Effects of Azadirachtin and Methoxyfenozide on some Biological and Biochemical Parameters of Cotton Leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract: Toxicity and sub-lethal effects of azadirachtin and methoxyfenozide on the 2nd instar larvae of cotton leafworm, *Spodoptera littoralis* were evaluated. Activity of chitinase, polyphenol oxidase (PPO), total proteases and α-amylase were evaluated in treated larvae by sublethal concentrations of azadirachtin and methoxyfenozide compared to untreated larvae. Azadirachtin (LC_{50} = 0.73, 0.36 and 0.17 mg L^{-1}) was approximately 20.8, 5.3 and 8.6 times more toxic than methoxyfenozide (LC_{50} = 15.18, 1.89 and 1.46 mg L^{-1}) after 48, 72 and 96hrs post-treatment, respectively. Treated 2nd instar larvae by LC_{10} and LC_{25} of azadirachtin and methoxyfenozide showed significantly ($P < 0.05$) reduced larval body weights, % pupation, pupal mean weight and % adult emergence rates. The average time to the pupation for larvae treated by concentrations equivalent to the LC_{10} and LC_{25} of azadirachtin and methoxyfenozide were significantly longer than those in the control. Fecundity and fertility were strongly reduced in all insecticide treatments compared to control. The chitinase, PPO, total proteases and α-amylase activities were partially inhibited in all insecticide treatments in a concentration dependent manner. These results suggest that sublethal concentrations of azadirachtin and methoxyfenozide may reduce population growth of *S. littoralis* by affecting its development and reproduction. From a practical point of view such effects are very important, because offspring can then be reduced and the insect population negatively affected.

Keywords: Spodoptera littoralis; azadirachtin; methoxyfenozide; Sublethal effects.

1. Introduction

Cotton leafworm, *Spodoptera littoralis* (Boisduval), is one of the most destructive agricultural lepidopterous pests of cotton and vegetable plants in Egypt **(Hatem *et al.*, 2009)**. The insect occurs during the whole cycle of cotton plants, causing considerable damage by feeding on leaves, fruiting points, flower buds and, occasionally, also on bolls. So, it requires several insecticide applications to control.

Commonly, control of this pest has largely been depending on the use of neurotoxic insecticides including organophosphates, carbamates and pyrethroids **(Ahmad *et al.*, 2009)**. The intensive application of these insecticides has led *S. littoralis* populations to be resistant to all of these groups **(Abou-Taleb, 2010)**. Also, environmental and human health concerns over excessive synthetic chemical insecticide use worldwide increasingly favor the development of alternative and safer methods for pest control **(Cherry *et al.*, 1997)**. Therefore, selective insecticides with modes of action different from those insecticide groups are highly desirable in integrated pest management (IPM) programs. Among these insecticides are azadirachtin and insect growth regulator; methoxyfenozide.

Azadirachtin; a tetranortriterpenoid compound derived from the neem tree, *Azadirachta indica* A. Juss (Sapindales: Meliaceae), has been effectively used against >400 species of insects, including many key crop pests (**Sahak *et al.*, 2010)**. This active compound has several biological properties including antifeedant effects, repellency **(Schmutterer 1990)** and insect growth regulator characteristics **(Ilio *et al.*, 1999)**. Methoxyfenozide is an ecdysone agonist **(Yanagi *et al.*, 2006)**. This compound mimics the biological function of the natural insect molting hormone 20HE, inducing a premature and lethal larval molt by binding directly to the ecdysteroid receptors **(Smagghe *et al.*, 2004)**. It is highly selective against lepidopteran larvae, with the result that other insects are often less affected by it **(Schneider *et al.*, 2008)**. Both azadirachtin and methoxyfenozide have been reported to be safer for beneficial organisms, mammals, and also for the environment, with minimal residual effects **(Pavela, 2009; Osorio *et al.*, 2008)**. Therefore, they have proven to be from the most promising for IPM programs.

