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Efficiency of one biopesticide and chitin *synthesis* inhibitor on toxicity, some biological and biochemical aspects of the cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract:

The present study, investigate the effect of the entomopathogenic fungi (EPF), *Metarhizium anisopliae* (Bioranza) and chitin *synthesis* inhibitor lufenuron (Wormatin 5% EC) on the fourth instar larvae of the cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) were investigated under laboratory conditions of 27 ± 1º C and 65 % ± 10 % R.H. The results revealed that the LC_{50} values obtained were 1.0677 and 0.0921 ppm for *S. littoralis* larvae treated with different concentrations of *M. anisopliae* and lufenuron respectively. The result recorded prolongation of larval duration, decreasing of both pupation % and pupal duration. The enzyme activities show a decrement of chitinase and elevation of protease. Also, the result reflects decrement in the total content (Lipid, protein, carbohydrate) for both treatments. Finally, it could be concluded that the used alternative insecticides have potentialities to reduce population density of *S. littoralis*, so could be used in combating the population of *S. littoralis*. Hoping that the obtained results may be of help in integrated pest management as it could be investigated in further researches.

Introduction

Lepidopterous insects are the most destructive pests of the cotton plant, namely the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) , a highly polyphagous lepidopteran pest found worldwide, and an important model system used in a variety of biological research (Legeai *et al.* , 2011).

The using synthetic chemical insecticides resulting in high control efficacy of *S. littoralis*, cause a negative effect on human health, agriculture, natural enemies as a result of chemical residue, environmental pollution and development of insect resistance to this chemical insecticides (Batta, 2016 and Fernandes *et al.* , 2010).

As a result, it is increasingly recognized that the biodiversity in agroecosystems delivers significant ecosystem services to agricultural production such as biological control of

pests. Entomopathogenic fungi, *Metarhizium anisopliae*, (Meyling and Eilenberg , 2007).

Also, the insect growth regulators (IGR,s); 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, among these IGR,s, chitin synthesis inhibitors (CSI's) was wormatin (Lufenuron) which interfere with the chitin deposition.

The aim of the present study was to evaluate the susceptibility of fourth instar larvae of the Egyptian cotton leaf worm *S. littoralis* is to fungi, bioranza and the CSI's lufenuron, and its effects on some biological and biochemical aspects on the 6th larval instar homogenate.

Materials and methods

1. Rearing technique of the Egyptian cotton leaf worm *Spodoptera littoralis*:

The colony of the cotton leaf worm *S. littoralis* was obtained from the division of the Cotton Leaf Worm, Plant Protection Research Institute, Agricultural Research Center larvae were reared for about 13 generations on castor bean leaves (*Ricinus communis* L) before any treatment as the method described by El-Defrawi *et al.* (1964).

2. Compounds used:

Bioranza a commercial product formulation of *M. anisopliae.* The international unit was 32,000 viable spores per mg (32 x 10^6 viable spore/mg). The active ingredient was 10% W.P. and the recommended application rate was 200 gm/100 liter water/feddan. Wormatin 5% EC (Lufenuron)**:** a commercial insect growth regulator product lufenuron. The active ingredient was 5% EC and the recommended application rate was 160 cm^3/feddan.

3. Toxicological studies:

Dipping method was used in this bioassay. 4th instar larvae were fed on treated castor bean leaves in different concentrations of the tested compounds, *M. anisopliae* and lufenuron for 3 seconds. Five concentrations were prepared for each compound which were 6, 4, 2, 1 and 0.5 gm/1litter for *M. anisopliae* and 0.75, 0.375, 0.1875, 0.0938 and 0.0469 ppm for lufenuron. 45 of 4th instars were divided into three replicates for each concentration and fed on treating leaves for 48hrs.

Also 30 4th instars used as control was performed using castor been leaves dipped in water, then the survived larvae were transferred to other clean jars and supplied daily fresh castor bean leaves for 7 days after treatment. The mortality percentage was recorded daily and corrected according to (Abbott, 1925) formula. Percentages of correcting mortalities were statistically analyzed according to (Finney, 1971) and the LC_{50} value was determined.

