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Toxicological and molecular Studies of newly compounds extracted from wastes against cotton leaf worm *Spodoptera littoralis.*

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ABSTRACT

An experiment was conducted under laboratory condition to evaluate the insecticidal activity of three newly compounds extracted from wastes from natural origin, Cyano acetyl urea (CAU), Benzimidazolyl acetyl urea (BAU) from urea and Cyano acetyl hydrolyzate (CAH) from rice straw, on cotton leaf worm (*Spodoptera littoralis*) through exposure of fourth instar larvae to castor bean leaves which immersed in different concentrations from tested compounds. In addition, the morphogenic abnormalities were recorded.

The other objective of this study is to evaluate the potential of the random amplified polymorphic DNA (RAPD) assay for the detection of genetic polymorphism between control and treated *S. littoralis* larvae, which have been exposed to the tested compounds at both LC_{25} and LC_{70}. Five primers namely: OP-01, OP-02, OP-03, OP-04, and OP-05 were used in this study.

The present findings clear that, LC_{25} estimates of fourth instar larvae ranged from 0.092 to 0.154 % of (CAU), 0.191 to 0.225 % of (BAU) and 0.009 to 0.021 % of (CAH) while, LC_{70} estimates of fourth instar larvae ranged from 1.289 to 2.009 % of (CAU), 1.445 to 2.159 % of (BAU) and 0.339 to 0.627 % of (CAH).

In addition, five primers OP-01, OP-02, OP-03, OP-04, and OP-05 generated a maximum of 26, 24, 20, 13 and 23 bands, respectively. RAPD profiles generated by these primers revealed differences between control and treated samples with visible changes in number and size of amplified DNA fragments. Polymorphism ranged from 44.4 to 100% as screened by the five primers among all samples. Taking all data together, higher polymorphism was recorded at LC_{70} (77.8, 84.4, and 86.4%) comparing corresponding values at LC_{25} (69.6, 80.3, and 79.4%) for larvae treated with CAU, BAU and CAH, respectively. Based on $LC_{70,}$ the highest polymorphism (86.4%) was observed in those treated with CAH comparing those either treated with CAU (77.8%) or with BAU (84.4%). Definitely, RAPD data confirmed the susceptibility test as well as the morphological study, and suggest that DNA damage and the possible occurred mutations may appear to be the main factor influencing the evident polymorphism between control and treated larvae.

Key words: - *Spodoptera littoralis*- urea derivatives - rice straw- insecticidal activity - RAPD (PCR).

INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* (Boisd.) is considered the most serious pest for Egyptian cotton plant. Its larvae feed not only on cotton leaves, but also they attack other economically important crops. Control program of the insect in Egypt mostly depends on the use of various conventional insecticides which has a

residual effect and increase the pest resistance. In order to avoid these hazards, there is a great need to develop alternative safe control agents with new modes of action.

Waste products from natural origin considered environmentally safe, less hazardous to non target biota, simple inexpensive and can be applied effectively by using techniques more suitable for developing countries (El-Maghraby *et al*., 2012).

Sublethal insecticide exposure can lead to physiological and behavioral changes in the organism (Hyne and Maher 2003). These responses can be measured using specific biomarkers that provide a measure of sublethal effects (McCarthy and Shugart 1990).

The Polymerase Chain Reaction (PCR) initially described by Saiki *et al.,* (1985) revolutionized the molecular biology and is still widely used for a variety of purposes. Classical PCR assay simply requires a target DNA sequence and two synthetic oligonucleotide primers complementary to opposite strands of the target DNA. Indeed, mutations may affect the annealing of the primers whereas DNA damage may interfere with the DNA polymerase activity, thus altering the number of newly synthesized amplicons. Hence, despite the advantages of using traditional PCR assays for detecting DNA alterations, there are a number of potential difficulties. First, the nucleotide sequence flanking upstream and downstream the target DNA needs to be determined (Newton and Graham, 1997). Second, the size of the PCR products play a crucial role in the detection of DNA damage because the amplification of short fragments (e.g. less than 300 bp) may be slightly reduced and possibly not inhibited at all even in case of extensive DNA damage. Third, it is conceivable that any reduction in the intensity of PCR reaction caused by factors other than DNA damage such as residual phenol after DNA extraction (Newton and Graham, 1997). Alternatively, if an amplicon entirely disappears, it could be argued that the PCR did not work at all, irrespective of the presence of DNA damage (Atienzar *et al.*, 2002b).

Such disadvantages can be eliminated by using the random amplified polymorphic DNA (RAPD-PCR) (Williams *et al.,* 1990). Generally, RAPD reactions are performed with a single 10 bp primer and multiple amplifiable fragments (from different loci) are usually present for each set of primers across each entire genome (Lynch and Milligan, 1994). The reactions generate a number of amplicons of variable lengths (e.g. between 100 and 4000 bp) (Atienzar *et al*., 2002b) and the number of loci that can be examined is essentially unlimited (Lynch and Milligan, 1994). RAPD-PCR is a versatile and inexpensive tool. Its main advantages lie in its rapidity and applicability to any organism (since no information on the nucleotide sequence, cell cycle, or chromosome complement is required) (Lushai *et al.,* 2000; Atienzar *et al*., 2001). Furhtermore, Allozyme patterns may represent an environmentally-induced phenotype expression of the genome. In contrast, RAPD and related fingerprinting methods infer the genetic variability directly at the genome level; provide a less-biased genomic sample (Fritsch and Riesberg, 1996).