The use of insecticides may result in multiple sublethal effects on insect pests, along with direct mortality, because of the different doses with which individual insects come into contact in the field **(Singh and Marwaha, 2000)**. Physiological effects may be manifested as reductions in life

span, development rate, fertility and fecundity **(Zalizniak and Nugegoda, 2006)**. These sublethal effects as well as mortality must be considered when examining the total effects of insecticides **(Yin *et al.*, 2008)**. Sublethal effects of azadirachtin and methoxyfenozide on life parameters (longevity, fecundity, and fertility) and progeny (pupal formation and adult emergence) of *S. littoralis* treated adults by ingestion were reported **(Pineda *et al.*, 2009)**. However, in Egypt, the larval stage is the target of insecticide spraying processes. Therefore, the main objective of this study is to investigate the sublethal effects of azadirachtin and methoxyfenozide on life table indices, when the 2nd instar larvae of *S. littoralis* were treated. This study also reports the effects of azadirachtin compared to methoxyfenozide on the total proteases, α-amylase, chitinase and polyphenol oxidase (PPO) activities, whereas these compounds have been recognized for their antifeedant and insect growth regulator properties.

2. Materials and Methods

2.1. Experimental insect:

A susceptible strain of the *S. littoralis* has been reared for many years in the Plant Protection Research Station, Alexandria, Egypt. Larvae were fed castor bean leaves under controlled laboratory conditions (25 ± 2 °C, RH 65%) for several years avoiding exposure to any pesticides according to the method of **Eldefrawi *et al.*, (1964)**.

2.2. Test insecticides:

Formulated azadirachtin (Neemforce® 0.15% EC) was produced by Arab company for pesticides and veterinary medicines manufacture. Methoxyfenozide (Runner®24%SC) was produced by Dow Agrosciences Co.

2.3. Bioassay and determination of sublethal concentrations:

A leaf dip bioassay method **(Eldefrawi *et al.*, 1964)** was used. Homogenous castor bean leaf pieces were dipped in six concentrations of each tested insecticide (prepared in water) for 10 sec., and dried at room temperature. A control containing pure water was also used with Triton X-100 as an emulsifier at 0.05% (vol:vol). Treated castor bean leaf pieces were introduced to ten 2nd instar larvae (2.3 ± 0.1 mg/larva), which had been starved for two hrs. The cups were covered with lids and maintained at 25 ± 2 °C. Each concentration was replicated five times. After 24 hrs, fresh untreated castor bean leaf pieces were added to each cup. Mortality was recorded after 48, 72 and 96hrs, corrected according to Abbott equation **(Abbott, 1925)** and subjected to probit analysis **(Finney, 1971)**. The median lethal concentrations, confidence limits and the slope were calculated. LC_{25} and LC_{10} equivalent concentrations (after 96h) were selected as sublethal concentrations for the subsequent experiments.

2.4. Latent effects of sublethal concentrationss of azadirachtin and methoxyfenozide against *S. littoralis:*

Castor bean leaves were soaked in the determined LC_{25} and LC_{10} equivalent concentrations of the tested insecticides. Four hundred 2nd instar larvae (2.3 ± 0.1 mg / larva) in 4 replicates was used for each treatment and provided with treated leaves. After 24hrs, surviving larvae were transferred to jars containing fresh untreated leaves and observed daily for pupation and emergence. Larval, pupal and adult durations were determined. Larval and pupal weights and percentages of adult emergence were recorded. Resulted adults were placed in plastic cups provided with a folded sheet paper as oviposition site. Two adult males were kept with one adult female to maximize the probability of successful mating. The sublethal effects of tested insecticides on fecundity (total number of eggs / female) and fertility (hatchability percentages of eggs) were determined. Initially, 12 mating were planned for each insecticide treatment as well as control. The mating cups were checked daily and egg masses were removed until female death. The total number of eggs /female for each mating and hatched eggs percentages were evaluated.

2.5. Total proteases activity assay:

Crude enzyme preparation: Surviving larvae after 96hrs of treatment with LC_{25} and LC_{10} equivalent concentrations of each insecticide were collected. Untreated larvae were used as control. One gram of larvae was homogenized in 5 ml 100 mM Tris-HCl buffer pH 7 using Polytron Kinemetica on ice. The homogenate was centrifuged at 4000 rpm for 15 min at 4ºC using IEC-CRU 5000 cooling centrifuge. The supernatant was used for total proteolytic activity estimation.

Enzyme activity determination: Total proteolytic activity was measured using azocasein as a substrate according to **(Olga *et al.*, 2002; Mohen and Gujar, 2003)**. The supernatant was incubated in a total volume 60 µl of assay buffer (100 mM Tris-HCl, pH 8) for 20 min at 37ºC before addition of 200 µl of 2% azocazein (w/v in assay buffer). The reaction was allowed to proceed for 180 min at 37ºC, and then stopped by addition of 300 µl cold 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 3000 rpm for 10 min IEC-CRU 5000 cooling centrifuge. Excess acidity was neutralized by adding 10 µl NaOH (10 N) to the reaction mixture and absorbance was measured at 440 nm using Sequoia-Turner Model 340 spectrophotometer. An assay mixture without enzyme was used as the blank.

