular Studies

Simplified procedure of electrophoretic separation of protein is the best method for the separation of isoenzymes. Protein molecules migration in an electric field depends on its net electric charge and size and pH of buffers. In alkaline solution proteins are negatively changed and travel towards anode. To visualize the location of enzyme bands on the gels, we follow

Aldehyde oxidase (AO)

After electrophoresis, the gel was rinsed in 100 ml tris/HCl, pH 8.5, which mixed with 20 mg NBT, 10 mg EDTA, 25 mg NAD, 100 mg KCl and 1ml

Benzaldehyde for 30min, then 5mg PMS were put. The gel was stained in dark place at (35- 37 ^{o}C) from (3 - 3.30 h.) After incubation, the gel was destained in 7% acetic acid **(**Ayala *et al*., 1972 and Pasteur *et al*., 1988).

α - glycerophosphate dehydrogenase (α-GPDH)

After electrophoresis, the gel was deposed in 100ml tris/HCl solution pH 8.5 which mixed with 20 mg NBT(Nitroblue tetrazolium salt), 10 mg EDTA (Ethylene diamenotetracitic acid), 25mg NAD, 400 mg α -Gph(α -glycerophosphate) for 30 min, then 5mg PMS (Phenazin methosulphate) were put. The gel was stained in dark place at 37^{o}C from (3-3.30h.). After incubation, the gel was destained in 7% acetic acid (Ayala *et al*., 1972 and Pasteur *et al*., 1988).

RESULTS AND DISCUSSION

Susceptibility of *Spodoptera littoralis* toward tested compounds

Susceptibility test on a laboratory strain of the 2nd and 4th larval instars of cotton leaf worm, *Spodoptera littoralis* was carried out at different concentrations of tested compounds. The mortalities were recorded after 120 hrs. post treatment . Data in Table 1 indicated that, 2nd and 4th larval instars of *S. littoralis* were susceptible to all tested coumpound, however, in different levels according to insecticide classes. The earlier instar was found to be more susceptible with lesser LC_{50} values and as the instars advances the values of LC_{50} was found to be increasing.

Table 1: Susceptibility of 2nd and 4th larval instars of *Spodoptera littoralis* toward tested compounds.

Tested compounds	LC_{50} after 120hrs (ppm)		95% fiducially limits		Slope
			Upper	Lower	
Indoxacarb	2nd	0.005	0.006	0.004	3.04± 0.07
	4th	0.45	0.54	0.36	1.79±0.04
Spinetoram	2nd	0.027	0.033	0.022	1.51±0.02
	4th	2.17	2.64	1.78	1.70±0.04
Methoxyfenozide	2nd	0.008	0.011	0.006	1.33±0.02
	4th	1.52	1.69	1.36	3.56±0.11

Indoxacarb was the most effective followed by methoxyfenozide and finally spinetoram. The LC_{50} values of indoxacarb, methoxyfenozide and spinetoram were 0.005, 0.008 and 0.027 ppm, for 2nd larval instars, while LC_{50} values were 0.45, 1.52 and 2.17 ppm, for 4th larval instars respectively. These results are in conformity with that obtained by (Ahmed *et al*., 2004; Abdou- Gehan and Abdalla, 2006; Ademczyk *et al*., 1999; Moulton *et al*., 2002).

Effect of Tested Compounds on *Spodoptera littoralis* Eggs.

Table (2) show that the three day old eggs are more affected than that of one or two days old in case of indoxacarb and spinetoram while the reverse was in the case of methoxyfenozide i.e. the highest ovicidal activity was showed at the early eggs.