4. Biological studies:

From the maintained insect culture, newly ecdysed Fourth instar larvae (30 larvae for each treatment 10 and 30 as control) were collected and fed on castor bean leaves treated with the determined LC_{50} value of *M. anisopliae* and lufenuron. Larvae were examined daily, and the following parameters were studied; larval duration, pupation %, pupal duration and pupal malformation %.

5. Biochemical studies:

5.1. 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 resulted supernatants were used directly for determination of enzymatic activity.

5.2. Determenation of the enzyme activities:

The activity of both protease and chitinase were determined according to the method of Ishaaya *et al.* (1971) and Ishaaya and Casida (1974), respectively.

5.3. Determination of the main components:

Total proteins, total lipids and total carbohydrates were determined in the total body homogenates according to the method of Bradford (1976), Singh and Sinha (1977) and Knight *et al*. (1972), respectively.

6. Statistical analysis procedure:

The significance of the main effects was determined by using analysis of variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range tests ($p<0.05$) and using student t-test. All analysis was preceded using a software package "Costat", a product of cohort software Ine. Berkley, California (Duncan, 1955).

Results and discussion

1. Toxicological studies of *Metarhizium anisopliae* and lufenuron against 4th larvae of *Spodoptera littoralis*:

Table (1) represents the efficiency of *M. anisopliae* and lufenuron on 4th instar larvae of *S. littoralis* . The LC_{50} as well as regression lines were calculated, the LC_{50} values recorded 1.0677 g/l and 0.0921 ppm for *M. anisopliae* and lufenuron respectively. Also, the slope values were 4.949 and 1.5387 for *M. anisopliae* and Lufenuron respectively, showing the homogeneity of the larvae.

The mode of action of *M. anisopliae* infection of the insect host with *M. anisopliae* starts with the unspecific adhesion of the fungal spores to the insect cuticle and penetrate into the insect haemolymph where extensive growth in the haemolymph and the production of toxins then to the insect tissue and the death occur.

These results agree with El Husseini (2019) where assumed the efficacy of the entomopathogenic fungus, *M. anisopliae* through applying different concentrations against *S. littoralis*. Also, Ramos *et al.* (2020) recorded that *M. anisopliae* caused the highest sporulation rates in controlling *S. frugiperda* with *B. bassiana* and *M. anisopliae*

While the larval mortality with lufenuron may be attributed to direct inhibition of chitin synthesis within the integument rather than to any indirect extracuticle effects on hormone levels and the ingestion of chitin synthesis inhibitor compounds by insect larva disturbed the endocuticular deposition during moulting process and abortive moulting because they block chitin synthesis.

Generally, treated larvae were observed to be less active in their movement with obvious muscle contractions and prior to their death larvae exhibited severe tremors followed by paralysis .These finding agree with Wahba and Shaker (2020) where studied the toxicity of lufenuron, against the 4th instar larvae of *S. littoralis*. These findings also agree with Abdel-Aal and El- Shikh (2012) and Maqsood *et al*. (2016) reported that lufenuron proved the most effective insecticide against *S. littoralis*.

Table (1): Toxicity values of and lufenuron against 4th instars *Spodoptera littoralis* larvae

Treated compound	LC_{50}	Confidential limit (95%) LC_{50}		Slope±S.E.	Accumulative mortality% (At the end of larval stage)
		Lower	Upper		
***Metarhizium anisopliae* gm/ 1litter**	1.0677	0.7194	1.4285	4.9489± 0.3094	51.2
Lufenuron (ppm)	0.0921	0.0561	0.1293	1.5387± 0.2188	50.5

