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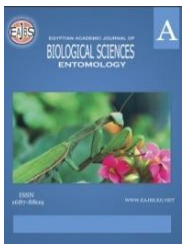

# Biological, Biochemical and Molecular Characterization Studies of Spinosad, Methoxyfenozide and Extrem on The Cotton Leafworm, *Spodoptera littoralis* (Boisd.).

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## ABSTRACT

This work aimed to study the effect of a bacterial bioagent Spinosad, an insect growth regulator Methoxyfenozide and Extrem their mixture (Spinetoram & Methoxyfenozide) were evaluated on 4$^{th}$ instar larvae of *Spodoptera littoralis* (Boisd.) under laboratory conditions. The results indicated that $LC_{50}$ values of three tested compound treatments on 4$^{th}$ instar larvae were determined as 7.28, 0.071 and 0.113 ppm, in Spinosad, Methoxyfenozide and extrem respectively. $LC_{50}$ of all treatments resulted in a prolongation of the larval duration and pupation rates were significantly decreased. The pupal weight recorded insignificant results for all treatments. Both Spinosad and Methoxyfenozide treatments insignificant differences in pupal duration, while significant differences were recorded when larvae were treated with $LC_{50}$ of Extreme**.** The biochemical studies in 6 days after treatment of fourth instar larvae showed a significant decrease in the activity of α-esterase enzymes, while β-esterase activity was significantly increased, acetylcholineterase enzyme showed a highly significant decrease in the treated with Spinosad, Methoxyfenozide, while it insignificant decrease when treated with Extreme. All treatments showed a non-significant difference in total protein contents of the whole-body homogenate of treated larvae. Results of SDS-PAGE analysis revealed that the protein profiles of *S. littoralis* changed after larval treatment with Spinosad, Methoxyfenozide and Extreme new proteins were observed in treated larvae.

## INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is one of the most economic insect pests in Egyptian fields. In Egypt, it is considered a destructive pest that causes economic losses not only for the cotton plants but also for other crops and vegetables Hosny *et al*. (1986), orchard trees, and ornamentals Mohamed *et al*. (2019). The extensive use of insecticides to control *S. littoralis* larvae has led to resistance to various classes of insecticides, residual toxicity, environmental pollution and negative effects on non-target organisms. Moreover, the use of chemical insecticides usually requires regular and frequent repetition which makes it a very expensive practice. In the search for alternative control agents both Spinosad, Methoxyfenozide.

Spinosad is a bioproduct from the naturally occurring soil actinomycete, *Saccharopolyspora spinosa* which was found to have high insecticidal activity. It is now commercially formulated under the name Spinosad (Sparks *et al.,* 1998). This formulation acts primarily on the insect's nervous system at the nicotinic acetylcholine receptor and also exhibits activity on the gamma-aminobutyric and receptor GABA (Sparks *et al.,* 2001). Insect growth regulators Methoxyfenozide is classified as a diacylhydrazine insecticide, also called Moulting Accelerating Compound (MACs) and acts as ecdysone agonists with enormous potential for development as insect-specific control agents with little or no effect on non-target species and provides effective control of a wide range of lepidopteran insects (Carlson *et al*., 2001). Extrem (Spinetoram and Methoxyfenozide) are considered promising candidates.

The objective of the present research was to evaluate the susceptibility of 4$^{th}$ instars larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) to Spinosad, Methoxyfenozide and Extrem (Spinetoram and Methoxyfenozide) and its effects on some biological aspects, some biochemical components and Electrophoretic patterns of protein profile of this insect.

## MATERIALS AND METHODS

**Insect Rearing:**

A laboratory strain (Lab) of cotton leafworm *S. littoralis* was obtained from the cotton leafworm department plant protection research institute (PPRI) that was reared under laboratory conditions for several years without exposure to insecticides. The colony was kept at a temperature of 27±2 ºC and 65±5 RH.) (El-Defrawi *et al.*, 1964). Larvae were reared on castor oil leaves (Ricinus communis L.), the 4th larvae selection for bioassays and biochemical assessments.