Table 2: Ovicidal activity of tested compounds on egg masses of *Spodoptera littoralis.*

Tested compounds	Conc. ppm	% of eggs hatching		
		0-24 hrs old eggs	24-48 hrs old eggs	48-72 hrs old eggs
Control	0.00	97.99±1.26	97.99±1.26	97.99±1.26
Indoxacarb	0.005	94.54±0.49	62.76±0.44	55.07±1.49
	0.45	86.71±1.25	54.73±1.08	29.64±0.59
Spinetoram	0.027	73.79±1.50	66.75±2.82	35.95±0.75
	2.17	55.77±0.60	8.77±4.69	1.14±1.14
Methoxyfenozide	0.008	75.25±0.73	85.07±0.98	94.01±0.81

	1.52	43.25±0.52	65.81±0.87	90.68±1.34

Laboratory data indicate that the probable mechanism of ovicidal activity of indoxacarb and spinetoram are due to adsorption of them into the chorion and subsequent oral uptake as the neonate chews through the chorion to hatch. At this point the larva ingests a dose of them that is sufficient to affect the larva to die within the egg. Sometimes, eggs can be seen with the head of the neonate larvae protruding (Plate 1-B,C and D). These findings are in full agreement with (Elbarky Nehad *et al*., 2008). In the case of methoxyfenozide, failure of the eggs hatching could be explained by the known mode of action of this compound, which act as ecdysone agonists. Lepidopterans eggs have high concentrations of ecdysteroids that are made available to developing embryos and prehatching larvae (Hoffman *et al*. 1980) thus, methoxyfenozide may accelerate embryo development lead to premature lethal molt (Plate 1- E and F).

Food Consumption and Utilization

Results in Table 3 reveal that food consumption of the 4th instar of *S. littoralis* larva was significantly reduced by LC_{50} treatments. The inhibitory effect of larval feeding on treated leaves revealed the superior action of indoxacarb. This reduction varied in case of other treatment. The data were always significant and showed that the activity of indoxacarb> methoxyfenozide> spinetoram in descending order Plate 2. This indicated that the tested compound, especially indoxacarb act as antifeedant. These results are in agreement with the findings of (Hussien, 2000; Abdel-Al and Abdel-Khalek, 2006) who revealed that, IGR_s act as antifeedant.

Table 3: Some nutritional values of 4th larval instar of *Spodoptera littoralis* treated with LC_{50} of tested compounds.

Physiological aspects	Control	Indoxacarb	Spinetoram	Methoxyfenozide
Weight of food ingested/ larva (mg)	16 ±1884	1008 ± 41**	1455 ± 100*	1320 ± 37*
Weight of food digested/ larva (mg)	1548 ± 25	528 ± 32**	1022 ± 26*	934 ± 33*
Approximate digestibility (%)	82.17± 0.39	52.32 ± 1.01**	70.23 ± 0.33*	70.72 ± 0.58*
Relative growth rate (%)	30.71 ± 0.13	18.43 ± 0.18*	20.83 ± 0.26*	19.35 ± 0.20*
ECI (%)	19.17 ± 0.09	22.54 ± 0.58*	21.23 ± 0.45*	19.71 ± 0.31
ECD (%)	23.30 ± 0.16	43.15 ± 1.86**	30.22 ± 0.76**	27.89 ± 0.65*

* = Significant at P< 0.05 **= highly Significant at P< 0.01

Data indicated that the treatments had significant reduced the digestibility this may be due to the high percent of excretion or food consumption by larvae as compared to the control. All tested compounds decrease the relative growth rate. The efficiency of conversion of ingested food to body tissue (ECI) increased in larvae fed on tested compounds. Also results showed a significant increase in the efficiency of conversion of digested food to body tissue (ECD).

Histopathological Studies:

Normal cuticle of *S. littoralis* larva composed of an outermost distinct layer, the epicuticle and the inner layer called procuticle. Procuticle, parts of which are hardened to form the exocuticle, while other parts remain flexible and colourless to form endocuticle . Finally there is commonly a distinct layer called hypodermal layer which composed of columnar or cuboidal cells (Plate 3-A).

Histopathological examination of the 6th larval instar of *S. littoralis* resulting from the 4th larval instar treated with LC_{50} of methoxyfenozide revealed a separation of the hypodermal cells from the endocuticle and the hypodermal cells showed several

mitotic divisions and some fissure. Distortion in the endocuticle was also quite visible. This distortion revealed blockage of its formation (Plate 3-B). Hegazy (1990) reported that benzoylphenyle ureas induced a great disturbance in cuticle deposition of *S. littoralis* larvae.

