EVALUATION OF THE EFFICACY OF TWO BIOAGENTS AGAINST THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.), USING MOLECULAR CHARACTERIZATION TECHNIQUES

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Abstract

Molecular studies have been carried out on 6th instar larvae of *Spodoptera littoralis* which treated in 2nd larval instars with LC_{50} of two bioagents named Viruset and Protecto at $1x10^3$ PIBs/ml and $3.2x10^2$ IU/ml., respectively. Four random primers were used in this study to generate a fragmenting pattern as a tool to investigate the molecular differences between treated samples and control. The numbers of unique and common fragments generated by using these primers (OPO1, OPO2, OPO3 and OPO4) was recorded. It has been found that primer OPO4 was the most powerful one in generating a unique informative fragmenting pattern; it gives 4 specific unique fragments. While the primer OPO1 was the poorest one in generating an informative fragmenting pattern.

INTRODUCTION

The Egyptian cotton leafworm, *S. littoralis,* is an important pest in Egypt and other countries in Africa and Asia causes extensive economic losses in many cultivated crops (Frank *et al.*, 1990). The extensive use of chemical for controlling *S. littoralis* caused negative side effects on humans, other living organisms and environment (Chantelli-Forti *et al.,* 1993 and Chaudhuri *et al.,* 1999). Furthermore, this insect acquired resistance to various classes of insecticides (Denholm *et al.,* 1998). The problems and hazards that have arisen as a result of using conventional insecticides were incentives for the search of alternative control agents. Microbial control agents are a primary means of biological control for insect pests. The use of microbial control agents is targeted for a particular pest species. The entomopathogens that have been mostly used in biological control include representatives of bacteria, fungi, viruses, nematodes and protozoa (Dent, 2000). This work was designed to study the differences between treated and untreated larvae using the RAPD-PCR techniques. El Gohary *et al.* (2000) and Abdel-Wahed *et al* (2013).

MATERIALS AND METHODS

1-Rearing technique of the Egyptian cotton leafworm, *S. littoralis*:

The original insect culture was obtained from the Cotton Leafworm Division, at Plant Protection Research Institute. Newly hatched larvae were transferred to clean glass jars covered with muslin held in position with rubber fragments and incubated under laboratory condition at 27°±2°C, 60 ± 5% RH, and 8:16 LD photoperiod. They were fed on castor oil leaves and examined daily. After pupation, pupae were collected; sexed and emerged moths were placed in pairs in breeding glass jars. These jars were supplied with leaves of Tafla, *Nerium oleander* (L.) as an oviposition site.

2-The tested compounds:-

The potency of two bioagents was evaluated for their effect on *S. littoralis* larvae as following:

2.1. *Spodoptera littoralis* Nuclear Polyhydrosis Virus (SLNPV), with the trade name: Viruset ®.

2.2. *Bacillus thuringiensis* var. *kurstaki* with the trade name Protecto®.

These two microbial agents were obtained from Insect Pathogen Production Unit Plant Protection Research Institute.

3- Bioassay:-

The insecticidal activity of the two bioagents was assessed on newly ecdysed 2nd instars of *S. littoralis* larvae as following:

a) Viruset: A series of dilution were prepared from 1 gm of the product obtained as a wettable powder, $1x10, 1x10^2, 1x10^3, 1x10^4, 1x10^5, 1x10^6$ and $1x10^7$ PIBs/ml. (PIBs= Polyhedral Inclusion Bodies)

b) Protecto The following dilutions were prepared from 1 gm of the product also obtained as a wettable powder, 3.2, $3.2x10$, $3.2x10^2$, $3.2x10^3$, $3.2x10^4$ and $3.2\ x10^5$ IU/ml. (IU= International Unit).

Treatment of larvae was conducted by the leaf dipping technique using a fresh castor oil leaves *Ricinus communis* (L) which were cleaned and immersed for 10 seconds in one of the prepared concentrations or dilutions of each one tested compound. The treated leaves were left to dry at room temperature before being offered to newly ecdysed 2nd instar *S. littoralis* larvae. Larvae were offered treated leaves for 48 hr and subsequently larvae were fed on untreated castor oil leaves for the following duration of the larval stage. Each treatment comprised 20 larvae and was replicated three times. The same number of larvae was considered as a control while larvae were offered castor oil leaves dipped in water.