2. Biological studies:

Table (2) illustrated that the duration of the *S. littoralis* treated as 4th instar with LC_{50} of *M. anisopliae* was 11.0 days, which was more than that of the control by 0.5 days, i.e. an increase of 4.76% than the control. Pupation % following treatment of 4th instar larvae was 49.5%, which was a reduction of nearly half its value in untreated insects , while the larval duration was lengthened by 2.17 days, i.e. 12.67 days as compared to 10.5 days in the control, making a 20.67% increase for lufenuron treatment. Meanwhile, the pupal duration was 8.67 days as compared to 11.0 days in the control, i.e. less than the control by 2.33 days making -23.9% reduction. Pupation % was reduced to 49.0 and 47.5 % for *M. anisopliae* and lufenuron respectively. Meanwhile, the percentage of malformed pupa was more evident when 4th instar larvae were treated with LC_{50} of lufenuron as it reached 33.33 %. The delayed effects of the insecticides which occurred during the development processes play an important role for their efficacy. The resulted data showed that, *M. anisopliae* and lufenuron caused a significant prolongation in the larval duration of *S. littoralis* larvae attributed to the slower metabolic rate of these larvae as a direct effect of insecticidal application. The feeding impairment of treated larvae could lead to prolongation of the larval instars and subsequently led to reduction in the percentage of pupation. These results were supported by El-Akad *et al*. (2016) when treated the newly hatched larvae of *P. gossypiella* with both of *B. bassiana* and *M. anisopliae* found changes in the different biological aspects. Also, Kimberly and Seow (2017) and Jennifer *et al.* (2014) *M. anisopliae* caused more effect against *Diatraea flavipennella* larval period. El-Sayed *et al*. (2017) after studying the toxicity of lufenuron and flufenoxuron against the 2nd instar larvae of *S. littoralis*, treated with the sublethal concentrations of it showed also significantly reduce of the larval duration and pupation%. The changes in larval and pupal durations may reflect metamorphic disruption that in harmony with the percentage of malformed pupae recorded for lufenuron in our results.

Table (2): Biological effects of LC_{50} of *Metarhizium anisopliae* and lufenuron on certain biological aspects of *Spodoptera littoralis* treated as 4th instar larvae:

Treated compound	Mean larval duration (days± S.E.)	Pupation %	Mean pupal stage (days± S.E.)	Pupal malformation %
Metarhizium anisopliae	$11.0^{b} \pm 1.04$ **(4.76)**	49.5	$8.67^{b} \pm 0.88$ **(23.9)**	4.04
Lufenuron	$12.67^{a} \pm 0.88$ **(20.67)**	47.5	$7.67^{c} \pm 0.2$ **(22.2)**	33.33
Control	$10.5^{b} \pm 0.57$	97.0	$11.0^{a} \pm 0.58$	0.0
F. Value	**21.973****		**85.369*****	
L.S.D.	**0.8389**		**0.6399**	

Numbers between brackets are percentages of reduction than the control.
Numbers of the same letters have no significant difference.

3. Biochemical studies:

3.1. Effect of *Metarhizium anisopliae* and lufenuron on specific activities of some enzymatic systems of *Spodoptera 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 *M. anisopliae* and lufenuron was determined. These enzymes represent a variety of hydrolases which are essential in physiological function of and hence in the metabolic pathway of a wide variety of a principal biochemical constituent in the targeted insect. When larvae were treated with *M. anisopliae* the activities of chitinase and protease were calculated to be 27.39 µg NAGA/min/g and 354.7 respectively, then those in untreated insects. These values were calculated to be a significant decrease than values of the control by 82.450 and 77.993%, respectively (Table 3 and Figure 1). Meanwhile, treatment of 4th instar *S. littoralis* larvae with LC_{50} of Lufenuron was significantly reduced as they were 49.96 µg NAGA/min/g and 245.3 µg casein/min for chitinase and protease, respectively (Table 3 and Figure 1). In untreated insects these values were 42.81µg NAGA/min/g and 282.6 µg casein/min to the respective mentioned enzymes which represent a significant decrease than the enzymatic activities in control insects by 116.702 and 86.801%.