A variety of bacteria, fungi, and insect species were successfully DNA typed, and closely related species were distinguished by the RAPD-PCR assay (Wilkerson *et al.,* 1995; Benecke., 1998; Lushai *et al.,* 2000; Amer *et al.,* 2008; Hamouda, 2008; Karam *et al.,* 2008; Kwon *et al.,* 2009; Ibrahim *et al.,* 2010; Mohamed *et al.,* 2010). In addition, RAPD assay proved to be a sensitive method to detect genotoxin-induced DNA damage and mutations (Atienzar *et al.,* 2001; Atienzar *et al.,* 2002 a&b; DeWolf *et al*., 2004).

The objectives of this study are to estimate the sublethal concentration of some waste extracts from natural origin: Cyano acetyl urea(CAU), Benzimidazolyl acetyl urea (BAU) and Cyano acetyl hydrolyzate (CAH), to record the morphogenetic

changes and to evaluate the potential of the RAPD-PCR assay for the detection of genetic polymorphism between control and treated *S. littoralis* larvae which have been exposed to tested compounds at both LC_{25} and LC_{70} .

MATERIALS AND METHODS

Maintenance of culture

Spodoptera littoralis larvae obtained from the laboratory culture of plant protection Research Institute, Agricultural Research Center (Cairo, Egypt). This strain was reared under constant laboratory conditions of 27±2°C and 65±5 % RH (EL-Defrawy *et al*., 1964).

Experimental compounds

Three newly compounds [Cyano acetyl urea (CAU), Benzimidazolyl acetyl urea (BAU) and Cyano acetyl hydrolyzate (CAH)] extracted from waste products, from natural origin, that prepared in National Research Center, El-Bohouth Street, Dokki, Egypt.

Insecticidal activity:

To assess the insecticidal activity of the tested compounds, different concentrations were prepared ranged from 2 to 0.025%. The larvae used in the experiments were freshly molted 4th instar larvae within 6 hrs after ecdysis. The leaf dipping technique was adopted, where freshly castor oil beans leaves were dipped for 30 second in one of the prepared concentrations. The treated leaves were left to dry for approximately 5 minutes at room temperature before being offer to *S. littoralis* larvae. Three replicates contained 20 larvae / jar were used for each treatment and also for the control experiment. The mortality percentages of treated larvae were corrected against those of the control by using Abbott's formula**,** (Abbott, 1925). Probit analysis was determined to calculate LC_{25s},LC_{50s}, LC70s and slope values of the tested compounds (Finney, 1971), through software computer program.

Extraction and RAPD-PCR analysis:

DNA was extracted from 6th larval instar (which had been treated with LC_{25} and LC_{70} of (CAU), (BAU) and (CAH) as 4th larval instar) according to the method of Sambrook *et al*. (1989) The extracted DNA was then used as an amplification template for RAPD-PCR analysis, using 5 primers (OP-01, OP-02, OP-03, OP-04, and OP-05). RAPD-PCR amplifications were performed in a total volume of 25 µl containing 10mM Tris-HC1 pH 8.3, 50 mM KC1, 1,5mM MgC12, 100 µM dNTP, 10 pM primer, 1.5 U Taq polymerase and 25 ng genomic DNA.

Amplification was performed using DNA thermal cycler (Progeny 30, Techno, Cambridge Ltd. Dux ford Cambridge, UK). The thermal conditions were as follows: 94 ºC for 5 min (initial denaturation), 40 cycles: 1 min denaturation at 94 ºC, 1min annealing at 40 ºC, 2 min extension at 72 ºC, and 7 min for final extension at 72 ºC Then the PCR reaction was kept at 4 ºC. The PCR products were subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Table (1): The code and sequences of five RAPD primers.

N0	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'
5	OPO5	5'- CCC AGT CAC T -3'

RESULTS

Insecticidal activity:

Insecticidal activity of three newly compounds extracted from waste products, Cyano acetyl urea (CAU), Benzimidazolyl acetyl urea (BAU) from urea and Cyano acetyl hydrolyzate (CAH) from rice straw against 4th larval instars of *Spodoptera littoralis* was recorded as shown in Table (2). Data revealed that CAH was the most effective compound followed by CAU and finally BAU. The LC_{25} values of CAH, CAU and BAU were 0.015, 0.123 and 0.191 % respectively, while The LC_{50} values of CAH, CAU and BAU were 0.101, 0.515 and 0.660 %. On the other hand, The LC_{70} values of CAH, CAU and BAU were 0.442, 1.569 and 1.730 %.

Table (2): Insecticidal activity of 4th larval instars of *Spodoptera littoralis* toward tested compounds.

Compound	Conc.		Lower limit	Upper limit	Slope
cyano acetyl urea	LC_{25}	0.123	0.092	0.154	1.083 +/- 0.081
	LC_{50}	0.515	0.447	0.594	
	LC_{70}	1.569	1.289	2.009	
benzimidazolyl acetyl urea	LC_{25}	0.191	0.156	0.225	1.252 +/- 0.083
	LC_{50}	0.660	0.583	0.752	
	LC_{70}	1.730	1.445	2.159	
Cyano acetyl hydrosylate	LC_{25}	0.015	0.009	0.021	0.816 +/- 0.072
	LC_{50}	0.101	0.083	0.121	
	LC_{70}	0.442	0.339	0.627	

Morphogenic abnormalities:

Treatment of 4th larval instar of *S. littorais* with CAU, BAU and CAH resulted in different morphogenic effects.

