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IDENTIFICATION AND EFFICIENCY OF BACTERIAL STRAINS ISOLATED FROM INFECTED LARVAE OF COTTON PINK BOLLWORM *Pectinophora gossypiella* AND SPINY BOLLWORM *Earias insulana* (LEPIDOPTERA: NOCTUIDAE)

[49]

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ABSTRACT

From infected pink and spiny bollworm larvae, collected from Qualyobia Governorate, Egypt, 13 bacterial isolates belonging to 7 species were detected. The efficiency of these bacterial isolates was evaluated on newly hatched pink and spiny bollworm larvae. Three bacterial species, namely, *Pseudomonas viridiflava, Serratia grimesii* and *Cellulomonas flavigena* had no efficiency. Meanwhile, four other entomopathogenic isolates bacterial species, *Pseudomonas pyrrocinia* (A1), *Serratia marcesens* (M3), *Serratia rubidaea* (E3) and *Bacillus thuringiensis* (S2) had noticeable efficiency. The efficiency of these isolates was compared to two commercial products, Dipel 2X and Protecto. Biochemical studies showed differences in total proteins bands patterns in uninfected and infected larvae.

Keywords: *Pectinophora gossypiella*, *Earias insulana*, Entomopathogenic bacteria.

INTRODUCTION

Protection of the environment from chemical pollution leads to the search of alternative methods for the control of pests in the field. Biological control is one of these methods and an important component in any integrated pest management program. In the last three decades, microbial control has proved to be a major tool in pest management, due to its specificity, nontoxicity or pathogenicity to nontarget organisms.

Bacteria species belonging to Pseudomonadaceae (*Pseudomonas*), Enterobacteriaceae (e.g., *Aerobacter, Cloaca, Serratia*) and Bacillaceae (*Bacillus, Clostridium*), are normally found in the soil and also occur in the gut of arthropods. These bacteria can become pathogenic, particularly in conjunction with other pathogens or when the host is physiologically stressed. Some commercial pest control agents against arthropods such as, *Serratia entomophila* and *Bacillus thuringiensis* were used in control measures,

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(Cherwonogrodsky, 1980). *Bacillus thuringiensis* (Bt) is a naturally occurring soil bacterium that produces poisons which cause disease in insects, and has been successfully used for the control of many insect species **(Eleazer *et al* 2003).**

The present work aims to identify and evaluate some bacterial strains isolated from infected cotton bollworm larvae *Pectinophora gossypiella* and *Earias insulana*. Total proteins pattern as induced by bacterial bioagents was determined in infected larvae and had compared to it's pattern in healthy larvae.

MATERIAL AND METHODS

1. Percentage of infected bollworm larvae with natural bacteria

During two successive cotton growing seasons, 2003 and 2004 cotton bolls were collected from cotton fields at the Agricultural Experiment Station of the Faculty of Agriculture, Ain Shams University at Qualyobia Governorate. Starting from 8 July weekly, bases on a 100 bolls were randomly picked and investigated in the laboratory. Percentages of pink and spiny bollworm larvae infected with bacteria were determined.

2. Isolation and identification of bacterial strains in infected bollworms

Isolation of bacterial strains from infected bollworm larvae was carried out and determined according to the method of **Schaad (1980)**. Biochemical characteristics and BIOLOG System were investigated. according to **Bochner (1989).**

3. Pathogenicity test

Pure cultures of isolated bacteria were grown on Nutrient agar medium (NA) for 2 days at 28°C. Bacterial growth was suspended in Sterile Distilled Water (SDW) and adjusted according to its optical density at A620 nm= 0.01 to 10^9 colony forming units (C.F.U)/ml. Bollworm artificial diet was prepared. 10 ml of each bacterial isolate per plate were added to 100 g artificial diet. Ten newly hatched larvae were fed on this treated diet for 48 hours and then transferred to untreated diet till pupation. The same number of larvae was reared on untreated diet and used as control. Each test was repeated 4 times and incubated at 26±1°C and 70±5% R.H.