**Tested Materials:**

One bio- pesticides Tracer® (Spinosad,24% SC) from Dow Agroscience Co., one insect growth regulator non-steroidal ecdysteroid agonists Runner® (Methoxyfenozide, 24% SC) from Dow Agroscience Co. and Extrem®, 36 % SC (Spinetoram 6% and Methoxyfenozide 30%) from Dow Agroscience Co.

**Bioassays for Tested Materials and LC $_{50}$ Calculation:**

The toxic effect of tested materials was evaluated against the larval stage, where newly fourth larval instar was fed on castor bean leaves dipped for 30 seconds in different five concentrations, one hundred larvae were divided into four replicates; every 25 larvae were used for each concentration (0.8, 0.4, 0.2, 0.1 and 0.05 ppm for Runner, 50, 25, 12.5, 6.25 and 3.12 ppm for Spinosad and 1, 0.5, 0.25, 0.125 and 0.0625 ppm for Extrem). A control experiment was performed using castor bean oil leaves dipped in water. The tested larvae fed on treated castor bean oil leaves for 48 hrs., then the survived larvae were transferred to other clean jars and supplied daily with untreated one until 7 days after treatment. The mortality was recorded daily. Mortality in treated treatments was corrected with the corresponding mortality in the untreated check according to Abbott's formula (Abbott, 1925) and subjected to Finney probit analysis (Finney, 1971). and the $LC_{50}$ value was determined.

**Biological Investigation:**

Newly moulted 4$^{th}$ instar larvae were treated with the $LC_{50}$ concentration of the three tested compounds (Spinosad, Methoxyfenozide, and Extrem). Treated larvae were examined daily to determine the post-treatment effects on those insects that survived the treatments, (*e.g*., the larval and pupal duration, larvae and Pupal weight). These parameters were compared with the untreated control larvae.

**Biochemical Studies:**

**1. Preparation of Samples for Biochemical Studies:**

Larvae were collected after six days following the treatment of the fourth instar, placed in ice containers and homogenized in appropriate buffer using a Teflon homogenizer surrounded with a jacket of crushed ice for 3 minutes. Homogenates were centrifuged at 8000 rpm for 10 minutes at 4°C (Biofuge 28RS Heraeus, Sepatech centrifuge). The resulting supernatants were used directly for the determination of enzymatic activity.

**2. Determination of the Enzyme Activities:**

The activity of both α and β non-specific esterases and acetylcholinesterase were determined according to the method of Van Asperen (1962) and Simpson *et al.* (1964), respectively.

**3. Determination of Total Soluble Proteins:**

Total protein content in larvae treated withLC50 homogenate was determined after 6th days post-treatment according to the method described by Bradford (1976).

**Total protein Electrophoresis:**

SDS-polyacrylamide gel electrophoresis was performed in 12 % acrylamide slab gels following the system of Laemmli (1970) to identify their protein profiles.

**Statistical Analysis Procedure:**

The significance of the main effects was determined by using an analysis of variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range tests ($p<0.05$). All analysis was preceded using a software package "Costat", a product of cohort software Ine. Berkley, California. (Duncan, 1955).

## RESULTS AND DISCUSSION

**Toxicological Studies:**

Table (1), presents the efficiency of three tested compounds: Spinosad, Methoxyfenozide and Extrem against the 4$^{th}$ instar larvae of *S. littoralis*. $LC_{50}$ values of three tested compound treatments on 4$^{th}$ instar larvae were determined as 7.28, 0.071 and 0.113 ppm, in Spinosad, Methoxyfenozide and extrem respectively. The Slope was 0.359, 0.866 and 2.069 in Spinosad, Methoxyfenozide and Extrem, respectively.