CAU treatment, induced 6th instar larva surrounded by old exuvium of 5th instar larva in the posterior part of the larva Figure (1.1). Beside, Larval pupal inter-mediate with larval head but the thorax has pupal structure. In addition shrinked pupa failed in shedding off the larval exuvium Figure (1.2). Pupal-adult intermediate was showed as head and thorax enclosed by old cuticle of pupa. Figure (1.3) revealed moth with poorly developed wings and distended abdomen.

In the case of BAU, it caused intermediate instar between 4th and 5th larval instars Figure (1.4) and pupal cuticle remained adhered to the moth's body at different sites Figure (1.5). The wing failed to spread Figure (1.6).

Also, CAH showed 6th instar larva surrounded by old exuvium of 5th instar larva in the middle parts of the larva Figure (1.7), shrinkage of the pupae Figure (1.8) and Pupal-adult intermediate, head and thorax enclosed by old cuticle of pupa Figure (1.9). In addition, Figure (1.10) showed moth with poorly developed wings.

In all cases the rate of malformations occurrence was directly proportional to the concentration used, the sequences of events were identical.

Molecular studies:

RAPD profiles generated by the different 5 primers revealed differences between control and treated *S. littoralis* 6th instar larvae, with visible changes in the number and size of amplified DNA fragments as the following: Primer OP-01 produced 26 scorable polymorphic bands (Fig. 2.1, Table 3). The control sample was identified by the presence of 3 unique bands (236, 306, and 433 bp). Samples treated with Cyano acetyl urea (CAU) were recognized by the 2 unique bands (774 and 1065 bp) for LC_{25} and any of unique bands for LC_{70}. On the other hand, one unique band (1694 bp) distinguished samples treated with benzimidazolyl acetyl urea (BAU) for LC_{25}. For samples treated with Cyano acetyl hydrolyzate (CAH) one unique band (959 bp) was detected for LC_{25} and 2 unique ones (266 and 318 bp) were detected for

LC_{70}. Absolutely, no bands were obtained by RAPD profiles for samples treated with BAU (for LC_{70}) using this primer.

Table (3): RAPD-PCR profile for *Spodoptera littoralis* 6th instar larvae treated by newly-extracted compounds from waste and amplified by primer OP-01

Rows	Rf	Control		Cyano acetyl urea				Benzimidazolylacetyl				Cyanoacetyl hydrosylate			
				LC_{25}		LC_{70}		LC_{25}		LC_{70}*		LC_{25}		LC_{70}	
		Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W
r_1	0.35							10.42	1694	-	-				
r_2	0.40	10.89	1261	13.97	1261					-	-				
r_3	0.41					15.49	1235	13.89	1235	-	-	14.52	1235		
r_4	0.44			13.20	1065					-	-				
r_5	0.45	16.69	1043			16.61	1043	17.42	1043	-	-	13.62	1043		
r_6	0.46									-	-	7.79	959		
r_7	0.47			12.02	919	10.29	919			-	-				
r_8	0.51	10.82	800			11.09	800			-	-				
r_9	0.52			17.56	774					-	-				
r_{10}	0.53							15.68	748	-	-			23.10	748
r_{11}	0.54	8.06	724							-	-	23.96	724		
r_{12}	0.55					8.21	700	7.54	700	-	-				
r_{13}	0.57			8.62	624			6.32	624	-	-				
r_{14}	0.58	6.82	612			6.86	612			-	-			9.79	612
r_{15}	0.60					11.00	558	7.71	558	-	-				
r_{16}	0.61	8.26	548	14.57	548					-	-	22.04	548	11.62	548
r_{17}	0.63	5.59	500							-	-			6.47	500
r_{18}	0.64					11.74	490	3.61	480	-	-				
r_{19}	0.65			10.23	461			8.39	452	-	-				
r_{20}	0.66	11.01	434							-	-				
r_{21}	0.67					8.68	408			-	-	18.05	408		
r_{22}	0.68			9.81	400			8.99	392	-	-			18.29	400
r_{23}	0.74									-	-			16.66	318
r_{24}	0.75	12.62	306							-	-				
r_{25}	0.77									-	-			14.04	266
r_{26}	0.80	9.21	236							-	-				

* No bands revealed upon RAPD-PCR

Figure 2.2 and Table (4) illustrate the RAPD profile generated by the primer OP-02. A sum of 24 polymorphic bands was generated by this primer from which, 15 were shared ones. The control sample was identified by the presence of two unique bands at about 879 and 648 bp. Samples treated with CAU were recognized by the presence of the three unique bands; at about 1882 bp (for LC_{25}) ; at 1288 and 1111 bp (for LC_{70}). On the other hand, three unique bands were distinguished samples treated with BAU; two bands at 1135 and 363 bp (for LC_{25}) and one at 560 bp (for LC_{70}). Only, one unique band at about 283 bp distinguished samples treated with CAH (for LC_{70}).

The primer OP-03 generated a total of 20 polymorphic bands (Fig. 2.3, Table 5) and two unique bands at about 983 and 322 bp were detected in the control sample. Two other unique ones were scored for those treated with CAU at about 1301 bp (for LC_{25}) and 1817 bp (for LC_{70}). No unique band characterized samples treated with BAU. Samples treated with CAH were recognized by the presence of the three unique bands at about 1635, 900, 770 bp (for LC_{25}) and one at 800 bp (for LC_{70}).