4- The Molecular study:-

The DNA was extracted according to the method of Sambrook *et al.* (1989). Before any analysis, it was important to determine the concentration and purity of isolated DNA; this was carried out by estimating UV absorbance at wave length of 260 and 280 nm using a spectrophotometer. DNA was subjected to PCR in order to generate the fragmenting profile. The random primers used were OPO1, OPO2, OPO3 and OPO4. Reactions were carried out in a thermocycler (Progeny 30, Techno, Cambridge Ltd. Dux ford Cambridge, UK). The PCR profile was as follows: 94 °C for 5 min, 94 °C for 1 min, 40 °C for 1min, 72 °C for 2 min, and final extension at 72 °C for 7 min. Then the PCR reaction was kept at 4 °C over night, till migration on agrose was occurred.

NO	Primer	Sequence
1	OPO1	5'- GGC ACG TAA G -3'
2	OPO2	5'- ACG TAG CGT C -3'
3	OPO3	5'- CTG TTG CTA C -3'
4	OPO4	5'- AAG TCC GCT C -3'

The gel was prepared with wells into which the DNA fragments are added and subemerged under an electrolyte buffer solution between a positive and a negative electrode. The DNA fragments are negatively charged so the wells containing them are placed closest to the negative electrode. When the current is turned on the DNA moves through the pores in the gel towards the positive electrode. PCR- DNA marker was used to determine the molecular weight of each fragment. The shorter fragments move faster because they are able to move through the pores of the gel more easily, whereas the longer DNA fragments move more slowly through the pores (Hurlbert, 1999).

5- Statistical analysis

1- Results were presented graphically as log/probit regression lines, and toxicity LC_{50} and LC_{90}values as well as the slope according to Finney, (1971) using "LdPLine®" software.

2- DNA sequences were analyzed using version 6 of the Gel-Pro Analyzer package of genetics computer program.

RESULTS AND DISCUSSION

1- Bioassay Test:

Data in Table (1) cleared that the efficiency of the two tested compounds, Viruset (*Spodoptera littoralis* Nuclear Polyhydrosis Virus, SLNPV) and Protecto (*Bacillus thuringiensis* var. *kurstaki*), were evaluated on 2nd instar larvae of *S. littoralis* (Boisd.). Viruset toxicity had an effect on 2nd instar larvae giving the LC_{90} and LC_{50} $1x10^7$ and $1x10^3$ PIBs/ml. Meanwhile the LC_{90} and LC_{50} of Protecto were $3.2x10^5$ and $3.2x10^2$ IU/ml., respectively. Our results was agreed with those reported by Abdel-Aziz, (2007) and Abdel- Wahed, *et al.* (2011).

Table 1. Susceptibility of *S. littoralis* 2nd instar larvae to Viruset and Protecto

Compound	Unit	LC_{90}	LC_{50}	Slope
Viruset	PIBs/ml	$1x10^7$	$1x10^3$	1.4599±0.2411
Protecto	IU/ml.	$3.2x10^5$	$3.2x10^2$	1.6531±0.2165

2- Molecular Studies:

This study has been carried out on 6th instar larvae of *S. littoralis* which treated in 2nd instar larvae with LC_{50} of Viruset and Protecto at $1x10^3$ PIBs/ml. and $3.2x10^2$ IU/ml., respectively.

Four random primers were used (OPO1, OPO2, OPO3 and OPO4) to generate the specific by which an informative conclusion could be summarized. The four primers used are shown in table (2) and fig. (1) along with their sequences.

Using primer OPO1, a distinguishing pattern was obtained when using a control, Viruset- treated, and Protecto-treated larvae as a source of DNA.