2.6. Amylase activity assay:

Crude enzyme preparation: Surviving larvae after 96hrs of treatment with LC_{25} and LC_{10} equivalent concentrations of each insecticide were collected. Untreated larvae were used as control. One gram of total larvae was homogenized in 5 ml glass distilled water using Polytron Kinemetica on ice. The

homogenate was centrifuged at 15000 rpm for 15 min at 4ºC using IEC-CRU 5000 cooling centrifuge. The supernatant was used for α-amylase activity assay.

Enzyme activity determination: Alpha-amylase activity was assayed by the dinitrosalicilic acid (DNS) according to **Bernfeld (1955)**, using 1% soluble starch solution as substrate. An assay mixture without enzyme was used as the blank. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35ºC.

2.7. Chitinase activity assay:

Crude enzyme preparation: Surviving larvae after 96hrs of treatment with LC_{25} and LC_{10} equivalent concentrations of each insecticide were collected. Untreated larvae were used as control. Larvae were homogenized in 0.1 M phosphate buffer, pH 7.0, using Polytron Kinemetica on ice, and then homogenates were centrifuged at 5000 rpm for 20 min at 0 ºC. The supernatants were used as enzyme source for chitinase activity assay.

Enzyme activity determination: Enzyme activity was measured according to **Monreal and Reese (1969)** method. One ml of colloidal chitin, as a substrate, in 0.05 M citrate phosphate buffer (pH 6.6) was mixed with 1 mL of enzyme extract. Colloidal chitin was prepared by the method that described by **Shimahara and Takiguchi (1988)**. Enzyme activity was assayed by measuring the amount of reducing sugar that produced by enzyme reaction **(Miller, 1959)**. Optical density (OD) was measured at 540 nm using Sequoia-Turner Model 340 spectrophotometer. The specific activity of chitinase was calculated as OD_{540} mg protein^{-1}.h^{-1}. An assay mixture without enzyme was used as a blank.

2.8. Polyphenol oxidase (PPO) activity assay:

Crude enzyme preparation: Surviving larvae after 96hrs of treatment with LC_{25} and LC_{10} equivalent concentrations of each insecticide were collected. Untreated larvae were used as control. Three grams of total larvae was homogenized in 10 ml of potassium phosphate buffer, pH 7.0 using Polytron Kinemetica on ice. The homogenate was filtered through two layers of cheesecloth. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 ºC using Cryofuge 20-3, Heraeus Christ centrifuge. The supernatant was used in protein determination and as the crude enzyme extract.

Enzyme activity determination: The activity of PPO was determined according to **Zhi-qing *et al.* (2008)** by mixing of 1.5 ml 0.2 mol/L pyrocatechol, 1.4 ml of 0.05 mol/L phosphate buffer (pH 6.8) and 0.1 ml enzyme extract, respectively. The mixture was incubated at 25 ºC for 30 min and the absorbance was measured at 420 nm using a Sequoia-Turner Model 340 spectrophotometer. The specific activity of PPO was calculated as OD_{420} /30 min/mg protein.

2.9. Protein measurements:

Lowry *et al.* (1951) method was used to determine protein content in the supernatant comparing to the standard curve of BSA.

2.10. Statistical analysis:

Estimates of LC_{50} and their 95% fiducial limits were obtained using the POLO program **(Russell *et al.*, 1977)** based on **Finney (1971)**. The criterion used to estimate the differences between LC_{50} values was non-overlap of their 95% confidence intervals. All other quantitative estimations were replicated four times and the values are expressed as mean ± standard error. The SAS 8.0 software was used for analysis of the data obtained from each experiment and the means were tested for significant differences using analysis of variance (ANOVA) test (LSD at $P < 0.05$) **(SAS Statistical software, 1999)**.

3. Results

3.1.Toxicity of azadirachtin and methoxyfenozide against 2nd instar *S. littoralis* larvae and determination of sublethal concentrations:

Toxicity of azadirachtin and methoxyfenozide against the 2nd instar larvae of *S. littoralis* after different exposure times were presented in Table (1). Azadirachtin (LC_{50} = 0.73, 0.36 and 0.17 mg L^{-1}) was approximately 20.8, 5.3 and 8.6 times more toxic than methoxyfenozide (LC_{50} = 15.18, 1.89 and 1.46 mg L^{-1}) after 48, 72 and 96hrs of exposure, respectively. The LC_{10} and LC_{25} values after 96 hrs of exposure are 0.02 and 0.05 mg L^{-1} for azadirachtin, 0.13 and 0.41 mg L^{-1} for methoxyfenozide (Table 1).