Indoxacarb caused abnormalities in the shape of the exocuticle and the hypodermal cells separated from the endocuticle (Plate 3-C). While spinetoram has slight effect on the cuticle as compared with other compounds. As shown in (Plate 3-D) the thickness of the cuticle decreased, some hypodermal cells became separated from the endocuticle. Elbarky *et al*. (2008) showed that, the amount of total carbohydrates, total proteins, carbohydrate hydrolyzing enzymes were significantly decreased when *S. littoralis* treated with spinetoram. These components are necessary for building cuticle and lake of them may lead to abnormal endocuticular deposition and abortive molting. These results accordance with the demonstrated by El-Sheikh *et al*. (2005).

Molecular Studies

The enzymes, which act on a substrate and tend to remove hydrogen from it are known as dehydrogenases while those involved in the oxidative chain are termed oxidases. These enzymes are rather specific. The alcohols are produced inside the living organism through the hydrogenation of aldehyde and ketones or through metabolism of fats (production of glycerol). The high concentration of alcohols inside the living organism causes high disturbance in different metabolic pathways, so the living organism must regulate these alcohols through alcohol metabolizing enzymes such as alcoholic dehyrogenase or aldehyde oxidase (AO) (Parkash and Shamina, 1994).

The present study refers to the pattern of aldehyde oxidase (Plate 4-A), which is recorded at higher diversity in each of control and treated samples Table 4. This may be due to the higher metabolic rate in these samples. Aldehyde oxidase isozyme patterns were studied in several insects; (Garcin *el al*., 1983) who demonstrated the capacity of (AO) in *D. melanogaster* to detoxify acetaldehyde and use it for energy production. (Barnes, 1983) who stated that the inversion-allozme polymorphism of (AO) is directly involved in the adaptation to a specific environmental component. Also, (AO) isozyme in adult *Hypera postica* can be influenced by developmental age and environmental conditions (Romero *et al*., 1986).

Table 4: Relative fragmentation R_f and amount percentage of haemolymph aldehyde oxidase pattern of *S. littoralis* after treated the 4th instar larvae with Tested compounds.

Row	Control		Indoxacarb		Spinetoram		Methoxyfenozide	
	Rf	%amount	Rf	%amount	Rf	%amount	Rf	%amount
r_1	0.04	33.01	0.04	29.79	0.04	29.32	0.04	25.22
r_2	-	-	0.05	38.62	-	-	0.05	43.56
r_3	0.06	37.23	-	-	0.06	38.89	-	-
r_4	-	-	0.08	31.54	-	-	-	-
r_5	0.09	29.86	-	-	0.09	31.92	0.09	31.41
Sum	28.2		19.5		30.6		24.1	

Dehydrogenases are important tools for the investigation of insect metabolic activities during the course of development. The relative activities of insect dehydrogenases may be related to the energy yielding (Dickinson and Sullivan, 1975).

The α- GPDH, is a soluble, very active enzyme which is heavily concentrated in the muscles to fulfill the energy utilization from high carbohydrate metabolic rate in such organs (Sacktor, 1974). It generates NAD^+ via reduction of dihydroxy acetone phosphate during glycolysis. Therefor, any rise in α- GPDH activity normally corresponds to the drastic fall in LDH activity (Sacktor, 1976).

The assayed α- GPDH activity in the present study Table 5 and (Plate 4-B) recorded high effect due to the treatment with methoxyfenozide . These results are agreement with many authors, (Mostafa, 1994) who stated that IGR_s lead to a high activity of α- GPDH in larvae of *M. domestica*. (Abdel-Moty et al., 1996) declared that IGR_s effect on the pattern of enzymes activity, quite fluctuation in the ontogenic spectrum of α- GPDH differing from the normal behavior.

Table 5: Relative fragmentation R_f and amount percentage of haemolymph α- GPDH pattern of *S. littoralis* after treated the 4th instar larvae with Tested compounds.

Row	Control		Indoxacarb		Spinetoram		Methoxyfenozide	
	Rf	%amount	Rf	%amount	Rf	%amount	Rf	%amount
r_1	-	-	0.2	56.90	0.2	65.45	0.2	79.21
r_2	0.25	60.00	-	-	-	-	-	-
r_3	0.42	24.54	0.42	16.07	0.42	24.72	0.42	20.79
r_4	0.56	15.46	0.56	27.04	0.56	9.84	-	-
Sum	55.0		52.5		53.0		39.0	

Finally, any unusual change of α- GPDH and AO patterns in treated and evoluted stages might be, on molecular levels and referred to depression or mutations of the regulating genes responsible for biosynthesis of polypeptide chain building these enzymes.