Table (4): RAPD-PCR profile for *Spodoptera littoralis* 6th instar larvae treated by newly-extracted compounds from waste and amplified by primer OP-02

Rows	Rf	Control		Cyano acetyl urea				Benzimidazolyl acetyl urea				Cyano acetyl hydrosylate			
				LC_{25}		LC_{70}		LC_{25}		LC_{70}		LC_{25}		LC_{70}	
		Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W
r_1	0.17	13.38	1922									9.31	1922		
r_2	0.18			14.90	1882										
r_3	0.19					18.36	1842	118.36	1842						
r_4	0.23	12.71	1590									7.66	1590		
r_5	0.24			8.96	1557			10.61	1557						
r_6	0.25					10.52	1288								
r_7	0.32	23.84	1159	26.19	1159					29.86	1184	22.44	1159	16.78	1184
r_8	0.33							20.45	1135						
r_9	0.34					22.20	1111								
r_{10}	0.38									22.50	939	17.49	939		
r_{11}	0.39							13.75	919					17.71	919
r_{12}	0.40			15.28	900	12.47	900								
r_{13}	0.41	15.29	879												
r_{14}	0.47									28.06	700	20.97	682	21.86	682
r_{15}	0.48			17.86	665	16.39	665	14.96	665						
r_{16}	0.49	17.93	648												
r_{17}	0.52									19.57	560				
r_{18}	0.54											14.54	535	23.36	523
r_{19}	0.55					10.99	512	13.80	512						
r_{20}	0.56	16.85	500	16.78	500										
r_{21}	0.57											7.56	464	9.85	464
r_{22}	0.59					9.05	431	7.60	442						
r_{23}	0.64							9.27	363						
r_{24}	0.70													10.41	283

Table (5): RAPD-PCR profile for *Spodoptera littoralis* 6th instar larvae treated by newly-extracted compounds from waste and amplified by primer OP-03

Rows	Rf	Control		Cyano acetyl urea				Benzimidazolyl acetyl urea				Cyano acetyl hydrosylate			
				LC_{25}		LC_{70}		LC_{25}		LC_{70}		LC_{25}		LC_{70}	
		Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W
r_1	0.2					10.00	1817								
r_2	0.2											9.75	1635		
r_3	0.2	7.99	1579	11.75	1579	9.64	1579	8.59	1607						
r_4	0.3	14.64	1348			19.16	1348	18.47	1372			14.45	1372		
r_5	0.3			22.67	1301										
r_6	0.3									22.50	1000	10.63	1018		
r_7	0.3	8.51	983												
r_8	0.4							13.50	949					25.62	932
r_9	0.4											14.92	900		
r_{10}	0.4	13.22	849	13.89	849	15.75	865	8.61	865						
r_{11}	0.4													16.61	800
r_{12}	0.4											11.51	770		
r_{13}	0.4					9.82	741	11.54	741						
r_{14}	0.4	10.44	727	13.65	713					39.14	727				
r_{15}	0.5											19.73	632	28.90	643
r_{16}	0.5					20.14	610	22.31	621						
r_{17}	0.5	19.72	600	22.82	600										
r_{18}	0.6											19.02	447	28.86	456
r_{19}	0.6	16.13	415	15.20	423	15.47	415	16.96	415	38.36	423				
r_{20}	0.7	9.31	322												

Primer OP-04 generated a total of 13 polymorphic bands. The lowest number (3) of unique bands was recorded by this primer (Fig. 2.4, Table 6). The first band was recorded at about 453 bp in the control sample (lane 1) while the second one was identified in the samples treated with BAU at about 400 bp. The third band (at 346bp) distinguished samples treated with CAH. No unique band was detected in samples treated with CAU.

Table (6): RAPD-PCR profile for *Spodoptera littoralis* 6th instar larvae treated by newly-extracted compounds from waste and amplified by primer OP-04

Rows	Rf	Control		Cyano acetyl urea				Benzimidazolyl acetyl urea				Cyano acetyl hydrosylate			
				LC_{25}		LC_{70}		LC_{25}		LC_{70}		LC_{25}		LC_{70}	
		Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W
r_1	0.13	16.04	1492	19.68	1492										
r_2	0.14					21.36	1461	19.19	1461			17.97	1461		
r_3	0.19	7.95	1209									7.05	1209		
r_4	0.24	17.77	1043	25.69	1043	21.51	1043	17.97	1021			19.61	1021		
r_5	0.29	16.91	859	18.19	859					41.43	859			23.54	859
r_6	0.30					21.94	839	17.21	839			20.33	839		
r_7	0.33			12.61	738									23.53	738
r_8	0.34	8.51	719			17.88	700	10.39	719	58.57	719	15.03	700		
r_9	0.38	9.83	613					16.14	613						
r_{10}	0.46	22.97	453												
r_{11}	0.47			23.83	442	17.30	431					20.01	431	37.35	442
r_{12}	0.49							19.08	400						
r_{13}	0.53													15.56	346

Primer OP-05 generated 23 polymorphic (Fig. 2.5, Table 7). One unique band was detected for the control (at 366 bp). Samples treated with CAU were distinguished by one unique band (for LC_{25}) at 1430.8 bp and 3 unique bands for LC_{70} (at 1804, 1590, and 1343 bp). On the other hand, 1 unique band (1766 bp) was determined for samples treated with BAU (for LC_{25}). No unique bands were determined for the same compound at $LC_{70.}$ As to individuals treated with CAH, 2 unique bands distinguished treated samples at LC_{25} (428, 719 bp) while 3 unique ones were detected for LC_{70} (at 400, 542, 1088 bp).