Chitinolytic enzymes are produce from cells in epidermis, gut, salivary glands or fat body of insects (Kramer and Koga, 1986). Chitinolytic enzymes secreted by the hypodermis and founded in the moulting fluid in the space between the old and the new cuticles during ecdysis (Kimura, 1976). Both enzymes chitinase and protease have role in digestion of old endocuticle in moulting process. So, any change in this enzyme activity may attributed to the inference of the insecticides (Hussain *et al*., 2010). The production of cuticle-degrading enzymes, chitinases, lipases and proteases effected and facilitating penetration of various fungi as well as providing nourishment for further development. Also, St Leger *et al.*, 1991 in *M. anisopliae* appressorium formation, hydrophobins, and the expression of cuticle degrading proteases are triggered by low nutrient levels. The resulting as Assar *et al*. (2016) recorded increase in activity of chitinase, Phenoloxidase, carbohydrates hydrolyzing enzymes by use some insect growth regulators and bioinsecticides against *S. littoralis*. Also, Abdel-Aal *et al*. (2009) found that some chitin synthesis inhibitors (CSI) increased chitinase activity of the late 6th instar larvae of *S. littoralis* and

recorded that chitinase and protease are essential for digestion of old endocuticle in the moulting process. So, any changes in these enzyme activities may attribute to the interference of the (CSI) in moulting process.

Table (3) : Enzymes activities of *Spodoptera littoralis* larvae after six days of treatment as 4th instar larvae with LC_{50} values of *Metarhizium anisopliae* and lufenuron.

Compound	Chitinase µg NAGA/min/g	Activity % according to control	Protease µg casein/min	Activity % according to control
Control	33.22 ± 0.107	**82.450**	454.4 ± 10.05	**77.993**
Metarhizium anisopliae	27.39** ± 0.284		354.7*** ± 2.188	
Control	42.81 ± 1.223	**116.702**	282.6 ±6.493	**86.801**
Lufenuron	49.96*** ± 1.335		245.3*** ± 5.099	

% Activity in the control= 100% less than 100% or more than 100% (decrease or increase)
****: moderately significant (p < 0.01) and ***: highly significant (p < 0.001), (student-t test).**

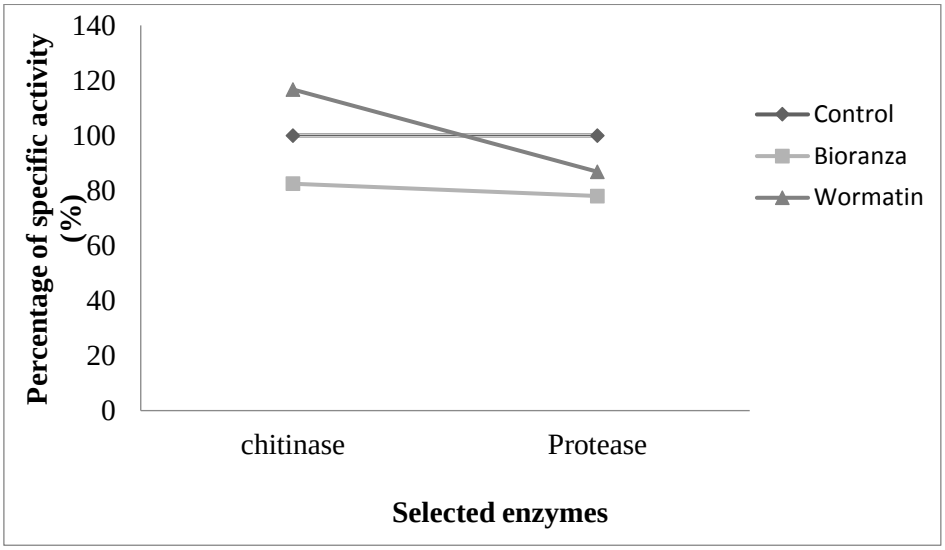

Figure (1): Effect of *Metarhizium anisopliae* and lufenuron at their corresponding LC_{50} values on the specific activities of chitinase and protease 6 days following treatment of 4th instar *Spodoptera littoralis* larvae.