In this primer, the treatment with Viruset induced the generation of a fragment with size of 979 bp. This fragment was absent in both control and Protecto-treatment larvae, the same finding was found in Viruset and Protecto regarding the induction of a fragment with a size of 452 bp and also this fragment was absent in control. On other hand, the missing fragments were noticed in both Viruset and Protecto treatments while it was present in control with size of 251 bp.

In primer OPO2, the treatment with tested compounds resulted in the presence of two fragment with size of 350 and 274 bp in casse of Protecto treatment while, this fragment was absent in control and Viruset treatments that may clarify the action of Protecto. The same findings was shown in fragments with size of 612, 500 and 408 bp that present in both control and Protecto while it was absent in Viruset treatment. On other hand, the missing fragments were noticed in both Viruset and Protecto treatments while it was present only in control with size of 770 bp. On the

other hand, fragments with size 1694, 1261 and 1065 bp were present in both control and Viruset treatments but it was absent in Protecto treatment.

In primer OPO3, the treatment with Protecto resulted in the absence of a fragment which was present in both control and Viruset treatments; this absence may be attributed to the treatment with Protecto. However, for the same treatment a fragment with size 576 bp was noticed while it was absent in both control and Viruset treatments. One fragment with size 358 bp was absent in the treatment in both Viruset and Protecto treatments and a fragment was observed in the control. However, the treatment with Protecto led to the absence of a fragment and this fragment was present in both control and Viruset treatments with size of 959 bp.

In primer OPO4, three fragments were noticed due to the treatment with Viruset, while, the same molecular size fragments were present in both control and Protecto treatment with size (1461,1021,839,431 bp).

On the other hand, a specific unique fragment was obtained (at molecular size of 859 and 510 bp) due to the treatment with Viruset. While this fragment was absent in the control and Protecto treatments.

RAPD-PCR technique clarified the DNA diversity among the 6th instar larvae of *S. littoralis* which was treated with LC_{50} of Viruset and Protecto. 43 DNA fragments were detected using four random primers. 17 fragments were common in treated and untreated larvae of *S. littoralis*; they represent 39.5 % of all detected fragments. On the other hand the RAPD-PCR technique shows 14 polymorphic amplified fragments represented 32.5%. This ratio is due to treatment with Viruset and Protecto. Treated and untreated larvae showed 12 unique fragments that represented 27.9 % of all detected fragments (Table 3). Finally, this study confirmed that Protecto was more effective on DNA generated than Viruset.

The previous results showed that primers number (OPO4) was the powerful one in generating a unique informative fragmenting pattern; it gave four specific unique fragments. While the primer OPO1 was the poorest one in generating an informative fragmenting pattern, it gives two specific unique fragments. Our results was agreed with those reported by El Gohary *et al.* (2000) who reported that the DNA fragments varied in intensity and ranged in size from (140-1500 bp) and (196 -1060 bp), respectively. Abd EL- Aziz, (2006) reported in his study that both proteins and RAPD-PCR markers could be used to give estimations of genetic variation and differentiation of different treated and untreated *S. littoralis* larvae with the selected bacterial strains MVPII and the best primers that can be used for developing a genetic marker to differentiate between the different strains were OPB-3 and OPA-18. Abdel-Ghany (2011) generate a banding pattern as a tool to investigate the molecular

differences between different treatments botanical extracts castor oil, gossypol on *S. littoralis* larvae. The numbers of unique and common bands generated by using these primers (C1, C4, C10, C13, C15, O5, O7, O14, and O10) was recorded. It has been found that primers O10, C4 were the most powerful one in generating a unique informative banding pattern.

Molecular genetic fingerprinting was carried out using 5 random primers on 2nd instar larvae of *S. littoralis* which treated with *Bt* and IGR. The obtained data suggested that primer OPO2 was the most powerful primers in regarding generating specific unique band. While the primer OPO4 was the poorest one in generating an informative banding pattern. Abdel- Wahed *et al* (2013).