From the above results, The assay disclosed that a maximum of 26, 24, 20, 13 and 23 DNA bands were generated by the primers OP-01, OP-02, OP-03, OP-4 and OP-05, respectively. The size of the amplified fragments ranged from about 1922 bp (OP-02) to 236 bp (OP-01) across the profiles generated by the five primers. The polymorphic ratios between control and treated larvae were also calculated (Table 8). RAPD data confirmed the insecticidal activity as well as the morphological study.

Table (7): RAPD-PCR profile for *Spodoptera littoralis* 6th instar larvae treated by newly-extracted compounds from waste and amplified by primer OP-05

Rows	Rf	Control		Cyano acetyl urea				Benzimidazolyl acetyl urea				Cyano acetyl hydrosylate			
				LC_{25}		LC_{70}		LC_{25}		LC_{70}		LC_{25}		LC_{70}	
		Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W	Amou.	M.W
r_1	0.29					9.55	1804								
r_2	0.30							7.18	1766						
r_3	0.32	6.91	1633	12.25	1633										
r_4	0.33					8.69	1589.8	8.95	1557			10.86	1590		
r_5	0.35			11.09	1431										
r_6	0.36	8.43	1401					9.29	1401			9.96	1401		
r_7	0.37					10.50	1343.1								
r_8	0.39			13.03	1209							9.23	1209		
r_9	0.40	9.65	1184			10.79	1158.9	14.91	1183						
r_{10}	0.42													15.81	1088
r_{11}	0.43			10.43	1021							10.07	1021		
r_{12}	0.44	9.68	1000			20.17	1000			36.42	1000				
r_{13}	0.45	10.91	938	8.73	959			21.39	939			6.77	959	12.13	939
r_{14}	0.51											14.31	719		
r_{15}	0.52	14.57	700	14.39	700	13.16	700			33.49	687			23.27	700
r_{16}	0.54	8.00	648	10.51	636	8.02	635.7	15.37	648			8.32	636		
r_{17}	0.56					6.23	587.9	8.66	588			9.87	588		
r_{18}	0.58													19.75	54
r_{19}	0.59									30.08	521	9.61	521		
r_{20}	0.60	20.74	510	19.55	510	12.85	510.2	14.21	510					10.68	500
r_{21}	0.64											10.96	428		
r_{22}	0.65													18.58	400
r_{23}	0.67	12.08	366												

Indeed, polymorphism ranged from 44.4 to 100% (Table 8) among all treated samples. Taking all data together, higher polymorphism was recorded at LC_{70} (77.8, 84.4, and 86.4%) comparing corresponding values at LC_{25} (69.6, 80.3, and 79.4) for larvae treated with CAU, BAU and CAH, respectively.

Based on $LC_{70,}$ the highest polymorphism (86.4%) was observed in those treated with CAH comparing those either treated with CAU (77.8%) or with BAU (84.4%). Thus, these results had confirmed the susceptibility test as well as the morphological study; a considerable percentage of larvae treated with CAH completely failed to transform to normal pupae, and moreover, the most malformed structures and morphological abnormalities were produced after its application.

Hundred percent polymorphism (100%) was noted for 4 samples. Out of them, 2 samples were amplified by primer OP-02: in individuals treated with CAU (for LC_{70}) and also for those treated with BAU (for LC_{25}). The third sample was amplified by primer OP-03 in larvae treated with CAH (for LC_{70}). The forth sample was treated with BAU and revealed no bands after screening by primer OP-01.

Table (8): Genotype polymorphism (% polymorphic)* among *Spodoptera littoralis* 6th instar larvae treated with newly-extracted compounds from waste and assayed by RAPD-PCR.

Primer	Cyano acetyl urea						Benzimidazolyl acetyl urea						Cyano acetyl hydrosylate					
	LC_{25}			LC_{70}			LC_{25}			LC_{70}			LC_{25}			LC_{70}		
	G.B	P.B	P.F	G.B	P.B	P.F	G.B	P.B	P.F	G.B	P.B	P.F	GB	P.B	P.F	G.B	P.B	P.F
OP-01	16	14	87.5	16	13	81.3	19	18	94.7	10	10	100	13	10	76.9	14	11	78.5
OP-02	10	8	80	13	13	100	14	14	100	9	8	88.9	10	7	70	11	10	90.9
OP-03	9	4	44.4	11	7	63.6	11	7	63.6	9	7	77.8	14	13	92.8	12	12	100
OP-04	9	6	66.6	10	8	80	10	7	70	7	5	71.4	10	7	70	10	9	90
OP-05	12	7	58.3	13	8	61.5	12	7	58.3	10	8	80	16	13	81.3	12	9	75
Total	**56**	**39**	**69.6**	**63**	**49**	**77.8**	**66**	**53**	**80.3**	**45**	**38**	**84.4**	**63**	**50**	**79.4**	**59**	**51**	**86.4**