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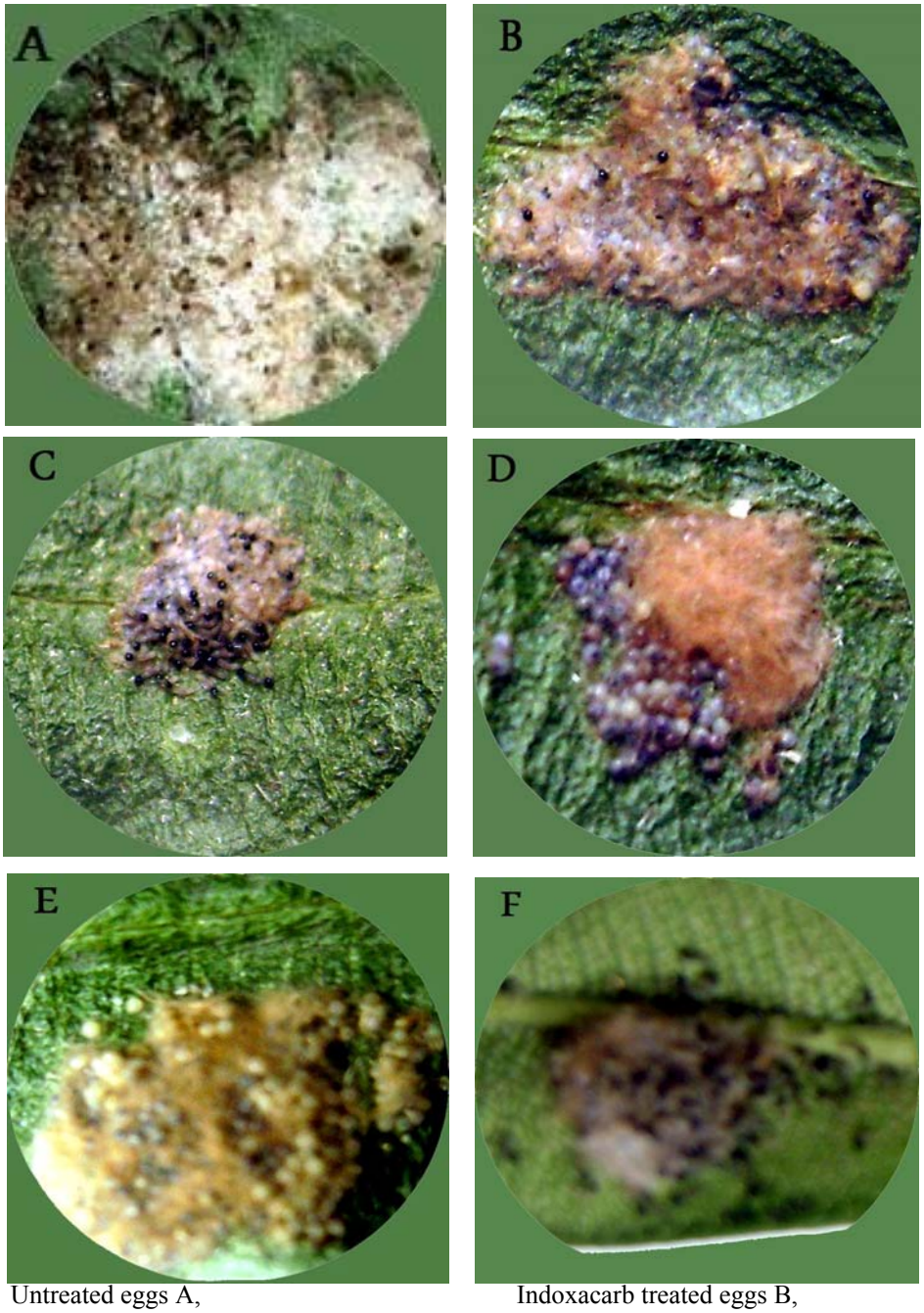