4. Total proteins electrophoresis of infected pink bollworm *Pectinophora gossypiella* and spiny bollworm *Earias insulana* larvae

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine total proteins in infected larvae with natural bacteria and in two commercial products of *B. thuringiensis* (Dipel 2x and Protecto). Proteins fractions were performed exclusively on vertical slab (19.8 cm x 26.8 cm x 0.2 cm) using the gel electrophoresis apparatus according to **Laemmli (1970)** and as modified by **Studier (1973).**

RESULTS AND DISCUSSION

1. Percentage of infected bollworm larvae with local bacteria strains

Total mean percentage of pink and spiny bollworm larvae collected from the

cotton fields infected with entomopathogeneic bacteria were 1.5 and 3% for the cotton growing season 2003 and 2004, respectively. The mean percentages of infected bollworm were 0, 1.3 and 4.7% in the months of July, August and September 2003, respectively and 1.8, 2.2 and 6.9% during July, August and September 2004, respectively **(Table, 1)**. The highest percentage of infection of cotton bollworm larvae was detected in the 1st week of September, 2004 reaching 7.5%.

2. Pathogenicity test

There were three bacterial species namely, *Pseudomonas viridiflava, Serratia grimesii* and *Cellulomonas flavigena,* which were non pathogenic to the newly hatched larvae of both pink and spiny bollworms, while there were four bacterial species, which were pathogenic, namely *Pseudomonas pyrrocinia* (A1), *Serratia rubidaea* (E3), *Serratia marcescens* (M3) and *Bacillus thuringiensis* (S2). Such results coincide with **Salama *et al* (1986)** who found that some isolates of *B. thuringiensis* were effected against *Spodoptera exigua* and *Heliothis armigera*. While, **Whitlock *et al* (1991)** isolated two new strains of *B. thuringiensis* from the soil as being highly pathogenic to *Spodoptera litura*.

All the isolated bacterial strains (13 isolates belonging to 7 species) were tested to evaluate their efficiency on the newly hatched larvae of pink and spiny bollworm **(Table, 2)**. The effects were studied on newly hatched larvae for pink and spiny bollworms, after feeding on artificial diet treated with different concentrations of isolated bacteria from infected larvae.

Using the highest concentration of 1 x 10^9 IU/ml., mortality percentages of the pink bollworms after 7 days were 23.2, 4.3, 18.9, 25.9 and 90.8%, while they were 10.8, 0, 9.7, 16.2 and 66.5% when a low concentration of 0.125 x 10^9 IU /ml was used of the following bacterial spp.; *P. pyrrocinia* (A1), *S. rubidaea* (E3), *S. marcescens* (M3), *B. thuringiensis* (S2) and [Diepl 2 x (D1)], respectively.

In case of Spiny bollworms, when a concentration of 1 x 10^9 IU/ml. was used, mortality percentages reached 17.6, 12.1, 16.5, 24.2 and 80.8%, while they were 0, 2.7, 9.9, 8.2 and 35.7% for a low concentration of 0.125 x 10^9 IU/ml from the above mentioned bacterial spp., respectively. Mortality among larvae was increased by increasing either the concentration or the period after treatment **(Table, 2)**. In similar experiments, **Zidan *et al* (1998)** tested *B. thuringiensis* against newly hatched larvae of *P. gossypiella* and *E. insulana*, they found that, at the range of the tested concentrations (0.0313-1.0 g/l.), a great percentage of mortality occurred mainly following the first two days from treatment. Most of treated larvae died within 5 days of treatment when a concentration of 1.0 g/l was used.

3. Electrophoresis patterns of total proteins of infected and uninfected pink bollworm *P. gossypiella* larvae

Electrophoresis patterns (SDS-PAGE) of total proteins of uninfected pink bollworm larvae and of that infected with isolated entomopathogenic bacteria, and of that treated with two commercial products (Dipel 2x, Protecto) are illustrated in **Table (3)**. A number of 41

bands were detected with molecular weight

Table 1. Percentage of infected bollworm larvae (*Pectinophora gossypiella* and *Earias insulana*) with local bacteria during cotton seasons 2003 and 2004 (Qualyobia, Egypt).