*Polymorphic ratio = no. polymorphic amplified fragments/total no. amplified fragments x 100
G.B = Generated bands P.B = Polymorphic bands P.F = Polymorphic frequency%

DISCUSSION

The principal criterion in this study is conversion of wastes to economic products as insecticides which uses on large scale in controlling pests in the entire world. Three newly compounds extracted from wastes from natural origin [Cyano acetyl urea (CAU), Benzimidazolyl acetyl urea (BAU) from urea and Cyano acetyl hydrolyzate (CAH) from rice straw] were evaluated as insecticide against 4th larval instars of *Spodoptera littorallis.* The tested compounds revealed differences in LC_{25}, $LC_{50,}$ LC_{70} values and the slope functions of the regression lines. In addition, remarkable variations in the potency of tested compounds which possess parallel regression lines of nearly equal slope values. This may suggest that these compounds have the same mode of action against the tested larvae. These results are in agreement with the findings of Mesbah *et al.,* (1996) who found that chlorfluazuron (urea derivatives) causes toxic effect, decreasing the pupation and adult emergence of *S. littoralis*. EL- Nemaky (2004) found that benzoylphenyl ureas were effective against one day old larvae of the sping bollworms. Similary, El-Maghraby *et al.,* (2012) observed the toxicity of rice straw derivatives on mosquito larvae.

In the present study, the application of either urea derivatives (CAU & BAU) or rice straw waste extract (CAH) induced molting disturbances, e.g. hanging larval exuvium, larval – pupal intermediates and pupal- adult intermediates. All morphogenetic effects indicate that these compounds may act as an insect growth regulator where (wright 1970) indicated that a pupal-adult intermediate is a true juvenile hormone effect. **(**Emam & Degheele, 1993) and (Hassanein & Zidan, 1996) tested benzoylphenyl ureas on fourth-instar larvae of *S. littoralis* and of *Plodia interpunctella* respectively,with sublethal doses. Ecdysis to the next instar was disrupted, with an extra moult being induced in some cases. Treatment resulted in malformations in pupae and adults. In addition Baker *et al.,* (2006) showed sever malformations when 4th larval instar of *S. littoralis* treated with rice bran extract.

The RAPD technique was very sensitive to detect genetic differences between the control and the treated *S. littoralis* 6th instar larvae. This conclusion was noted also by other authors who tested different toxicants (Atienzar *et al.,* 2001; 2002 a&b; Abd-Allah *et al.,* 2003; Atienzar and Jha, 2004; Barreto *et al.,* 2005).

The present data suggest that DNA damage, and the possible occurred mutations may appear to be the main factor influencing RAPD patterns; the different polymorphism between control and treated larvae. Definitely, RAPD data confirmed the results about insecticidal activity as well as the morphological abnormalities.

Indeed, resulting DNA profiles, gain and loss of bands among individuals depending on 1- presence/absence of priming sites 2-priming complementary completeness/incompleteness or 3- the distance between priming sites (Fritch and Riesberg, 1996). Hence, some important DNA effects must have occurred due to CAU, BAU and CAH treatments. These DNA effects (DNA adducts, DNA breaks, point mutations, genomic rearrangements, insertions and deletions) affect the presence/absence of primer sites, their complementarities to primers and /or the distance between priming sites. RAPD bands of different molecular weights are interpreted as separate loci which are scored on a present (amplification) or absent (non-amplification) basis ((Fritch and Riesberg, 1996). Thus, the new PCR products could be amplified because some sites become accessible to the primers after structural changes or because some mutations have occurred in the genome (Atienzar et al., 2001). Even a single point mutation within the primer site can generate significant changes in RAPD patterns (Williams *et al.,* 1990). It is also well known that DNA repair, and replication of damaged DNA can lead to point mutations (Livneh et al., 1993). Disappearance of amplified products could be a result of extensive DNA damage (Atienzar *et al.,* 2000), since DNA lesions are expected to have detrimental effects on RAPD profiles, they not only can induced structural change, but also can reduce the polymerization of DNA and / or block the *Taq* DNA polymerase (Nelson *et al.,* 1996). These reasons may explain that, in this study, absolutely no bands were obtained at in RAPD profiles at LC_{70} for larvae treated by BAU using primer OP-01. Again, DNA damage and mutations are the main factors that influenced RAPD patterns and gain or loss of bands (Atienzar *et al.,* 2000; 2002 a&b).

RAPD data revealed that the highest polymorphism was observed in larvae treated with Cyano acetyl hydrolyzate and screened by the five mentioned primers, which in turn confirms the susceptibility test. Furthermore, a considerable percentage of larvae treated with this compound completely failed to transform to normal pupae. Moreover, the most malformed structures and morphological abnormalities were produced after its application.

Several Cyano-derivatives used as pesticides, known to induce DNA damage (Villarini *et al.,* 1998; Undeger and Basaran, 2005; Patel *et al.,* 2006). This may reflect the observed polymorphism between control and larvae treated with either CAU or CAH. On the other hand, CAU and BAU are urea derivatives. Many urea-derived insecticides are in wide use also in controlling insect pests. Whatever the mode of action, the inhibition of chitin synthesis (Hajjar and Cassida, 1979; Hassal, 1990) is considered as the obvious ultimate effect of urea-derived compounds (Abd-Allah *et al,* 2003). Urea derivatives have the property of DNA adduct formation (Lutz, 1979; Miller and Miller, 1983). In the present study, changes in genomic DNA may lead to the inhibition of chitin synthesis, and thus the observed malformations and mortality, which was also reported by Abd-Allah *et al.,* (2003). Merzendorfer *et al.,* (2012) examined the effect of diflubenzuron (urea-derivative) on *Tribolium castenatum*. They revealed that unexpectedly, genes encoding enzymes involved in chitin metabolism were unaffected, but many genes encoding cuticle proteins were affected. However, RAPD data must be considered as preliminary, semi-quantitative, and give an overview of DNA effects (Atienzar *et al.,* 2001; De Wolf *et al.,* 2004). Hence, a more specific method may be needed in further research.