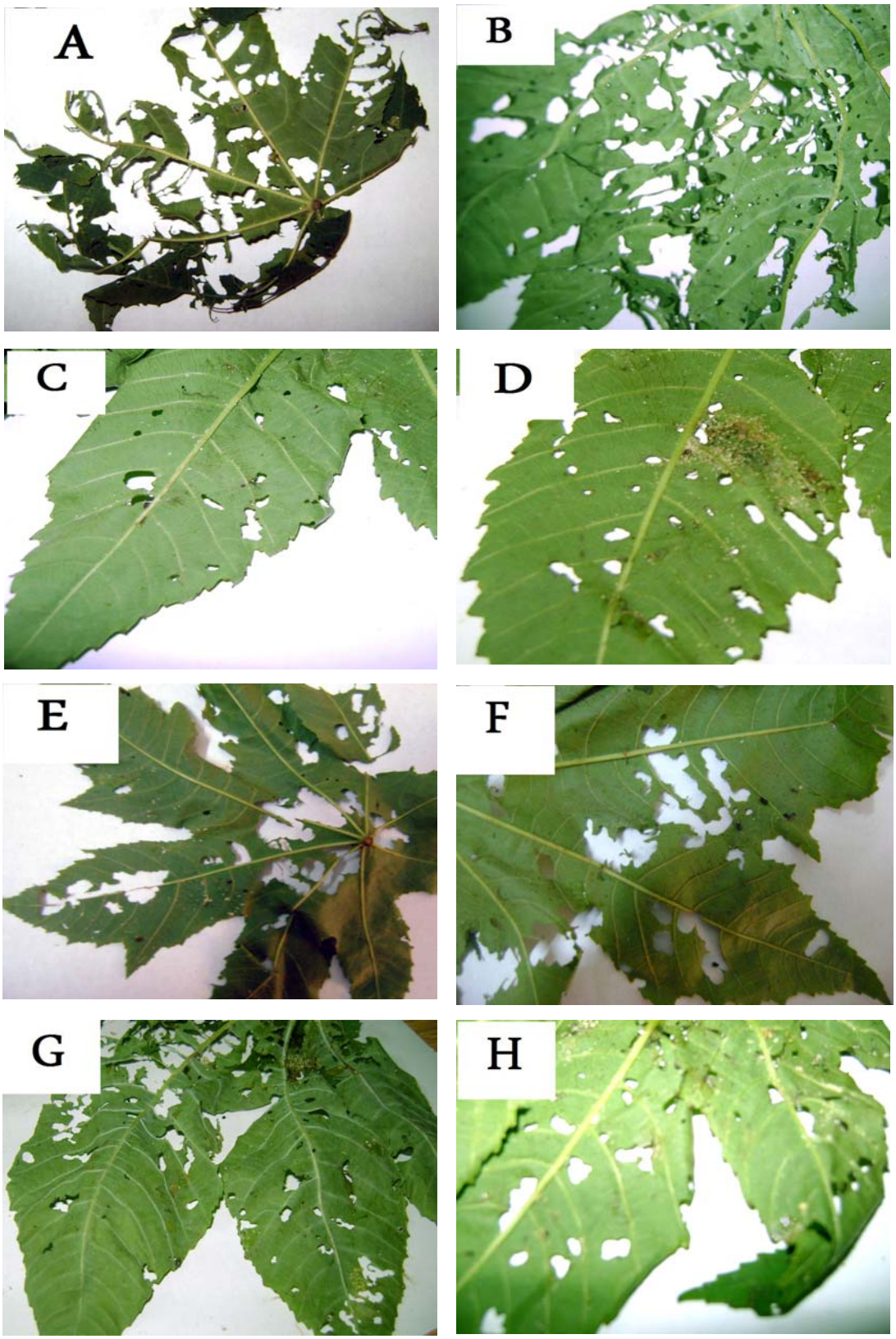
Plate (1)