3.2. Sublethal effects of azadirachtin and methoxyfenozide on some biological aspects of *S. littoralis*:

Effects of sublethal concentrations of tested insecticides on some biological parameters of *S. littoralis* are presented in Tables (2, 3 and 4). The average weight of treated larvae was decreased significantly compared to control during the observation period. It is clear that, the higher concentration of all tested insecticides (LC_{25}) were significantly the highest in the effect on the larval weight. When larvae were treated with azadirachtin at LC_{25} (0.05 mgL^{-1}), the larval weight averages were 30.3, 112.5 and 323.5 mg / larva compared to 58.9, 237.3 and 784.1 mg / larva in control after 4, 8 and 12 days of treatment, respectively. The larval weight averages were 34.7, 147.3 and 475.0 mg / larva when larvae were treated with LC_{25} of methoxyfenozide (0.41 mgL^{-1}), after 4, 8 and 12 days of treatment, respectively, (Table2).

Table (1): Toxicity of azadirachtin and methoxyfenozide against 2nd instar larvae of *S. littoralis* after different exposure times:

Insecticide	Time after exposure (hr)	LC_{50} (mg L^{-1}) (95% CL)	LC_{25} (mg L^{-1}) (95% CL)	LC_{10} (mg L^{-1}) (95% CL)	Slope ± SE
Azadirachtin	48	0.73 (0.57 – 1.02)	0.25 (0.19 - 0.31)	0.10 (0.06 - 0.13)	1.45 ± 0.19
	72	0.36 (0.30 - 0.44)	0.13 (0.10 - 0.16)	0.05 (0.03 - 0.07)	1.5 ± 0.14
	96	0.17 (0.14 - 0.21)	0.05 (0.04 - 0.07)	0.02 (0.01 - 0.03)	1.3 ± 0.11
Methoxyfenozide	48	15.18 (8.85 – 40.69)	2.12 (1.40 - 3.03)	0.36 (0.12 - 0.65)	0.79 ± 0.14
	72	1.89 (1.55 - 2.32)	0.56 (0.41 - 0.71)	0.19 (0.12 - 0.27)	1.3 ± 0.11
	96	1.46 (1.17 - 1.87)	0.41 (0.29 - 0.54)	0.13 (0.07 - 0.20)	1.23 ± 0.13

Table (2): Effect of azadirachtin and methoxyfenozide when applied to the 2nd instar larvae of *S. littoralis* on the larval weight gain, larval duration and %pupation:

Insecticide	Conc. (mg L^{-1})	Mean weight (mg/larva) (± SE) after different days of treatment			Larval duration (days) ± SE	Pupation (%) ± SE
		4	8	12		
Control	-	58.9a ± 2.3	237.3a ± 8.1	784.1a ± 8.5	16.5c ± 1.5	94.7a ± 3.2
Azadirachtin	0.02	37.3c ± 2.6	131.4d ± 4.9	470.3c ± 9.5	19.7b ± 0.7	73.0c ± 1.5
	0.05	30.3d ± 1.8	112.5e ± 1.6	323.5d ± 10.7	19.3b ± 1.5	51.2e ± 2.3
Methoxyfenozide	0.13	43.1b ± 1.4	184.2b ± 3.7	556.3b ± 9.2	22.4a ± 1.4	79.8b ± 1.6
	0.41	34.7cd ± 1.2	147.3c ± 4.3	475.0c ± 7.8	23.1a ± 0.8	68.7d ± 1.9

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05.

The average time to the pupation for larvae treated by concentrations equivalent to the LC_{10} and LC_{25} of azadirachtin and methoxyfenozide treatments were significantly longer than those in the control treatment. These times were 19.7 and 19.3 days for azadirachtin and 22.4 and 23.1 days for methoxyfenozide at LC_{10} and LC_{25}, respectively, where it was 16.5 days for control (Table 2). Sulethal concentrations of tested insecticides had a considerable effect on pupation. The LC_{25} treatment of azadirachtin caused the lowest pupation percentage 51.2% compared to 94.7% in control. Also, significant decrease in pupation percentage was achieved with the LC_{25} of methoxyfenozide (68.7%), the LC_{10} of both azadirachtin and methoxyfenozide (73.0 and 79.8%, respectively) compared to control treatment (94.7%), (Table 2).