In conclusion, newly compounds extracted from wastes, Cyano acetyl urea(CAU), Benzimidazolyl acetyl urea (BAU) and Cyano acetyl hydrolyzate (CAH),

are effective chemicals to control cotton leaf worm, however, they must be improve the insecticidal characters to increase its toxicity and its stability to can use them in IPM program. In addition, RAPD assay could be considered as a relatively cheap but quick method to preliminary screen the polymorphism induced in *S. littoralis* 6th instar larvae after treatment with tested compounds. However, the RAPD-PCR assay is a semi-quantitative tool until preliminary data further documented by a more specific method.

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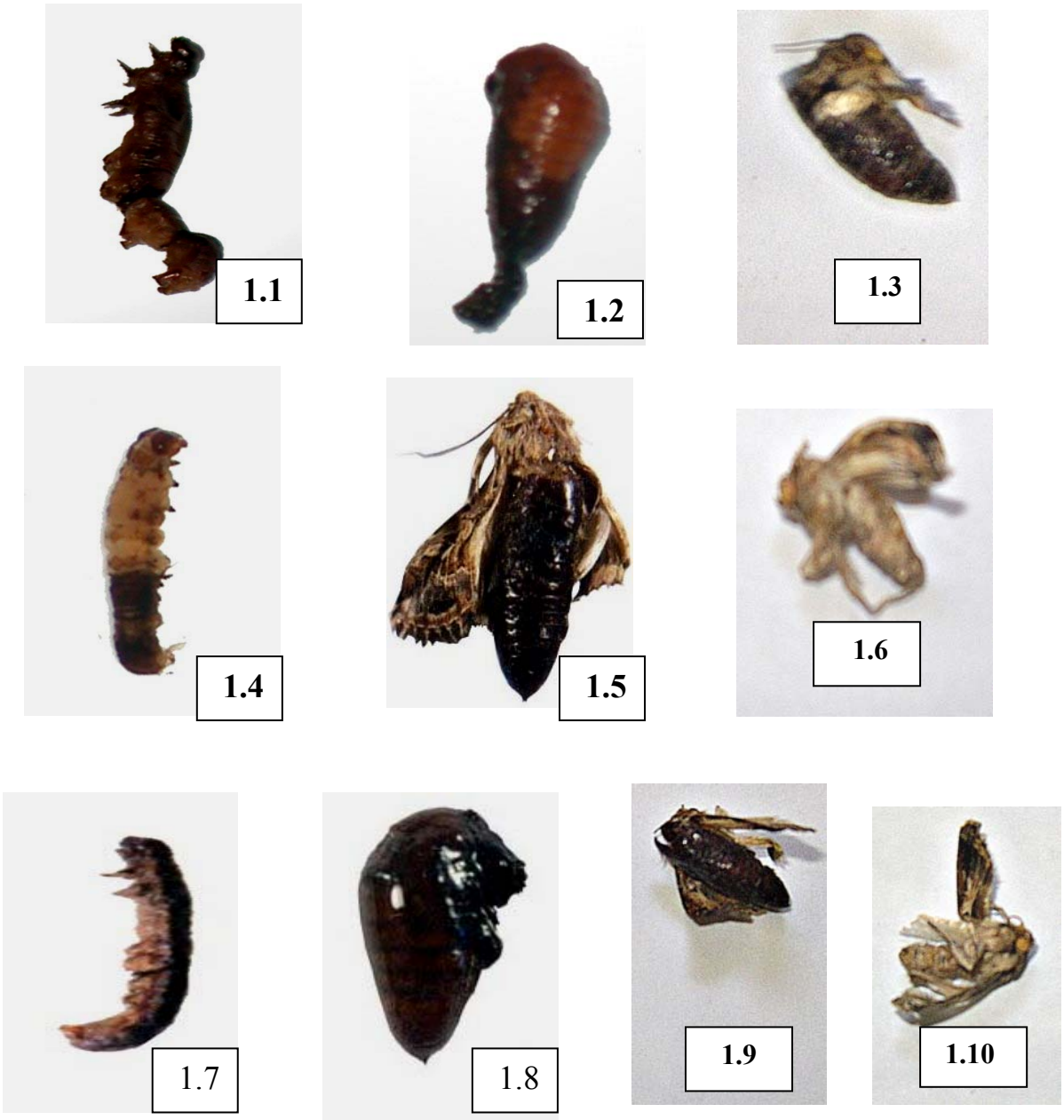

Figure (1) shows the morphological abnormalities produced by tested compounds.
1.1& 1.2& 1.3 the morphological abnormalities produced by Cyano acetyl urea (CAU)
1.4& 1.5 & 1. 6 the morphological abnormalities produced by Benzimidazolyl acetyl urea (BAU)
1.7& 1.8 &1.9 & 1.10 the morphological abnormalities produced by Cyano acetyl hydroslyzate (CAH)

