BIOLOGICAL AND BIOCHEMICAL EFFECTS OF *SERRATIA MARCESCENS* (EUBACTERIALES: *ENTEROBACTERIA*) AS MICROBIAL AGENT AND THE CHITIN SYNTHESIS INHIBITOR LUFENURON ON THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.) (LEPIDOPTERA: NOCTUIDAE)

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INTRODUCTION

In Egypt, as in many other countries, the control of cotton leafworm, *Spodoptera littoralis* depends mainly on the extensive use of conventional insecticides. However, the use of these conventional insecticides usually requires regular and often frequent and extensive replication for an indefinite period. This is certainly very expensive in addition to the development of many other problems such as residual toxicity and environmental pollution (Frank *et al.,* 1990), negative effects on non–target organisms (Franz, 1974) and resistance against these toxicants (Tabashink *et al.,* 1987). The public concern over the harmful effects of chemical pesticides on the environment and human health has enhanced the search for environmentally safe and friendly control alternative methods, using the entomopathogenic microbial control agents such as bacteria, viruses, fungi, protozoa, and nematodes (Lacey *et al.,* 2001). Pathogenic effect of *Serratia marcescens* is, however, due to its rapid multiplication in the hemocoel which results in death through one to three days when ingested. Its ability to secrete chitinase can affect insects when this enzyme conacted the cuticle of larve or pupae (Sikorowski, 1985). The use of insect growth regulators during a sensitive period of insect development might result in some morphological and physiological abnormalities, as well as death of treated insects. The preceding criteria of toxicity may play effective role in the control of *S. littoralis* which is considered one of the most economic pests.

MATERIAL AND METHODS

Rearing technique

The stock culture of the cotton leafworm, *Spodoptera littoralis* (Boisd.) was obtained from a laboratory strain maintained in the Cotton Pest Research Dept, Plant Protection Research Institute, Agricultural Research Centre, Dokki, Giza, for several generations without any insecticidal pressure. The insect was reared on castor-oil leaves, *Ricinus communis*, under laboratory conditions at 25 ± 2 °C and 60 ± 5 % R.H. Late 6th instar larvae were used in the current work.

Control agents

1. Biopesticide

Serratia marcescens: Chitinase-producing bacterial strain belongs to *Enterobactieraceae* isolated from Egyptian Soils. The isolated bacterial strain was formulated as a biocontrol agent for controlling parasitic nematodes. It was produced by Soils, Water and Environ. Res. Inst. ARC, and distributed on a commercial scale (trade name, Nemaless)

2. IGR

Common name: Lufenuron. Trade name: Match® 5%. This chemical was obtained from Syngenta Agro S.A.E.

Bioassay

Preliminary tests were carried out using series of concentrations (in water) for each of the bio-agent, *Serratia marcescens* (10^4, 10^5, 10^6, 10^7, 10^8. 10^9 colony forming unit/ml (cfu/ml) and the chitin synthesis inhibitor lufenuron (0.1, 0.25, 0.5, 2, 4 ppm) were prepared using the commercial formulation. Sawdust was treated with *Serratia marcescens* and offered to late 6th instar larvae in a wettable form, while, in case of lufenuron left to dry at room temperature before using (50 ml of the aforementioned product /50 gm sawdust). The late 6th instar larvae were exposed to the treated sawdust in glass jars. Three replicates were carried out for each concentration; each replicate consisted of 20 larvae. The pupal weight, adult emergence and adult malformation were determined. Larvae kept in untreated sawdust were considered as control. Percent mortality of pupae was corrected against those of the control using Abbott's formula (Abbott, 1925). The data were then subjected to probit analysis (Finney, 1971) to obtain the LC_{50} values.

Biochemical determinations

Preparation of samples for biochemical analysis:

1-, 8-, and 13-day old pupae were homogenized in distilled water using a Teflon homogenizer surrounded with a jacket of crushed ice for 3 minutes. Homogenates were centrifuged at 6000 r.p.m. for 10 minutes at 5°C. The supernatant was divided into small aliquots (0.5 ml) and stored at –20 °C until analysis. Three replicates were used for each biochemical determination.