The high toxicity of Spinosad against 4$^{th}$ instar larvae of *S. littoralis* was recorded by Mohamed *et al.* (2015) when different host plants were used for feeding. On the other hand, the toxicity of Spinosad against the cotton leafworm, *S. littoralis* was increased by adding 0.3% mint oil Marwa (2019). Also, Jixiang *et al.* (2019) recorded an increase in toxicity of methoxyfenozide when mixed with lufenuron against *Spodoptera exigua*.

**Table 1:** Lethal toxicity values (ppm) of Spinosad, Methoxyfenozide and extrem tested against *S. littoralis* 4$^{th}$ instar larvae.

| Treated Compound | $LC_{50}$ (ppm) | Confidential limits for (95%) | | Slope±S.E. |
|---|---|---|---|---|
| | | Lower | Upper | |
| Spinosad | 7.28 | 3.759 | 14.125 | 0.359 ± 0.24 |
| Methoxyfenozide | 0.071 | 0.015 | 0.128 | 0.866 ±0.26 |
| Extreme | 0.113 | 0.076 | 0.146 | 2.069 ±0.34 |

**Biological Studies.**

The data in Table 2 indicated that $LC_{50}$ of all treatments resulted in a prolongation

of the larval duration compared to control, on the other hand. Pupation rates were also significantly decreased where it recorded 0.28, 0.24, and 0.29 gram for the treatments; Spinosad, Methoxyfenozide and extrem compared to the control (0.40 gram). The pupal weight recorded insignificant results for all treatments, where it recorded 0.36, 0.35 and 0.33 gram for Spinosad, Methoxyfenozide and extrem, respectively, while it was 0.36 gram in the untreated control. Also, the results recorded insignificant differences in pupal duration when larvae were pretreated with Spinosad, Methoxyfenozide, while significant differences were recorded when larvae were treated with $LC_{50}$ of Extreme compared to the untreated control. These results agree with Seham (2020), who showed significantly reduce mean d pupal weight, prolonged larval duration after treating *S. littoralis* with spinosad. Methoxyfenozide showed a significant ($P < 0.05$) reduction in pupal weight of *S. littoralis* pretreated with LC25 of methoxyfenozide Hamdy (2016).

**Table 2:** Larval duration, pupal duration and pupal weight of *S. littoralis* treated as 4th instar larvae with $LC_{50}$ of Spinosad, Methoxyfenozide and Extreme.

| Treated Compound | Mean larval duration (days ± S.E.) | Mean pupal weight (gm ± S.E.) | Mean pupal duration (days ± S.E.) |
|---|---|---|---|
| **Spinosad** | $12.0^{a} \pm 0.6$ | $0.36 \pm 0.01$ | $9.2^{b} \pm 0.12$ |
| **Methoxyfenozide** | $11.0^{ab} \pm 0.4$ | $0.35 \pm 0.02$ | $9.4^{b} \pm 0.24$ |
| **Extrem** | $12.0^{a} \pm 0.2$ | $0.33 \pm 0.01$ | $11.0^{a} \pm 0.18$ |
| **Control** | $10.0^{b} \pm 0.4$ | $0.36 \pm 0.03$ | $9.1^{b} \pm 0.18$ |
| **F value** | $.1123^{*}$ | $.5473^{ns}$ | $.0002^{***}$ |
| **L.S.D.** | 1.8828 | 0.0471 | 0.5803 |

Numbers of the same letters have no significant difference

**Biochemical Studies:**

**- Effect of Spinosad, Methoxyfenozide and Extreme on Specific Activities of Some Enzymatic Systems and Total protein content of *S. littoralis*.**

The activities of some enzymatic systems in 4th instar *S. littoralis* larvae, on the 6th-day post-treatment with the determined $LC_{50}$ value of Spinosad, Methoxyfenozide and Extreme was determined. These enzymes represent a variety of hydrolases that are essential in the physiological function of and hence in the metabolic pathway of a wide variety of principal biochemical constituents in the targeted insect.

The activity of α-esterase recorded 24.94, 15.24 and 20.68 µg α- naphthyl acetate/ min/ mg protein for Spinosad, Methoxyfenozide and Extreme respectively show a significant decrease than values were calculated to control 41.41 (Table 3).