As shown in Table (3), all treatments significantly suppressed the pupal mean weight compared to control treatment. The weight averages of pupae were 215.3 and 192.4 mg / pupa in the LC_{10} and LC_{25} azadirachtin treatments, respectively, compared to 307.2 mg / pupa in the control treatment. In case of LC_{10} and LC_{25} methoxyfenozide treatments, pupae weight averages were 269.8 and 233.8 mg / pupa, respectively. However, pupal duration did not changed significantly in all treatments compared to control (Table 3). Reduction in the adult emergence rates were significantly achieved by all treatments. Methoxyfenozide at LC_{25} achieved the higher reduction in the adult emergence rates, where it was 63.4% compared to 96.0% in the control. The adult emergence rate was 70.2% at the LC_{25} azadirachtin treatment (Table 3).

Table (4) represents the effect of insecticides treatments on the adult fecundity, fertility and longevity. Azadirachtin and methoxyfenozide at LC_{25} have the highest effect on the adult fecundity where the average number of eggs laid / female were 302.2 and 311.5 / female, respectively, compared to 589.0 / female in control treatment. Also, azadirachtin and methoxyfenozide at the LC_{10} significantly decreased the adult fecundity where the average number of eggs laid / female were 377.7 and 383.4 / female, respectively. Fertility (percentages of egg hatch) was significantly decreased under all insecticides treatments. Methoxyfenozide at LC_{25} achieved the highest effects on the fertility where percentage of egg hatch was 69.7% compared to 97.0% in control. On the other hand, adult longevity did not differ significantly in treatments compared to control (Table4).

Table (3): Effect of azadirachtin and methoxyfenozide when applied to the 2nd instar larvae of *S. littoralis* on the pupal weight gain, pupal duration and %adult emergence:

Insecticide	Conc. (mg L^{-1})	Pupal mean weight (mg/pupa) ± SE	Pupal duration (days) ± SE	%Adult emergence ± SE
Control	-	307.2a ± 3.4	7.6a ± 0.5	96.0a ± 2.5
Azadirachtin	0.02	215.3d ± 6.9	7.2a ± 0.4	80.4b ± 2.1
	0.05	192.4e ± 8.6	7.3a ± 0.3	70.2c ± 1.5
Methoxyfenozide	0.13	269.8b ± 6.1	7.5a ± 0.4	78.3b ± 2.1
	0.41	233.8c ± 4.4	7.4a ± 0.5	63.4d ± 1.7

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05.

Table (4): Effect of azadirachtin and methoxyfenozide when applied to the 2nd instar larvae of *S. littoralis* on adult fecundity, fertility and longevity:

Insecticide	Conc. (mg L^{-1})	Fecundity (No. eggs laid / female)± SE	fertility (%egg hatch) ± SE	Adult longevity (days) ± SE
Control	-	589.0a ± 36.5	97.0a ± 2.1	5.5a ± 0.3
Azadirachtin	0.02	377.7b ± 43.2	82.5b ± 3.5	5.1a ± 0.2
	0.05	302.2c ± 25.5	83.8b ± 4.2	5.2a ± 0.4
Methoxyfenozide	0.13	383.4b ± 19.1	79.6b ± 1.8	5.8a ± 0.5
	0.41	311.5c ± 21.9	69.7c ± 2.6	5.4a ± 0.2

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05

3.3. *In vivo* effects of azadirachtin and methoxyfenozide on the total proteases, α-amylase, chitinase and PPO activities in the *S. littoralis* larvae:

The specific activity of total proteases (OD_{440} / mg protein / hr) in *S. littoralis* 2nd instar larvae after 96hrs of exposure to two concentrations of azadirachtin and methoxyfenozide is significantly decreased compared to control (Table 5). The highest enzyme activity inhibition was occurred by azadirachtin at LC_{25}, where the inhibition percentage was 40.6%. Azadirachtin also, at LC_{25} caused the highest α-amylase inhibition, where the inhibition percentage was 35.5% (Table 6). Data in Table (7) showed that all treatments significantly inhibit the chitinase activity compared to the chitinase activity in control. The highest inhibition was recorded with LC_{25} of methoxyfenozide followed by LC_{25} of azadirachtin and LC_{10} of methoxyfenozide with inhibition percentages of 50.3, 32.3 and 27.9, respectively. Also, methoxyfenozide at LC_{25} achieved the highest PPO inhibition, where the inhibition percentage was 61.8%, (Table 8).