3.2. Effect of *Metarhizium anisopliae* and lufenuron on total soluble protein content, total carbohydrate and total lipid in treated *Spodoptera littoralis* larvae:

Larvae treated by LC_{50} of *M. anisopliae* and lufenuron induced a significant reduction in total soluble protein content 6th day post treatment, i.e. 235.0 mg/g and 396.8mg/g, respectively, then those in untreated insects. These values were calculated to be a significant decrease than values of the control by 32.413 and 32.413 %, respectively (Table 4 and Figure 2). Similarly, the activity of the total carbohydrates was significantly reduced as they were 273.3 mg glucose/larva and 318.4 mg glucose/larva for mg glucose/larva, respectively. In untreated insects these values were 313.4 and 365.7 mg glucose/larva to the respective mentioned compound which represent a significant decrease than the total carbohydrates in control insects by 12.795 and 12.934 % (Table 4 and Figure 3). The data in Table showed a

highly significant in the total lipid contents in the tested compound, *M. anisopliae* and lufenuron comparing with control. They were calculated to be 254.8 and 261.8 mg oleic/larva which represent a marked decrease in the total lipid contents of the respective mentioned compound untreated insects to 23.872 and 38.846 % (Table 4 and Figure 4).

Table (4) : The amounts of total protein, total carbohydrate and total lipid in *Spodoptera littoralis* larvae after six days of treatment as 4th instar larvae with LC_{50} values of *Metarhizium anisopliae* and lufenuron.

Compound	The biochemical components		
	Total protein mg/larva	Total carbohydrate mg glucose/larva	Total lipid mg oleic/larva
Control	347.7 ± 20.19	313.4 ± 7.818	334.7 ± 6.332
Metarhizium anisopliae	235.0*** ± 5.863 **(32.413)**	273.3*** ± 4.573 **(12.795)**	254.8*** ± 5.82 **(23.872)**
Control	459.8 ± 6.347	365.7± 4.53	428.1 ± 6.851
Lufenuron	396.8*** ± 3.602 **(13.702)**	318.4*** ± 4.126 **(12.934)**	261.8*** ± 6.572 **(38.846)**

Numbers between brackets presented % decrease or increase in case the biochemical components
***: highly significant (p < 0.001), (student-t test).**

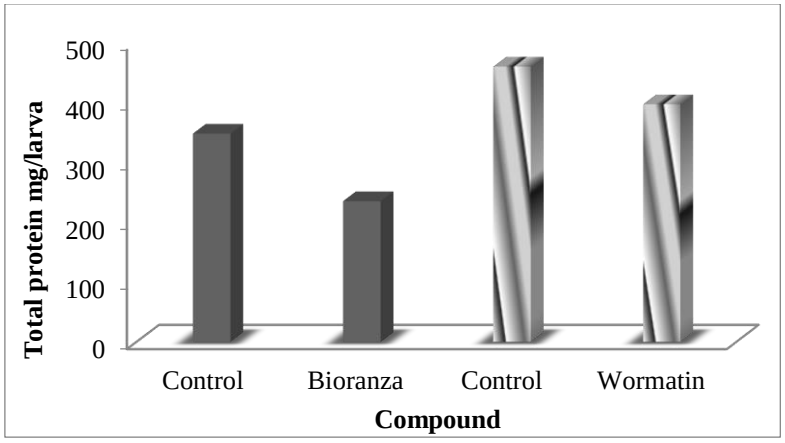

Figure (2) : Total protein content in *Spodoptera littoralis* larvae after six days of treatment as 4th instar larvae with LC_{50} values of *Metarhizium anisopliae* and lufenuron