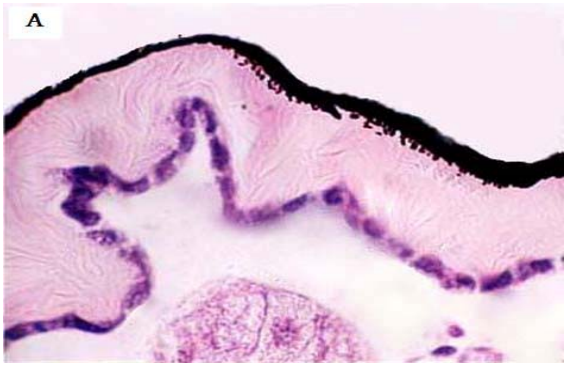
Untreated eggs A, Indoxacarb treated eggs B,
Spinetoram treated eggs C& D, Methoxyfenozide treated eggs E &F.

Plate (2)

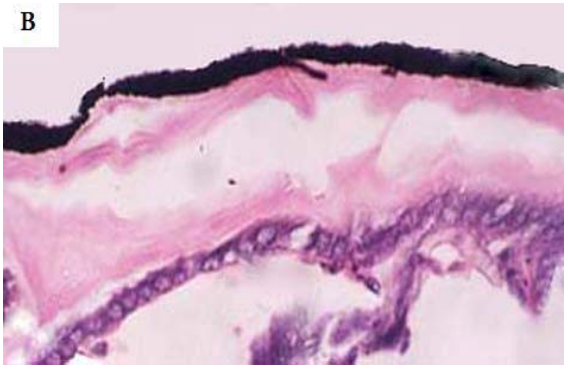


Untreated leaves A& B
Indoxacarb treated leaves C & D
Spinetoram treated leaves E& F
Methoxyfenozide treated leaves G & H

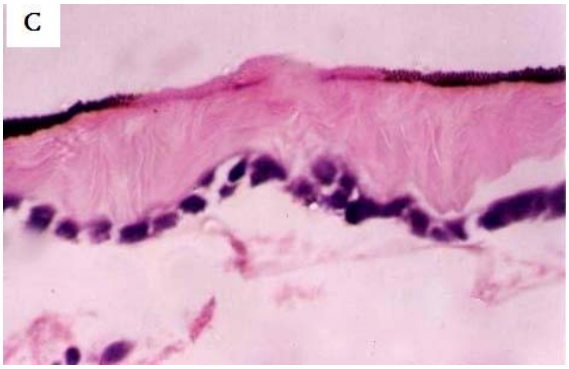
Plate (3)



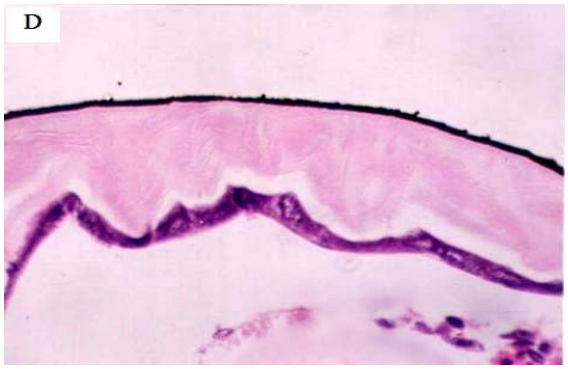
Cross section of normal cuticle of *S. littoralis* larva.



Cross section in the cuticle of *S. littoralis* larva treated with methoxyfenozid.



Cross section in the cuticle of *S. littoralis* larva treated with indoxacarb.



Cross section in the cuticle of *S. littoralis* larva treated with spinetoram

Plate (4)

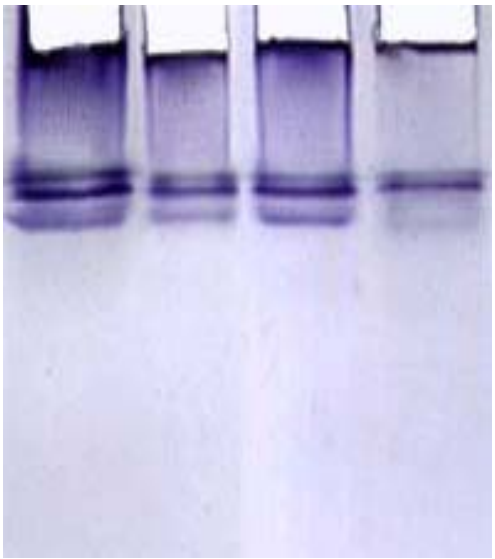


Plate (4-A) Aldehyde oxidase pattern of larval haemolymph of *S. littoralis* after treated the 4th larval instar with the tested compounds.