Table (5): *In vivo* effect of azadirachtin and methoxyfenozide on the 2nd instar larvae of *S. littoralis* total proteases activity after 96hrs of exposure:

Insecticide	Conc. (mg L^{-1})	Specific activity (OD_{440}/mg protein/hr) ± SE	Activity (%control)	%Inhibition
Control	-	0.138 a ± 0.005	100 ± 3.6	0.0
Azadirachtin	0.02	0.103 c ± 0.003	74.6 ± 2.9	25.4
	0.05	0.082 d ± 0.004	59.4 ± 4.9	40.6
Methoxyfenozide	0.13	0.119 b ± 0.005	86.2 ± 4.2	13.8
	0.41	0.107 c ± 0.006	77.5 ± 5.6	22.5

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05

Table (6): *In vivo* effect of azadirachtin and methoxyfenozide on the 2nd instar larvae of *S. littoralis*α-amylase activity after 96hrs of exposure:

Insecticide	Conc. (mg L^{-1})	Specific activity (µmol maltose/min/mg protein) ± SE	Activity (%control)	%Inhibition
Control	-	1.07 a ± 0.07	100 ± 6.5	0.0
Azadirachtin	0.02	0.804 c ± 0.04	75.1 ± 5.0	24.9
	0.05	0.690 d ± 0.04	64.5 ± 5.8	35.5
Methoxyfenozide	0.13	0.960 b ± 0.06	89.7 ± 6.3	10.3
	0.41	0.782 c ± 0.05	73.1 ± 6.4	26.9

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05.

Table (7): *In vivo* effect of azadirachtin and methoxyfenozide on the 2nd instar larvae of *S. littoralis* chitinase activity after 96hrs of exposure:

Insecticide	Conc. (mg L^{-1})	Specific activity (OD_{540}/mg protein/hr) ± SE	Activity (%control)	%Inhibition
Control	-	3.84 a ± 0.10	100 ± 2.6	0.0
Azadirachtin	0.02	3.12 b ± 0.12	81.3 ± 3.8	18.7
	0.05	2.60 c ± 0.08	67.7 ± 3.1	32.3
Methoxyfenozide	0.13	2.77 c ± 0.09	72.1 ± 3.2	27.9
	0.41	1.91 d ± 0.05	49.7 ± 2.6	50.3

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05.

Table (8): *In vivo* effect of azadirachtin and methoxyfenozide on the 2nd instar larvae of *S. littoralis* PPO activity after 96hrs of exposure:

Insecticide	Conc. (mg L^{-1})	Specific activity (OD_{420}/mg protein/hr) ± SE	Activity (%control)	%Inhibition
Control	-	15.2 a ± 0.6	100 ± 3.9	0.0
Azadirachtin	0.02	11.5 b ± 0.4	75.7 ± 3.5	24.3
	0.05	9.7 c ± 0.2	63.8 ± 2.1	36.2
Methoxyfenozide	0.13	9.1 c ± 0.2	59.9 ± 2.2	40.1
	0.41	5.8 d ± 0.1	38.2 ± 1.7	61.8

Within a column, means possessing the same letter do not differ significantly at *P* = 0.05.

4. Discussion

Insect management strategies must be directed towards the use of insecticides that are none or less toxic to all environmental components including the beneficial arthropods. Neem derived insecticides are being given a thought to replace synthetic insecticides in insect pest management programs **(Sami, 2014)** because they are not toxic to humans and many beneficial arthropods. Also, IGRs which act as chitin synthesis inhibitors or juvenile hormone analogs have been regarded as excellent integrated control insecticides because of their specificity to target pests and their general safety to vertebrates **(Deakle *et al.*, 1982)**. In field application of insecticides, some insects may expose to sublethal concentrations of the applied insecticide. Many sublethal effects on insect pests can result from that exposure.