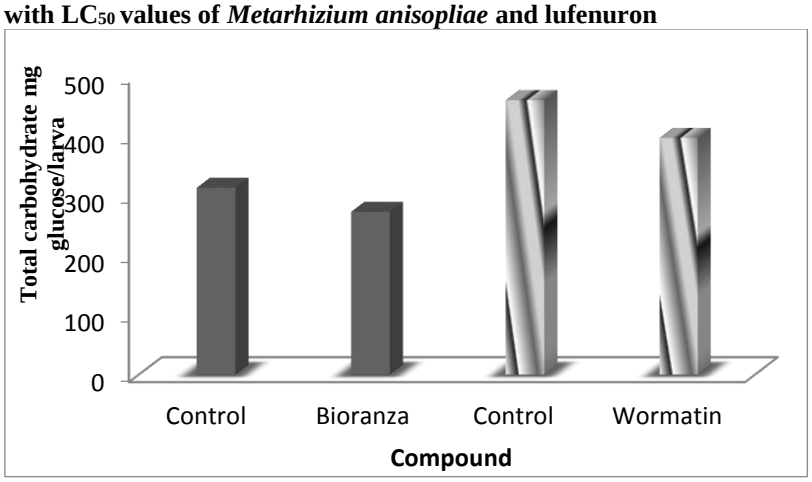

Figure (3) : Total carbohydrate contents of *Spodoptera littoralis* larvae after six days of treatment as 4th instar larvae with LC_{50} values of *Metarhizium anisopliae* and lufenuron.

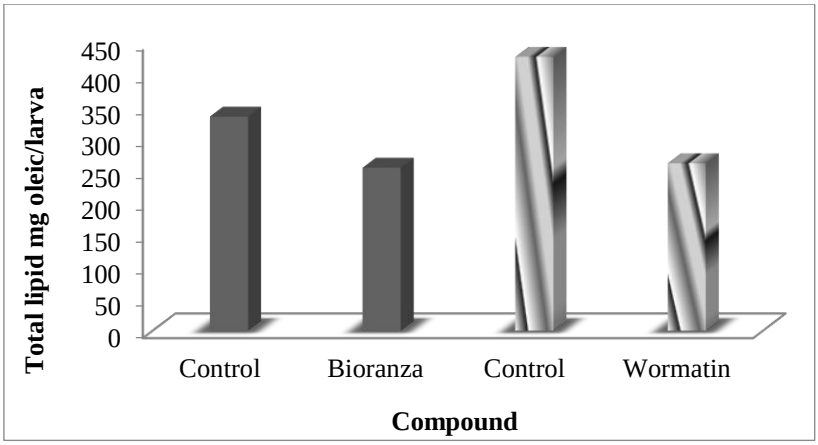

Figure (4) : Total lipid contents of *Spodoptera littoralis* larvae after six days of treatment as 4th instar larvae with LC_{50} values of *Metarhizium anisopliae* and lufenuron.

Proteins are the most important compounds found in cells of all living organism, including many substances like enzymes and hormones that necessary for the main function of the living organisms (Fagan *et al*., 2002). Carbohydrates providing the energy wanted for the growth of the cell (Lee *et al*., 2002). Lipids are significant source of energy (Ali, 2011). The significant reduction in the total protein may be due to binding with foreign substances, as any insecticides (Ahmed *et al*., 1985). The reduction of carbohydrates may due to the effect of anti-feedent and increased metabolism under toxicant stress (Remia *et al.*, 2008). The present results agree with El-Badawy *et al*. (2018) where found that *P. lilacinum* isolate caused significant increase in total carbohydrates and total lipids and reduction in total protein content of *S. littoralis*. Also, Nirupama (2015) the total protein of silkworm, *Bombyx mori* was reduced gradually with the fungal infection. Also, our results were proved by Vidhya *et al.* (2016) where infection of the army worm *S. litura* (Fabricius) by *B. bassiana, M. anisopliae* showed significant decreased of total protein content. Also our results were proved by Sobhi *et al.* (2020) they recorded inhibition of total lipid and total protein of *S. littoralis* treated with essential camphor oil while reduction in total carbohydrates they observed are in contrast to our findings as we reported stimulation of total carbohydrates after treatment with essential *C. cyminum* oil. Kungreiliu Panmei *et al*. (2021) the results support the reduction in the body nutrients of *Ae. aegypti* larvae infection by lufenuron where the metabolic disturbances affected the growth and development of larvae. Aliabadi *et al*. (2016), reported the effects of sub-lethal concentration of lufenuron has been reported in *Glyphodes pyloalis*. The reduction of hemolymph and body homogenate total carbohydrate contents of the treated larvae (2nd and 4th instar) of *S. littoralis* may due to starvation, damage of the alimentary canal by the tested IGRs (Saleh and Abdel-Gawad, 2018).

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