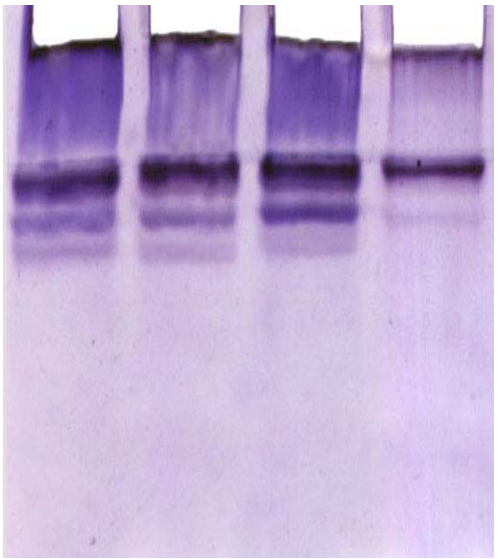


Plate (4-B) α -GPDH pattern of larval haemolymph of *S. littoralis* after treated the 4th larval instar with the tested compounds.

ARABIC SUMMARY

كفاءة بعض المبيدات الحشرية الجديدة على المستوى الفسيولوجي والنسيجي والجزيئي لدودة ورق القطن

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اجريت الدراسة لتقييم كفائة بعض المبيدات الحشرية الجديدة (الاندوكساكارب و السبنوترام و الميثوفينوزيد) على دودة ورق القطن وذلك من خلال تعرض كل من العمر اليرقى الثانى والرابع لاوراق الخروع المعاملة بتركيزات مختلفة من المركبات المختبرة. وقد تم تحديد التركيزالنصفى المميت لكل من العمر اليرقى الثانى والرابع. وقد تراوح التركيز المميت للنصف للعمر اليرقى الثانى بين ٠٠٤، و٠٠٦، جزء فى المليون لمركب الاندوكساكارب وبين ٠٢٢، و ٠٣٣، جزء فى المليون لمركب السبنوترام وبين٠٠٦، و ٠١١، جزء فى المليون لمركب الميثوفينوزيد. بينما تراوح التركيز المميت للنصف للعمر اليرقى الرابع بين ٣٦، و٥٤، جزء فى المليون لمركب الاندوكساكارب وبين ١,٧٨ و ٢,٦٤ جزء فى المليون لمركب السبنوترام وبين١,٣٦ و ١,٦٩جزء فى المليون لمركب الميثوفينوزيد. كما تم دراسة تأثيرتلك المركبات على النشاط الابادى للبيض وأوضحت الدراسة ان اكثر المركبات تأثيرا كان مركب السبنوترام. كما اوضحت الدراسة ان معاملة العمر اليرقى الرابع لدودة ورق القطن بالتركيز النصفى المميت ادى الى نقص فى معدل استهلاك الغذاء والنمولحشرة المختبرة وكان اكثر المركبات تأثيرا مركب الاندوكساكارب. ومن ناحية اخرى وجد ان معاملة الطور اليرقى الرابع لدودة ورق القطن بمركب الميثوفينوزيد ادى الى تغيرات هستولوجية فى جليد يرقات العمر السادس الناتج من تلك المعاملة حيث تسبب فى تلف لتكوين الجليد الداخلى بأكملة بينما كان هذا التأثير اقل وضوحا فى المعاملة بالاندوكساكارب و السبنوترام. كما سجلت التغيرات فى أنماط أنزيمى الألدهيد أكسيديزو الألفاجلسروفوسفات ديهيدرجينيز لعينات الدم للطور اليرقى السادس بين كل من اليرقات المعاملة وغير المعاملة وقد اوضحت النتائج ان مركب الميثوفينوزيد كان الاكثر تأثيرا ثم يلية الاندوكساكارب واخيرا السبنوترام على انماط الانزيمين المختبريين.