Inspections date	Bollworm larvae				
	No. of larvae collected	No. of healthy larvae	No. of infected larvae	% healthy larvae	% infected larvae
Season 2003					
8/7/2003	5	5	0	100	0
15/7	10	10	0	100	0
22/7	15	15	0	100	0
29/7	15	15	0	100	0
July-mean	11.3	11.3	0	100	0
5/8	35	34	1	97.14	2.9
12/8	43	43	0	100	0
19/8	22	22	0	100	0
26/8	41	40	1	97.56	2.4
August-mean	35.3	34.8	0.5	98.7	1.3
3/9	34	32	2	94.12	5.9
10/9	34	32	2	97.43	3.6
September mean	45	43	2	95.3	4.7
General mean	27.6	27	0.6	98.5	1.5
Season 2004					
10/7/2004	10	10	0	100	0
17/7	17	17	0	100	0
24/7	14	13	1	92.9	7.1
31/7	12	12	0	100	0
July-mean	13.3	13	0.3	98.2	1.8
7/8	15	15	0	100	0
14/8	29	28	1	96.6	3.4
21/8	25	25	0	100	0
28/8	54	51	3	94.4	5.6
August-mean	30.8	29.8	1	97.8	2.2
5/9	53.0	49	4	92.5	7.5
12/9	48.0	45.0	3.0	93.7	6.3
September-mean	50.5	47.0	3.5	93.1	6.9
General mean	27.7	26.5	1.2	97.0	3.0

Table 2. Corrected mortality percentages among the newly hatched larvae of the bollworm (*Pectinophora gossypiella* and *Earias insulana*) when left feeding on artifical diet treated with different concentrations of natural isolated bacteria and Dipel 2x.

Treatment	Concentration ($-x10^9$)	Pink bollworm			Spiny bollworm		
		After 3 days	After 5 days	After 7 days	After 3 days	After 5 days	After 7 days
*A1	1	15.1	23.2	23.2	8.2	17.6	17.6
	0.5	12.4	18.9	18.9	0	11.0	11.0
	0.25	2.7	10.8	10.8	0	0	0
	0.125	5.4	10.8	10.8	0	0	0
E3	1	4.3	4.3	4.3	6.5	9.9	12.1
	0.5	2.7	2.7	2.7	2.7	2.7	9.3
	0.25	1.6	1.6	1.6	0	2.7	9.3
	0.125	0	0	0	0	2.7	2.7
M3	1	10.8	17.8	18.9	11.0	11.0	16.5
	0.5	9.7	17.8	17.8	1.1	8.2	12.1
	0.25	13.5	21.6	21.6	8.2	13.7	13.7
	0.125	4.3	4.3	9.7	9.9	9.9	9.9
S2	1	15.1	24.3	25.9	24.2	24.2	24.2
	0.5	4.3	16.2	24.3	2.7	13.7	13.7
	0.25	12.4	23.2	23.2	0.0	16.5	16.5
	0.125	4.3	16.2	16.2	0.0	3.8	8.2
D1	1	74.6	90.8	90.8	52.2	70.3	80.8
	0.5	66.5	85.4	85.4	34.1	54.9	54.9
	0.25	48.6	74.6	83.8	19.2	38.5	42.3
	0.125	23.2	66.5	66.5	12.1	35.7	35.7

Table 3. Relative mobility values and molecular weight of SDS protein bands detected in body of treated and untreated pink bollworm *Pectinophora gossypiella* larvae