Azadirachtin and methoxyfenozide at the two tested concentrations (LC_{10} and LC_{25}) significantly decreased the average weight of treated larvae, reduced pupation percentage, suppressed the pupal mean weight and reduced adult emergence rate compared to control during the experimental period. Similarly, **Martinez and van Emden (1999)** mentioned that sublethal concentrations of azadirachtin incorporated into artificial diet prolonged larval instars, reduced food intake and severely reduced growth of *S. littoralis* larvae. Also, our results are in agreement with **Wondafrash *et al.* (2012)**, they reported that water extracts of neem seed and leaf have significantly reduced the growth and development of *Helicoverpa armigera* larvae and pupae as compared to the control and this was clearly manifested by the significantly reduced larval and pupal weight recorded from treated larvae. Similar observations were reported by **Charleston (2004)** who found that the

development of *Plutella xylostella* was significantly prolonged when fed on neem treated plants. Prolongation of larval stage after treatment of *S. littoralis* larvae with sublethal concentrations of methoxyfenozoide have been also reported by **(Pineda *et al.*, 2004 and 2007)**. Similar observation in *Agrotis ipsilon* has also been reported by **(Fahmy, 2014)**.

Studies on the effects on reproductive parameters caused by azadirachtin **(Riba *et al.* 2003, Grisakova *et al.* 2006)** and methoxyfenozide **(Sun *et al.* 2000, Saenz-de-Cabezon *et al.* 2005)** have been documented in several important pests. In the current study, the fecundity and fertility of *S. littoralis* were adversely affected when the 2nd instar larvae were treated with two sublethal concentrations (LC_{10} and LC_{25}) of these compounds. Several lepidopteran pests have been reported previously to suffer reduced fecundity and fertility after exposure to ecdysone agonists, either through topical application or by ingestion. **Pineda *et al.* (2009)** reported that azadirachtin and methoxyfenozide negatively affected the longevity and reproductive parameters of *S. littoralis* adults treated orally. These effects may be a result of interference of azadirachtin **(Tanzubil and McCaffery, 1990)** and methoxyfenozide **(Pineda *et al.* 2007)** with vitellogenin synthesis, its uptake, or both by developing oocytes. It is well known that the maturation of insect eggs is dependent on proteins, lipids, and carbohydrates, which are required for embryogenesis **(Kanost *et al.* 1990)**. Taking into account that azadirachtin **(Medina *et al.* 2004)** and methoxyfenozide **(Pineda *et al.* 2006)** can disrupt ecdysteroid-regulated events in insects, **Pineda *et al.* (2009)** propose that both compounds could interfere with the accumulation of proteins in the eggs, which also might explain the oviposition delay in *S. littoralis* females. **Tanzubil and McCaffery (1990)** and **Lawrence (1993)** observed lower protein levels in ovaries from *S. exempta* and *Anastrepha suspensa* (Diptera: Tephritidae) fed with azadirachtin and RH-5849 (an analog of methoxyfenozide), respectively, suggesting a reduced level of vitellogenesis and development compared with untreated insects.

Possible contribution of males to the fertility reduction was indicated by the study of **Shimizu (1988)**, who showed that *in vitro* exposure of testes from the diapausing pupae of *Mamestra brassicae* to 3 ppm azadirachtin caused degeneration of the spermatocysts. The sterilizing action of azadirachtin *in vivo*, however, may rather be due to disturbances in the neuroendocrine regulation of reproduction **(Sayah *et al.*, 1998)**.

Chitinases are enzymes with a specific hydrolytic activity directed towards chitin. Insects periodically shed their old exoskeletons and either continuously or periodically shed their peritrophic membranes and resynthesize new ones **(Lehane, 1997)**. This process is mediated by the elaboration of chitinases in the molting fluid that accumulates in the space between the old cuticle and the epidermis and in the gut tissue. Chitinase plays an essential role during ecdysis chitin. This enzyme is vital to moult in insects, and may also affect gut physiology through their involvement in peritrophic membrane turnover. Phenoloxidases are related to critical steps of melanization reactions, which in insects are crucial for the sclerotization of a new cuticle after ecdysis **(Andersen, 2005)** and for the encapsulation of pathogens in the hemolymph **(Soderhall and Cerenius, 1998)**. Chitinases and PPO of insects might constitute a useful target site for insecticides. In our study, the two concentrations of azadirachtin and methoxyfenozide significantly inhibit chitinases and PPO of treated *S. littoralis* larvae in a concentration dependent manner. **Nasr *et al.* (2010)** recorded same results with pyriproxyfen and buprofezin against *S. littoralis* larvae. They mentioned that, the inhibition of PPO of *S. littoralis* by pyriproxyfen and buprofezin was concentration dependent. In general, further investigations were needed to clarify the interaction of azadirachtin and methoxyfenozide with insect chitinases and PPO activity and its impact on the insect growth.