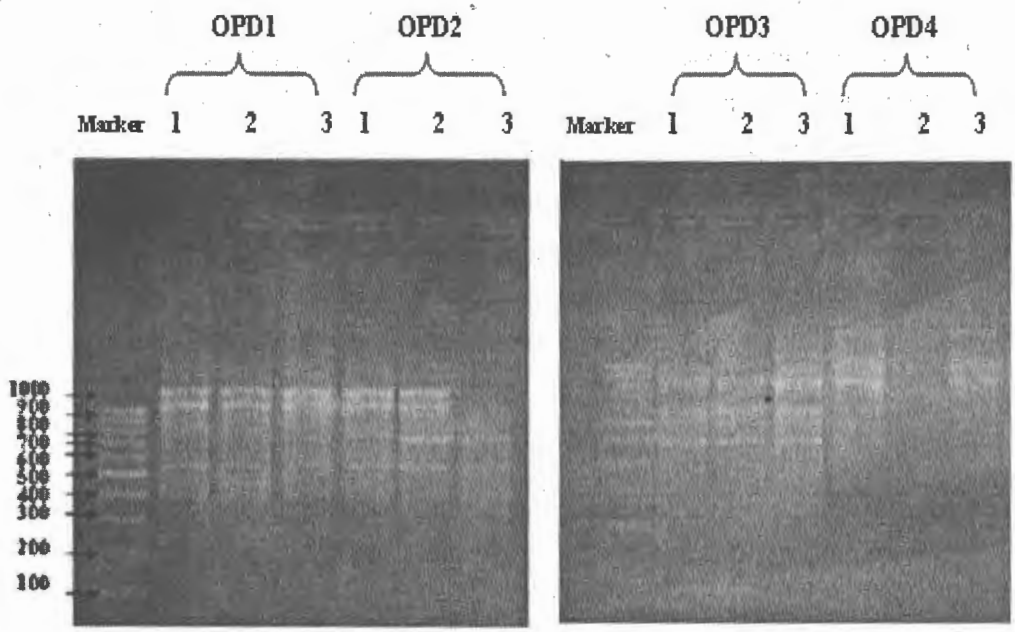

Fig. 1. Molecular fingerprinting using RAPD DNA for pattern for samples treated with Viruset, Protecto and control.

M=Marker 1=Control 2=Viruset 3=Protecto

Table 2. RAPD-PCR Products in 6th instar larvae of *Spodoptera littoralis* which treated in 2nd instar larvae with LC_{50} of Viruset and Protecto compared with control using random primers.

Lanes	Primer 1: OPO1									Primer 2 : OPO2									Marker	
	Control			Viruset			Protecto			Control			Viruset			Protecto				
Rows	M.w	Amount	Rf	M.w	amount	Rf	M.w	Amount	Rf	M.w	amount	Rf	M.w	Amount	Rf	M.w	amount	Rf	M.w	amount
r1										1694	8.687	0.514	1694	10.248	0.564					
r2	1288	11.774	0.561	1288	14.921	0.561	1288	12.851	0.561	1261	13.384	0.564	1261	16.824	0.593					
r3	1088	12.943	0.589	1088	12.732	0.589	1065	15.08	0.593	1065	18.846	0.593	1065	10.631	0.607				1000	8.936
r4				979	10.524	0.607													900	5.779
r5	785	14.526	0.645	785	15.311	0.645	849	13.082	0.631	770	12.638	0.648							800	6.888
r6	713	8.342	0.662				713	9.681	0.662	687	9.303	0.669	727	23.352	0.659	741	23.33	0.655	700	7.85
r7	612	8.303	0.69	624	11.632	0.686	600	6.956	0.693	612	6.707	0.69				600	9.897	0.693	600	9.834
r8	548	8.665	0.711	548	13.564	0.711	548	16.489	0.711	548	9.442	0.711	548	16.279	0.711	538	10.131	0.714		
r9										500	5.136	0.729				509	8.204	0.725	500	11.447
r10				452	10.309	0.746	452	7.844	0.746	452	6.348	0.746	434	22.832	0.754					
r11	408	14.58	0.764	400	11.021	0.768	400	9.839	0.768	408	9.875	0.764				400	21.822	0.768	400	11.639
r12	324	11.88	0.807				343	8.214	0.796							350	18.137	0.793		
r13																			300	13.479
r14	251	9.025	0.85													274	8.483	0.836		
r15																			200	11.713
r16																			100	12.437
r17																				
r18																				

Continued Table (2).....