Bands No.	Relative front	Molecular weight (KDa)	% of relative concentration of SDS protein bans							
			M	P	D	S2	M3	E3	A2	C
1	0.21	151.58	-	-	+	-	-	-	-	-
2	0.24	149.52	-	+	-	+	+	+	+	+
3	0.26	130.50	-	-	-	-	-	+	-	-
4	0.29	111.06	-	-	-	-	-	+	-	-
5	0.31	100.0	+	-	-	-	-	-	+	-
6	0.34	87.75	-	+	-	-	-	+	-	+
7	0.35	82.54	-	-	-	-	-	-	+	-
8	0.36	75.63	-	+	-	-	-	-	-	-
9	0.37	73.96	-	-	-	-	+	-	-	+
10	0.39	66.00	+	-	-	-	-	-	-	-
11	0.40	58.79	-	+	-	-	-	-	-	-
12	0.41	54.13	-	-	+	-	-	-	-	-
13	0.42	52.37	-	-	-	+	+	-	-	+
14	0.43	48.33	-	+	-	-	-	-	-	-
15	0.44	46.97	+	-	-	-	-	+	+	-
16	0.45	43.00	-	+	+	+	-	-	-	-
17	0.46	40.83	-	-	-	-	-	-	+	-
18	0.47	39.39	-	+	-	-	-	-	-	-
19	0.48	37.08	-	-	+	+	+	+	-	-
20	0.49	35.79	-	-	-	-	-	-	+	+
21	0.52	30.64	-	+	-	-	-	-	-	-
22	0.53	29.76	-	-	-	-	-	+	-	-
23	0.56	25.50	-	-	-	-	-	-	+	-
24	0.58	23.56	+	+	-	-	-	-	-	-
25	0.60	21.00	-	-	+	+	+	-	-	-

Table 3. Cont.

Bands No.	Relative front	Molecular weight (KDa)	% of relative concentration of SDS protein bans							
			M	P	D	S2	M3	E3	A2	C
26	0.61	20.58	-	+	-	-	-	+	+	-
27	0.62	18.92	-	-	-	-	+	-	-	-
28	0.63	18.88	-	-	-	-	-	-	+	-
29	0.67	14.86	-	-	-	-	+	-	-	-
30	0.72	12.18	-	+	-	-	+	-	-	-
31	0.73	11.76	-	-	-	-	-	+	-	-
32	0.74	11.00	-	-	+	+	-	-	-	-
33	0.75	10.69	-	+	+	-	+	-	-	-
34	0.76	10.37	-	+	-	-	-	+	-	-
35	0.78	9.24	-	-	-	-	-	-	+	-
36	0.79	8.89	-	-	-	-	-	-	-	+
37	0.82	7.66	-	-	-	-	-	-	-	+
38	0.85	6.75	-	+	-	+	+	+	-	-
39	0.86	6.43	-	-	-	-	-	-	+	+
40	0.88	5.89	-	-	-	-	+	-	+	-
41	0.89	5.71	-	-	-	+	-	+	-	+
Total no. of bands			4	14	7	8	11	12	12	9

S2 = *B. thuringiensis*, M3 = *Serratia marcescens*, E3 = *Serratia rubidaea*, A2 = *Pseudomonas pyrrocinia*, M = Marker, P = Protecto, D = Dipel 2 x, C = Control

(MW) ranged between 151.58 and 5.71 number of bands between untreated and treated larvae, ranged between 9 bands in control and 14 bands in larvae which were treated with Protecto, 7 bands in that treated with Dipel 2x, while S2 isolate, has 8 bands, M3 isolate, has 11 bands. Both E3 isolate and A2 isolate, had the same number of bands (12 bands).

As shown from Lan. 8 in **Table (3)**, bands with mobilities (0.79 and 0.82) and with MW of 8.89 and 7.66 KDa, respectively appeared only in control larvae so they were considered as normal bands for the healthy larvae.

Protecto or Diepl 2x treated larvae shared two bands with mobilities of 0.45 and 0.75 and with MW of 43.00 and 10.69 KDa, respectively.

Therefore, these bands were considered as specific bands for infection with *B. thurigiensis* var. *kurstaki* on which based on Protecto or Dipel 2x.