The results in Table 3 recorded that β-esterase activity was significantly increased as they were 41.24, 60.62 and 66.53 µg β-naphthyl acetate/ min/ mg protein for Spinosad, Methoxyfenozide and Extreme, respectively compared to 12.34 µg β-naphthyl acetate/ min/ mg protein in control.

The activity of the acetylcholinesterase enzyme of untreated *S. littoralis* larvae was 397.41 µg acetylcholine bromide/ min/ mg protein. The results showed a highly significant decrease in the treated larvae with Spinosad, Methoxyfenozide being 133.12 and 291.88 µg acetylcholine bromide/ min/ mg protein, respectively, while it recorded an insignificant decrease when larvae were treated with Extreme 382.02 acetylcholine bromide/ min/ mg protein compared to control (Table 3). Our results agree with Ibrahim (2008) and Marwa (2019) who demonstrated that Spinosad showed a significant decrease in the activity of AChE enzyme of *S. littoralis*. While Barrania (2020) reported that

methoxyfenozide increased the AChE from 3rd instar of *S. littoralis* head capsules.

Larvae treated by $LC_{50}$ of Spinosad, Methoxyfenozide and Extreme induced insignificant decrease in total soluble protein content 6th-day post-treatment, i.e., 9.796, 8.791 and 8.791 mg/g body weight, respectively, as compared to 9.372 mg/g body weight in untreated insects

Our results are in harmony with both Mary *et al.* (2019) and Saleh and Abdel-Gawad (2018) they recorded that the total protein show an insignificant increase of *S. littoralis* larvae treated with spinosad.

**Table 3:** Effect of Spinosad, Methoxyfenozide and Extreme at $LC_{50}$ values on the specific activity of some hydrolytic enzymes and Total protein content on 6th day following treatment of 4th instar *S. littoralis* larvae.

| Compound | Specific activity of the selected enzymes | | | Total protein content mg/g body weight ± S.E. |
|---|---|---|---|---|
| | α-Esterase | β-Esterase | AChEase | |
| | µg α- naphthyl acetate/ min/ mg protein | µg β-naphthyl acetate/ min/ mg protein | µg Acetylcholine Bromide/ min/ mg protein | |
| **Spinosad** | $24.94^{b} \pm 1.12$ | $41.24^{b} \pm 4.06$ | **133.12c ± 18.32** | **9.796± 0.52** |
| **Methoxyfenozide** | $15.24^{c} \pm 0.87$ | $60.62^{ab} \pm 5.69$ | **291.88b ± 32.29** | **8.791 ± 0.36** |
| **Extreme** | $20.68^{bc} \pm 1.34$ | $66.53^{a} \pm 5.72$ | **382.02ab±20.76** | **8.791 ± 0.09** |
| **Control** | $41.41^{a} \pm 3.0$ | $12.34^{c} \pm 0.56$ | **397.41a ± 35.32** | **9.372 ± 0.58** |
| **F value** | **0.0000*** ** | **0.0013** ** | **0.0013**** | **0.0420** |
| **L.S.D.** | **9.6725** | **20.859** | **112.961** | **2.125** |

Numbers of the same letters have no significant difference

**Separation of Protein Patterns by Using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS–PAGE) Technique of *S. littoralis* Treated with Spinosad, Methoxyfenozide and Extreme.**

Sodium dodesyl sulfate (SDS-PAGE) revealed that the proteins in the tissue of control were separated into 9 bands with molecular weights ranging between (25.00–245.00) kDa while, it separated into 11 bands in Spinosad treatment with molecular weights ranging between ((25.00–245.00), in case of Methoxyfenozide treatment separated into 9 bands with molecular weights ranging between (25.00-196.91) kDa and 10 bands for Extrem treatment with molecular weights ranged between and (30.34-244.28) kDa (Fig.1 and Table 4).