Lanes	Primer 3 : OPO3									Primer 4 : OPO4									Marker	
	Control			Viruset			Protecto			Control			Viruset			Protecto				
Rows	M.w	Amount	Rf	M.w	amount	Rf	M.w	Amount	Rf	M.w	amount	Rf	M.w	amount	Rf	M.w	amount	Rf	M.w	amount
r1							1730	9.552	0.15											
r2	1624	7.471	0.169	1658	9.821	0.163	1557	8.699	0.181											
r3	1431	9.116	0.206	1461	11.435	0.2	1372	10.504	0.219	1461	19.192	0.138				1461	17.972	0.138		
r4	1235	7.751	0.25	1235	13.930	0.25	1209	10.803	0.256							1209	7.1509	0.194		
r5																				
r6	1021	10.47	0.306	1043	12.352	0.3	1021	20.17	0.306	1021	17.974	0.244				1021	19.612	0.244	1000	8.351
r7	959	8.57	0.325	959	9.379	0.325							859	22.713	0.294				900	4.89
r8										839	17.21	0.3				839	20.326	0.3	800	7.282
r9	716	15.751	0.406	716	15.510	0.406	716	13.1661	0.406	719	10.406	0.338	719	25.304	0.338	700	15.027	0.344	700	7.182
r10	641	8.651	0.438	641	10.834	0.438	655	8.023	0.431	613	16.138	0.381								
r11							576	6.232	0.469										600	8.299
r12	510	20.8	0.506	510	16.923	0.506	510	12.857	0.506				510	52.042	0.421					
r13																			500	9.861
r14										400	19.089	0.494				431	20.011	0.475	400	13.214
r15	358	11.446	0.606																	
r16																			300	15.3
r17																			200	11.508
r18																			100	14.138

Table 3. DNA diversity among *S. littoralis* treated with Viruset and Protecto using RAPD-PCR.

Primers	Polymorphism				Genetic markers (bp)*		
	TAF	MAF	PAF	Unique	Control	Treated with Viruset	Treated with Protecto
OPO1	11	6	3	2	251	979	-
OPO2	12	3	6	3	770	-	350 - 274
OPO3	11	7	1	3	358	-	1730 – 576
OPO4	9	1	4	4	613	859-510	1209
Total	43	17	14	12	5	3	5

bp---------- size of genetic marker (unique).

TAF---------- total amplified fragments.

MAF---------- monomorphic amplified fragments (common).

PAF---------- polymorphic amplified fragments.

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تقييم فاعلية مركبين حيويين ضد دودة ورق القطن باستخدام تقنية التوصيف الجزيئي

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معهد بحوث وقاية النباتات – مركز البحوث الزراعية – دقي – جيزة – مصر.

اجريت هذه الدراسة علي يرقات العمرالسادس لدوده ورق القطن التي عوملت في العمر الثاني بالتركيز النصف مميت لكلا من مركبي الفيروست والبروتكتو والتي كانت ١×١٠^{٣} PIBs/ml و ٣.٢×١٠^{٢} IU/ml على التوالي. تم استخدام اربع بادئات عشوائية (OPO1, OPO2, OPO3, OPO4) لإنتاج نموذج حزمى مميز كآدة لدراسة التباينات الجزيئية بين مختلف المعاملات. وتم حصر وعد الحزم المميزة والحزم المشتركة التى تم انتاجها بواسطة استخدام هذه البادئات الاربع، وقد وجد أن البادئ OPO4 هوأقوى البادئات فى انتاج حزم مميزة معبرة، حيث أعطى ٤ حزم مميزة متفردة بينما كان البادىء OPO1 أقل البادئات المستخدمة قدرة على انتاج حزم معبرة .