Several enzymes based on food materials have critical roles in nutritional indices of the insect pest population. In the current study, effects of azadirachtin and methoxyfenozide on the activity of total proteases and α-amylase were used as a parameter for studying of their effect on the nutritional indices. Alpha-amylase is an enzyme that degrades starch by hydrolyzing α-1,4-glucan bonds. Proteases hydrolyze proteins to amino acids **(Pascual-Ruiz *et al.* 2009)**. Results in this study indicated that, azadirachtin and methoxyfenozide significantly inhibit total proteases and α-amylase of treated *S. littoralis* larvae in a concentration dependent manner. These results are in agreement with results of many previous authors. The α-amylase activity on polyacrylamide gel showed a weak enzymatic activity in larvae of *Plodia interpunctella* fed azadirachtin indicating a severe reduction in α-amylase activity **(Rharrabe *et al.*, 2008)**. **Khaled and Farag (2015)** showed that treatment with methoxyfenozide caused considerable reduction compared to the control in α-amylase; invertase and trehalase activity. In a study of the effects of azadirachtin on the proteases activity, **Khosravi and Sendi (2013)** showed that azadirachtin caused a reduction of the protease activity in *Glyphodes pyloalis*. Also it is reported that, methoxyfenozide significantly reduced the *S. littoralis* total protease activity **(Sabry and Khedr, 2014)**.

Finally, our results verified the lethal and sublethal effects of azadirachtin and methoxyfenozide on the larval stage of *S. littoralis*. The sublethal concentrations of these insecticides negatively affect fertility and fecundity of *S. littoralis*. These effects are very important from a practical point of view, because offspring can then be reduced and the insect population can be negatively affected. Fortunately, the mode of action of azadirachtin and methoxyfenozide is different from pyrethroid, carbamate and organophosphate insecticides. So, azadirachtin and methoxyfenozide can be used for *S. littoralis* control and in resistance management programs.

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الملخص العربى

تأثير الأزاديراختين والميثوكسيفينوزيد على بعض العوامل البيولوجيه والبيوكيميائيه لدودة ورق القطن

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تم تقدير سمية وتأثير تركيزات منخفضة الأزاديراختين والميثوكسيفينوزيد على العمر اليرقى الثانى لدودة ورق القطن. تم تقدير نشاط إنزيمات الكيتينيز والبوليفينول أوكسيديز والإنزيمات المحلله للبروتينات والألفاأميليز فى يرقات تم تعريضها لتركيزات منخفضه من الأزاديراختين والميثوكسيفينوزيد بالمقارنه بيرقات غير معامله. وجد أن سمية الأزاديراختين (التركيزات اللازمه لموت ٥٠% من اليرقات المعامله = ٠,٧٣ ، ٠,٣٦ ، ٠,١٧ مجم / لتر) كانت ٢٠,٨ ، ٥,٨ ، ٨,٦ مره قيمة سمية الميثوكسيفينوزيد (التركيزات اللازمه لموت ٥٠% من اليرقات المعامله = ١٥,٨، ١,٨٩ ، ١,٤٦ مجم / لتر) ضد العمر اليرقى الثانى لدودة ورق القطن ، على الترتيب. يرقات العمر الثانى المعامله بالتركيزات اللازمه لموت ١٠ و ٢٥% من اليرقات المعامله أظهرت إنخفاض معنوى فى متوسط وزن اليرقات ، نسبة تحول اليرقات إلى عذارى ، متوسط وزن العذارى ونسبة تحول العذارى إلى حشرات كامله. الوقت اللازم لتحول اليرقات إلى عذارى كان أطول بصوره معنوية عند معاملة يرقات العمر الثانى بالتركيزات اللازمه لموت ٢٥% مقارنة بالكونترول. متوسط عدد البيض الذى تم وضعه / أنثى وخصوبته إنخفضت فى المعاملات بصوره معنوية مقارنة باليرقات الغير معامله (كنترول). نشاط إنزيمات الكيتينيز ، البوليفينول أوكسيديز ، والإنزيمات المحلله للبروتين ، والألفاأميليز تم تثبيطها بصوره جزئيه معتمدا على التركيز فى اليرقات المعامله مقارنة باليرقات الغير معاملة.

من هذه النتائج يتضح أن التركيزات المنخفضه من الأزاديراختين والميثوكسيفينوزيد لها تأثير فى خفض تعداد ونمو دودة ورق القطن وذلك كنتيجة للتأثير على تطورها وتكاثرها. من الناحية العملية هذه التأثيرات مهمة جدا لأن الأعداد الناتجة من الممكن أن تنخفض وبالتالى فإن تعداد الحشرات سيتأثر سلبيا.