1. Determination of Chitinase activity

Chitinase was assayed using 3, 5-dinitrosalicylic acid reagent to determine the free aldehydic groups of hexoaminase liberated on chitin digestion according to the method described by Ishaaya and Casida (1974).

2. Determination of protease and the carbohydrate hydrolyzing enzyme amylase.

The method was based on the digestion of starch, according to the method described by (Ishaaya *et al.*, 1971).

3. Determination of phosphatases activity

Acid and alkaline phosphatases activities were measured according to the method of Laufer and Schin (1971). Acid buffer pH (4.8) and an alkaline buffer of pH 10.5 (5 ml of 0.2 M glycine +3.86 ml 0.2 N NaOH and then diluted with 20 ml distilled water) were, respectively, used with the two enzymes . The activity was then measured specrophotometrically at 400 nm.

4. Determination of total carbohydrates

Total carbohydrates were determined as described by Singh and Sinha (1977).

Statistical analysis

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

RESULTS AND DISSCUSION

Biological effects

Table (1) reveals the LC_{50} values of the tested compounds againest the late 6^{th} instar larvae, recording $90x10^7$ cfu and 0.303 ppm for *S. marcescens* and lufenuron, respectively. The data in Table (2) show the changes in pupal weight, adult emergence % and malformation % of *S. littoralis* after pupation in sawdust treated with *S. marcescens* and lufenuron. Treatment with *S. marcescens* caused significant increase in the pupal weight compared with control. On the other hand, lufenuron significantly decreased the pupal weight. Adult emergence and malformation percentages significantly decreased and increased after pupation in sawdust treated with the LC $_{50}$ of *S. marcescens* and lufenuron ,respectively. The increase in pupal weight caused by *S. marcescens* may be attributed to the rapid multiplicity of bacteria inside the pupa, which may add more weight to pupae.

TABLE (I)

Susceptibility of *Spodoptera littoralis* (Boisd.) late 6th instar larvae to *S. marcescens* and lufenuron

Treatments	LC_{50}	95% Fiducial limits		Slope ± S.E.	X^2 (*df*)
		Lower	Upper		
S. marcescens *(cfu)	90 x 10^7	84 x 10^7	96 x 10^7	1.46 ± 0.18	$6.61_{(4)}$
lufenuron (ppm)	0.303	0.176	0.534	1.22 ± 0.270	$0.162_{(3)}$

*Cfu: colony forming unit

TABLE (II)

Effect on pupal weight , adult emergence % and malformation % of *Spodoptera littoralis* after pupation in sawdust treated with LC_{50} values of *S. marcescens* and lufenuron.

Treatments	Mean pupal weight (mg) ± S.E.	Mean adult emergence % ± S.E.	Mean malformation % ± S.E.
S. marcescens	385.12 ± 2.19 a	65.67 ± 2.73 a	40.33 ± 3.72 a
lufenuron	305.17± 5.28 b	51.18 ± 4.92 b	29.42 ± 4.18 b
Control	325.67 ± 4.32 c	98.33 ± 1.45 c	2.67 ± 0.34 c
L.S.D.	12.34	11.62	8.14

Values in a column followed by the same small letter are not significantly different ($P < 0.05$; Duncan's multiple rang test).

These results are similar to those obtained by Abd El-Salam *et al.* (1979) who found that feeding of *Agrotis ipsilon* larvae on castor-oil leaves dipped in diflubenzeron decreased the pupal weight in a concentration depending mannar. Shaurub *et al.* (1999) and El-Sheikh (2002) used flufenoxuron against *A. ipsilon* and found decrease in pupal weight and adult emergence % and increase in malformation %. Similar effectwere found by Abdel-Aal (2003) using chlorfluazuron and flufenoxuron against late 6th instars of *S. littoralis*. Furthermore, Tolba (2006) found an increase in pupal weight and malformation % and decrease in pupal weight and adult emergence% of *A. ipsilon* after pupation in sawdust treated with *S. marcescens* and flufenoxuron, respectively.