Furthermore, bands with mobilities of 0.36, 0.40, 0.43, 0.47 and 0.52 and with MW of 75.63, 58.79, 48.33, 39.39 and 30.64 KDa, respectively, (Lan. 2), appeared only in larvae treated with Protecto. Therefore, these bands were considered as specific bands for Protecto.

While bands with mobilities of 0.21 and 0.41 and with MW of 151.58 and 54.13 KDa, respectively, (Lan. 3), appeared only in larvae treated with Dipel 2x.

Therefore, these bands were considered as specific bands for Dipel 2x. Larvae treated with isolate M3 showed two bands with MW of 18.92 and 14.86 KDa, respectively. It appeared only in larvae infected with M3 isolate. While bands with mobilities of 0.26, 0.29, 0.53 and 0.73 and with MW of 130.50, 111.06, KDa. There were clear variations in the 29.76 and 11.76 KDa, respectively, appeared only in larvae infected with E3 isolate. Also, bands with mobilities of 0.35, 0.46, 0.56, 0.63 and 0.78 and with MW of 82.54, 40.83, 25.50, 18.88 and 9.24 KDa, respectively, appeared only in larvae infected with A2 isolate.

4. Total proteins electrophoresis of uninfected and infected spiny bollworm *E. insulana* larvae

Electrophoresis patterns (SDS-PAGE) of total proteins of uninfected spiny bollworm larvae and of that infected with isolated entomopathogenic bacteria, and of that treated with two commercial products Dipel 2x, Protecto, are illustrated in **Table (4)**. A number of 27 bands were detected with molecular weight (MW) ranged between 153.46 and 7.71 KDa. There were clear variations in the number of bands between untreated and treated larvae ranged between 13 bands in control and 9 bands in larvae treated with Protecto or with S2 isolate. Both Dipel 2x, E3 and A2 isolates had the same number of bands (10 bands). While 12 bands are found in larvae treated with M3 isolate.

As shown (Lan. 2) in **Table (4)**, bands with mobilities of 0.04, 0.06, 0.15 and 0.19 and with MW of 153.46, 133.58, 76.69 and 61.42 KDa, respectively, appeared only in control larvae so they were considered as normal bands for the healthy larvae.

Protecto or Dipel 2x treated larvae showed two bands with mobilities of 0.18 and 0.25 and MW of 63.15 and 44.56 KDa, respectively. Therefore, these bands were considered as specific bands for Protecto or Dipel 2x.

Table 4. Relative mobility values and molecular weight of SDS protein bands detected in body of treated and untreated larvae of the spiny bollworm *Earias insulana.*

Bands No.	Relative front	Molecular weight (KDa)	% of relative concentration of SDS protein bands							
			M*	C	D	P	A2	E3	M3	S2
1	0.03	156.0	+	-	-	-	-	-	-	-
2	0.04	153.46	-	+	-	-	-	-	-	-
3	0.05	144.72	-	-	-	-	-	-	+	-
4	0.06	133.58	-	+	-	-	-	-	-	-
5	0.09	113.14	-	-	-	-	-	-	+	-
6	0.10	101.13	-	+	-	-	-	+	+	+
7	0.11	95.81	-	-	-	-	+	-	-	-
8	0.12	93.92	-	+	+	-	+	+	-	-
9	0.13	83.67	-	-	-	-	-	-	-	+
10	0.14	81.11	-	-	+	-	-	-	-	-
11	0.15	76.69	-	+	-	-	-	-	-	-
12	0.16	76.23	-	-	-	+	-	-	-	-
13	0.17	67.18	-	-	-	-	+	+	+	+
14	0.18	63.15	-	-	+	+	-	-	-	-
15	0.19	61.42	-	+	-	-	-	-	-	-
16	0.23	46.00	+	+	-	-	+	-	+	-
17	0.24	44.20	-	-	-	-	-	+	-	-
18	0.25	44.56	-	-	+	+	-	-	-	-
19	0.26	42.36	-	+	-	-	-	-	+	-
20	0.30	40.01	-	+	+	+	+	+	+	+
21	0.33	37.90	-	+	+	+	+	+	+	+
22	0.50	28.05	-	+	+	+	+	+	+	+
23	0.52	28.00	+	-	-	-	-	-	-	-
24	0.57	21.13	-	+	+	+	+	+	+	+
25	0.68	15.06	-	+	+	+	+	+	+	+
26	0.71	14.0	+	-	-	-	-	-	-	-
27	0.90	7.71	-	-	+	+	+	+	+	+
Total no. of bands			4	13	10	9	10	10	12	9