Generally, all treatments led to the detection of new bands and disappeared some bands compared to control. It is concluded that the treatments have efficacy on the soluble protein in insect body when the 2nd instar larval of *S. littoralis* with chlorphyrifos, spinosyns group and lufenuron) produced some differences in SDS protein patterns (PAGE). Mery *et al.* (2019) Also Ibrahim (2016) studied the effect of teflubenzuron, methoxyfenozide and pyriproxyfen on SDS-separated protein of the whole-body tissues of 6th instar larvae of *S. littoralis* treated as 4th larval instar. In each selected generation with three insect growth regulators bands expressed by one or more unique or polymorphic ones in treated larvae that were different from one to another according to the tested compounds. In our results detection of new bands, and disappeared some bands compared to control may be attributed to that the insects used this new band in detoxification of the insecticides used.

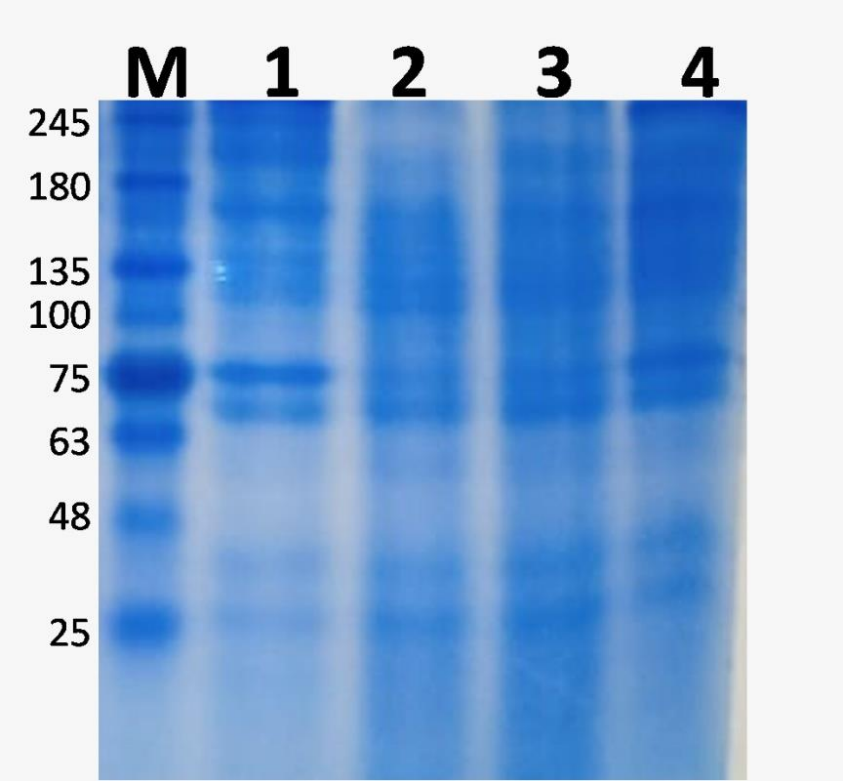


**Fig. 1:** Electrophoretic SDS - PAGE protein patterns for the whole body tissues of *S. littoralis* larvae untreated (lanes 1) and 6th instar larvae treated as 4th instar with Spinosad (lanes 2), Methoxyfenozide (lanes 3) and Extreme (lanes 4).
M=Marker

**Table 4:** Molecular weight (KDa) and amount percentage of native electrophoretic protein patterns for the whole-body tissues of *S. littoralis* as control and treated samples with Spinosad, Methoxyfenozide and Extreme.