Biochemical Effects:

The activity of chitinase, protease and amylase in the pupal stage of *S. littoralis* formed on sawdust treated with *S. marcescens* and lufenuron is presented in Table 3.

Effect on chitinase activity:

A significant increase in chitinase activity after 3, 8 and 13 days of pupation in sawdust treated with lufenuron. With *S. marcescens,* significant increase in chitinase activity took place at the 13th day only. Similar results were reported by Lee *et al.* (1994) who found an increase in the chitinase activity of larvae of *Hyphantria cunea* treated with the (chitin synthesis inhibitors) diflubenzuron and chlorofluazuron. Moreover, the chitinase activity was markedly increased, when 4th instar larvae of *S. littoralis* was treated with diflubenzuron. (Farag, 2001). Also, Tolba (2006) found an increase in chitinase activity in pupae of *A. ipsilon* treated with *S. marcescens* and flufenoxuron .The increase in chitinase activity in pupae of *S. littoralis* treated with *S. marcescens* could be attributed to the bacterial ability to secrete chitinase, which leads to physiological changes in treated insect individuals.

In addition, the increase in chitinase activity of pupae treated with lufenuron, could be attributed to the secondary effect of chitin synthesis inhibitor. The primary effect involves blocking of incorporation of uridine 5'-diphospho-N-acetylglucoseamine into chitin. Chitin deposition is carried out through this polymerization step (Verloop, 1977). Moreover, the increase in chitinase activity may be a secondary affect for the reduced activity of β-ecdysone metabolizing enzymes, followed by β-ecdysone accumulation which resultes in hyperchitinase activity (Yu and Terriere, 1977).

Effect on protease activity

It is obvious that a significant increase in protease activity was observed after 3 (logarithmicphase), 8 and 13 (stationary phase) days of pupation in sawdust treated with *S. marcescens* that secretes chitinase. On contrary, lufenuron significantly decreased protease activity at the 3rd, 8th and 13th days (Table 3). The obtained results agree with Farag (2001) who reported that protease activities were markedly decreased after treatment of *S. littoralis* with three IGRs, and with Ibrahim (2006) working on *S. littoralis* and found that the protease activity was significantly increased after treatment with *B. thuringiensis* and decreased due to treatment with flufenoxuron and hexaflumuron.

TABLE (III)

Changes in the mean of chitinase, protease and amylase in pupal stage of *Spodoptera littoralis* after pupation in sawdust treated with LC_{50} values of *S. marcescens* and lufenuron.

Time in days	Mean chitinase (ug N-acetyl-D- glucos amine/min./ml.) ± S.E.				Mean protease (ug protein/min./ml.) ± S.E.				Mean amylase (ug glucose/min./ml.) ± S.E.			
	Control	*S. marcescens*	lufenuron	L.S.D	Control	*S. marcescens*	lufenuron	L.S.D	Control	*S. marcescens*	lufenuron	L.S.D
3	7.57 ± 0.45 Aa	8.32± 0.18 Aa	30.53 ± 1.72 Bd		47.98 ± 5.86 Aa	53.55 ± 3.91 Ba	37.41 ± 3.79 Ca		110.23 ± 8.65 Aa	132.58 ± 3.89 Ba	182.37 ± 7.16 Ca	
8	9.37 ± 1.1 Aa	10.41 ± 0.79 Aa	39.19 ± 2.61 Be	6.95	58.90 ± 2.41 Ab	71.41 ± 4.49 Bb	51.62 ± 2.82 Cb	4.19	177.8 ± 3.16 Ab	213.15 ± 6.95 Bb	289.42 ± 9.97 Cb	17.58
13	14.39 ± 1.34 Ab	24.44 ± 2.19 Bb	42.74 ± 2.68 Cf		± 4.36 Ac	98.18 ± 3.89 Bc	65.48 ± 4.76 Cc		264.43 ± 8.43 Ac	123.68 ± 2.78 Bc	315.46 ± 6.34 Cc	
L.S.D	4.61				7.32				22.19			

Values in a raw followed by the same capital letter are not significantly different and values in a column followed by the same small letter are not significantly different ($p<0.05$; Duncan's multiple rang test).