S2= *B. thuringiensis*, M3= *Serratia marcescens*, E3= *Serratia rubidaea*,
A2 = *Pseudomonas pyrrocinia*, M = Marker, P = Protecto, D = Dipel 2 x,
C = Control

Furthermore, band with mobilities of 0.16 and with MW of 67.23 KDa, appeared only in larvae treated with Protecto. Therefore, this band was considered as specific band for Protecto treated larvae.

A band with MW of 81.11 KDa, (Lan. 3) appeared only in larvae treated with Dipel 2x. Therefore, this band was considered as specific band for Dipel 2x treated larvae.

Isolate A2 showed one band with MW. of 95.81 KDa and appeared only in larvae infected with A2 isolate. Also, one band with MW of 44.20 KDa, appeared only in larvae infected with E3 isolate. Two bands with mobilities of 0.05 and 0.09 and with MW of 144.72 and 113.14 KDa, respectively, appeared only in larvae infected with M3 isolate. While one band with mobility of 0.13 and with MW of 83.67 KDa appeared only in S2 isolate infected larvae.

The synthesized proteins are electrolyte macromolecules, varying in molecular weight and charge. These molecules could be separated electrophoretically based on molecular weight by SDS-PAGE technique. Total proteins for uninfected and infected pink and spiny bollworm larvae were electrophoretically separated. The obtained patterns of electrophoretic bands patterns could be used for the differentiation between the uninfected and infected pink and spiny bollworm larvae either with *B. thuringiensis* var. *kurstaki* (Dipel 2x and Protecto) or entomopathogenic isolated bacteria.

The absence of the most *B. thuringiensis* proteins bands in *B. thuringiensis* infected larvae indicated that killing occurred via toxicity rather than bacterial growth. This result is in agreement with that reported by **Hodgman *et al* (1993).** On the other hand, the presence of most *Serratia* proteins bands in *Serratia* infected larvae indicated that the killing action was due to the growth of bacteria i.e. via lysis of larvae tissues, which is in agreement with results that had been obtained by **Villalobos *et al* (1997).**

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تعريف وتقدير فعالية البكتريا المعزولة من يرقات دودة اللوز القرنفلية والشوكية المصابة في الطبيعة

[49]

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تناولت هذه الدراسة تعريف بعد العزلات البكتيرية والتى تم عزلها من يرقات ديدان اللوز القرنفلية والشوكية في الطبيعة بهدف استخدامها كبديل حيوى لمكافحة ديدان اللوز، حيث تم عزل سبعة أنواع بكتيرية ثلاثة منها غير فعالة وهى:

Pseudomonas viridiflava, Serratia grimesii, Cellulomonas flavigena

وأربعة منها لها فاعلية نسبية علي اليرقات حديثة الفقس لكل من دودة اللوز القرنفلية والشوكية بالمقارنة بالمبيد الحيوى البكتيرى Dipel 2x, Protecto .

Pseudomonas pyrrocinia, Serratia marscesns, Serratia rubidaea, Bacillus thuringiensis

تم إجراء دراسة المحتوى البروتينى في اليرقات الغير معاملة واليرقات المعاملة بالعزلات البكتيرية المختلفة والمركب البكتيرى التجارى دايبل 2 أكس، بروتكتو عن طريق التفريد الكهربى وأظهرت الدراسة وجود اختلافات بينهما فى الأجزاء المختلفة للمركبات المكونة للبروتينات .

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