| Band No. | Marker | | Control Lane 1 | | Spinosad Lane 2 | | Methoxyfenozide Lane 3 | | Extreme Lane 4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Amout % | M.wt | Amoun % | M.wt | Amout % | M.wt | Amout % | M.wt | Amout % | M.wt |
| 1 | 5.72 | 245.00 | 5.72 | 245.00 | 5.72 | 245.00 | 4.72 | 196.91 | 5.41 | 244.28 |
| 2 | 4.55 | 180.00 | 4.85 | 214.45 | 4.91 | 195.15 | 1.35 | 164.00 | 1.35 | 196.03 |
| 3 | 8.70 | 135.00 | 2.33 | 164.26 | 2.01 | 162.62 | 0.51 | 137.30 | 2.77 | 164.47 |
| 4 | 3.15 | 100.00 | 0.51 | 139.25 | 1.32 | 140.83 | 0.07 | 108.42 | 0.51 | 133.45 |
| 5 | 2.98 | 75.00 | 0.07 | 113.85 | 2.07 | 125.97 | 0.67 | 74.67 | 0.59 | 108.12 |
| 6 | | 63.00 | 0.67 | 73.70 | 0.67 | 97.81 | 2.05 | 66.26 | 2.05 | 76.49 |
| 7 | 0.62 | 48.00 | 1.65 | 67.99 | 0.62 | 72.90 | 0.96 | 58.18 | 5.93 | 69.08 |
| 8 | 2.85 | 25.00 | 2.18 | 36.81 | 2.05 | 67.70 | 3.15 | 33.97 | 0.96 | 59.96 |
| 9 | | — | 1.24 | 25.00 | 0.96 | 56.76 | 1.53 | 25.00 | 0.32 | 44.67 |
| 10 | | — | | — | 1.01 | 34.26 | | — | 0.32 | 30.34 |
| 11 | | | | — | 1.56 | 25.00 | | — | | — |
| Total bands | | 8 | | 9 | | 11 | | 9 | | 10 |

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## ARABIC SUMMARY

**التغيرات البيولوجية والكيميائية الحيوية والجزيئية للسبينوساد، ميثوكسي فينوزيد وإكستريم على يرقات دودة ورق القطن**

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مركز البحوث الزراعية- معهد بحوث وقاية النباتات- قسم بحو دودة ورق القطن

يهدف هذا البحث إلى دراسة تأثير مركب حيوي بكتيري سبينوساد، ومنظم نمو حشرات ميثوكسي فينوزيد وإكستريم خليط من (السبينوتورام & ميثوكسي فينوزيد) وتم تقييمهما ضد يرقات العمر الرابع لدودة ورق القطن تحت الظروف المعملية. أشارت النتائج إلى أن قيم التركيز النصفي المميت لثلاث المركبات المختبرة على يرقات العمر الرابع تم تحديدها على أنها 7.28 و0.071 و0.113 جزء في المليون لل سبينوساد وميثوكسي فينوزيد وإكستريم على التوالي. أدى التركيز النصفي المميت لجميع المعاملات إلى إطالة مدة العمر اليرقي وانخفضت نسبت التعذر بشكل كبير. سجل وزن العذراء نتيجة غير معنوية لجميع المعاملات. كما ادت المعاملة بالسبينوساد والميثوكسي فينوزيد الي حدوث فروقا طفيفة في فترة طورالعذراء ، بينما سجلت فروق معنوية عند معاملة اليرقات بـالتركيز النصف مميث لإكستريم. كما أظهرت الدراسات البيوكيميائية بعد 6 ايام من معاملة يرقات العمر الرابع انخفاضًا معنويًا في نشاط إنزيمات ألفا إستريز، بينما زاد نشاط بيتا إستريز بشكل كبير، أظهر إنزيم أسيتيل كولين إستيريز انخفاضًا معنويًا مرتفعًا في حالة معاملات اليرقات بالسبينوساد وميثوكسي فينوزيد، بينما انخفض بشكل ضئيل عند المعاملة بمركب الاكستريم. أظهرت جميع المعاملات اختلافًا غير معنوي في محتوى البروتين الكلي في اليرقات المعاملة. أظهرت نتائج تحليل SDS-PAGE أن خصائص البروتين في يرقات دودة ورق القطن قد تغيرت بعد معاملة اليرقات بالسبينوساد والميثوكسي فينوزيد والاكستريم وظهور حزم بروتينه جديدة في اليرقات المعامله.