TABLE (IV)

Changes in the mean acid and alkaline phosphatases activity and total carbohydrates content in pupal stage of *Spodoptera littoralis* after pupation in sawdust dust treated with LC_{50} values of *S. marcescens* and lufenuron.

Time in days	Mean acid phosphatase (ug phenol/min./ml.) ± S.E.				Mean alkaline phosphatase (ug phenol/min./ml.) ± S.E.				Mean total carbohydrates (mg glucose/ml) ± S.E.			
	Control	*S. marcescens*	lufenuron	L.S.D.	Control	*S. marcescens*	lufenuron	L.S.D.	Control	*S. marcescens*	lufenuron	L.S.D.
3	4.15 ± 0.26 Aa	9.17 ± 0.86 Ba	14.27 ± 0.98 Ca		13.32 ± 1.12 Aa	16.86 ± 0.92 Ba	5.35 ± 0.08 Ca		12.31± 0.76 Aa	10.82 ± 0.46 Ba	9.64 ± 0.13 Ca	
8	9.38 ± 0.28 Ab	13.81 ± 0.49 Bb	14.46 ± 0.47 Ba	3.24	5.77 ± 0.45 Ab	3.12 ± 0.72 Bb	2.41 ± 0.25 Bb	0.94	9.74 ± 0.75 Ab	5.62 ± 0.32 B b	9.18 ± 0.23 Ca	0.48
13	11.73 ± 1.12 Ac	10.45 ± 0.47 Aa	17.15 ± 0.79 Bb		3.60 ± 0.36 Ac	2.34 ± 0.07 Bb	1.16 ± 0.02 Cc		4.48 ± 0.73 Ac	2.16 ± 0.12 Bc	3.14 ± 0.09 Cb	
L.S.D.	1.76				1.12				2.11			

Values in a raw followed by the same capital letter are not significantly different and values in a column followed by the same small letter are not significantly different ($p < 0.05$; Duncan's multiple rang test).

Effect on amylase activity

Data show that contaminating insect body with *S. marcescens* resulted in a significant increase in amylase activity during the 3rd and 8th days of pupation. A sharp decrease in amylase activity was observed at the end of the 13th day. Significant increase in amylase activity during the whole period was found in lufenuron treatment.

The increase in amylase activity during the 3rd and 8th days of pupation of *S. littoralis* pupated on sawdust treated with *S. marcescens* was similar with the results obtained by Afifi (2001) who demonstrated a significant increase of amylase activity in *Pectinophora gossypiella* treated with *Bacillus thuringiensis*. Farag (2001) reported that diflubenzuron induced an acceleration in amylase activity in 6th instar larvae of *S. littoralis*. Abdel-Aal *et al.* (2004) detected significant increase in amylase activity of *S. littoralis* larvae treated with flufenoxuron or *Bacillus thuringiensis*. Tolba (2006) found an increase in amylase activity in pupae of *A. ipsilon* treated with flufenoxuron or *S. marcescens* and sharp decrease in its activity at stationary phase. The increase in amylase activity of pupae treated with *S. marcescens* or lufenuron could be attributed to the accumulation of glucose as a result of chitin digestion or suppression in bulding up of a new cuticle ,respectively.

Effect on acid and alkaline phosphatase activities

Data in Table (4) show that *S. marcescens* caused a significant increase in the acid phosphatase activity in pupal stage through the period of 3 to 8 days. *S. marcescens* caused a significant increase in the alkaline phosphatase activity at 3rd day of pupation, while, a significant decrease in the enzyme activity was obtained at 8th and 13th days of pupation as compared with the control. On the other hand, lufenuron significantly increased acid phosphatase activity during the whole period, whereas significant decrease took place with alkaline phosphatase activity.

The increase in acid phosphatase activity with *S. marcescens* was similar to the data obtained by Vincent *et al.* (1993) who reported that acid phosphatase activity was increased in the larvae and adults of *Melanoplus sanguinipes* after infection with *Beauveria bassiana*.

The decrease in alkaline phosphatase activity in *S. marcescens* was similar to the data obtained by El-Sweerki (1994) who noticed a high reduction in the acid and alkaline phosphatases activity of the haemolymph of the 4th larval instar of *S. littoralis* immediately after treatment with LC_{50} of *B. thuringiensis*. Sokar (1995) reported that acid phosphatase activity was significantly reduced in the haemolymph of the 4th larval instar of *S. littoralis* at different intervals (48, 72, 96 and 120 hrs.) after treatment with LC_{50} of *B. thuringiensis* var. *kurstaki* (Dipel-2X). The activity of alkaline phosphatase in the normal larvae was decreased by the time prolongation. Moreover, Tolba (2006) found an increase in acid phosphatase and decrease in alkaline phosphatase activities in *A. ipsilon* following pupation in sawdust treated with flufenoxuron.

Acid and alkaline phosphatases have been shown to be associated with insect development especially in relation to nutrition and egg maturation. Acid phosphatase has received considerable attention in developmental studies because of its association with histolysis. This latter criterion is appreciable at the metamorphic moults of holometabolous species to which *S. littoralis* belongs (Tsumuki and Kanehisa 1984).

An increase in acid and alkaline phosphatase activities following treatment with *S. marcescens* may be due to the bacterial growth inside the insect body, this process required more energy and nutrients, so the insect increases acid and alkaline phosphatase activities to compensate the reduction in energy and in tissues development.

The increase in acid phosphatase activity may be due to the role of acid phosphatase in the immune response at which the bacterial infection stimulates the insect immune system haemocytes, indicating that the enzyme is released from the haemocytes. These observations are also discussed in terms of the possible role of acid phosphatase in the immune response (Vincent *et al.*, 1993). The above explanations may elucidate the high morphogenetic efficiency (malformation %) of these compounds toward *S. littoralis* in the present work.

Total carbohydrate contents

The results in Table (4) reveal that both *S. marcescens* and lufenuron caused a significant decrease in total carbohydrate contents through the pupation period. Similar results were recorded for the carbohydrate contents of the 6th instar larvae of *S. littoralis* treated with abamectin (natural products derived from *Streptomyces avermitilis*) (Ahmed 2001) and the 2nd larval instar of *P. gossypiella* treated with LC_{50} of *B. thuringiensis* (Afifi 2001) and *A. ipsilon* treated with flufenoxuron and *S. marcescens* Tolba (2006) .

Carbohydrates are of vital importance since they can be utilized by the insect for energy production. In most insects, carbohydrate reserves are present as glycogen and trehalose which can be readily converted into glucose for the support of all life processes. Metamorphic changes in insects are usually accompanied by substantial depletions of their carbohydrate reserves. During this period, glycogen and trehalose supply glucose which provides an energy source and a substrate for the synthesis of pupal and adult tissues, especially the cuticle. *Serratia marcescens* caused a high decrease in total carbohydrates because bacteria utilize carbohydrates as source for energy and building a new cell. This may decrease the available carbohydrates in treated insect especially glucose which plays an important role in energy supply, adult maturation (sperm and egg development) and building up of a new chitin.

SUMMARY

The biological and biochemical activities of the entomopathogenic bacterium, *Serratia marcescens* that produced chitinase and the chitin synthesis inhibitory insecticide Lufenuron were determined using LC_{50} values against *S. littoralis* (Boisd.). Treatment of sawdust as pupation medium with *S. marcescens* caused a significant increase in the pupal weight while, lufenuron significantly decreased it. Both adult emergence and malformation were significantly changed. Significant increase in chitinase and amylase activities was observed after 3 and 13 days of pupation in lufenuron- treated sawdust. *S. marcescens* caused a significant increase in the acid and alkaline phosphatases activity in pupal stage at the 3^{rd} day of pupation. Both *S. marcescens* and lufenuron caused a significant decrease in total carbohydrate contents during the pupal stage.

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