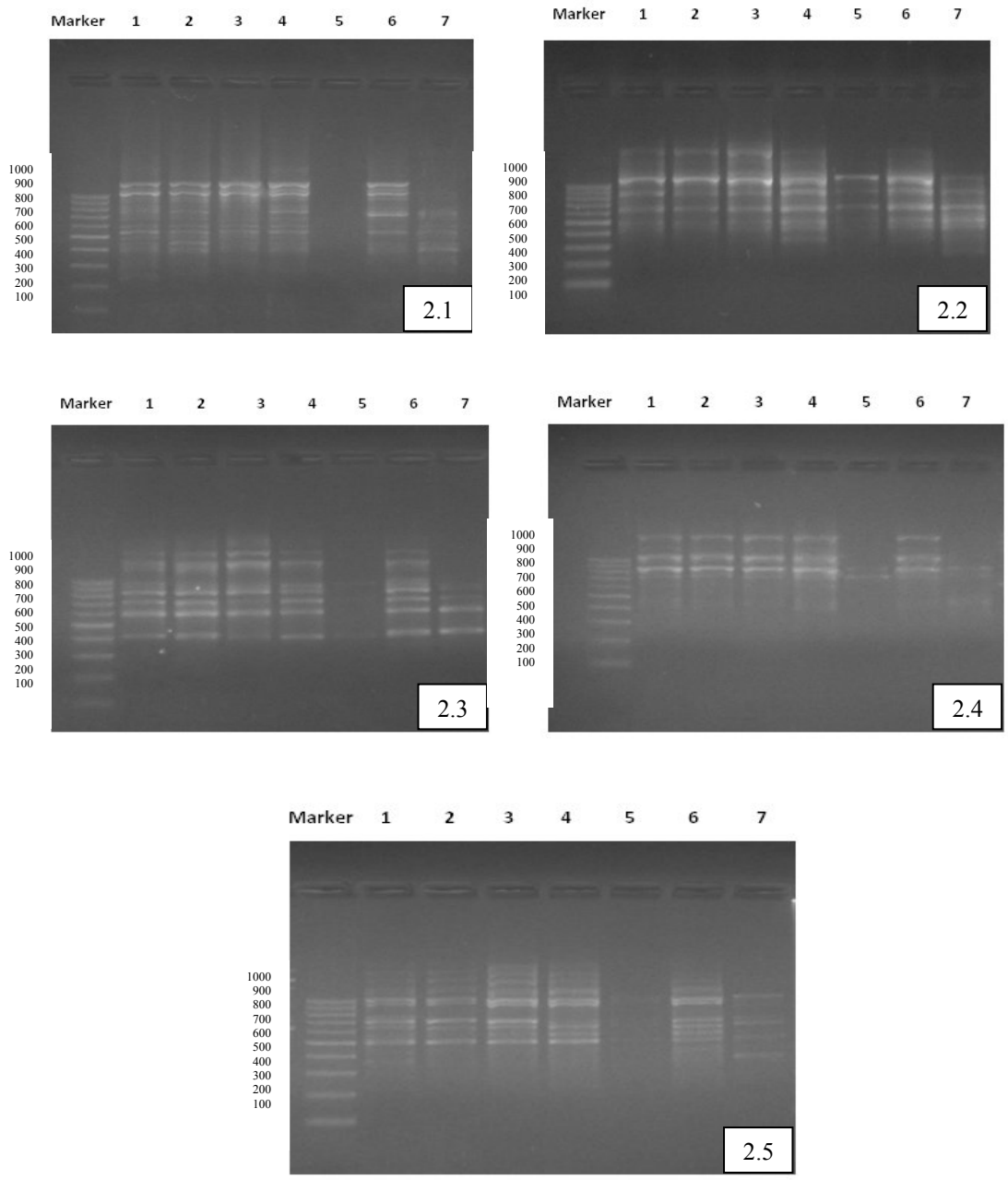

Figure (2): RAPD-PCR profiles of *Spodoptera littoralis* 6th instar larvae treated with the waste product newly-extracted compounds and generated by the OP-01(2.1), OP-02 (2.2), OP-03 (2.3), OP-04 (2.4) and OP-05 primers (2.5).

1=control, 2,3= Cyano acetyl urea (LC_{25}, $LC_{70,}$ respectively), 4,5= Benzimidazolyl acetyl urea (LC_{25}, $LC_{70,}$ respectively), 6,7= Cyano acetyl hydroslyzate (LC_{25}, $LC_{70,}$ respectively.

ARABIC SUMMARY

الدراسات السمية و الجزيئية لمركبات حديثه مستخلصه من النفايات ضد دودة ورق القطن (سبودوبتراليتورالز).

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الهدف الرئيسي من هذه الدراسة هو تحويل النفايات ذات الاصل الطبيعى إلى منتجات اقتصادية كالمبيدات الحشرية التي تستخدم على نطاق واسع في مكافحة الآفات في العالم بأسره. وقد أجريت التجربه تحت الظروف المعملية لتقييم نشاط ثلاثة مركبات حديثة مستخرجه من النفايات و هي:(سيانو أسيتيل يوريا و بنزداميدوذوليل أسيتيل يوريا وهما مستخلصين من اليوريا) و (سيانو أسيتيل هيدروليزيت وهو مستخلص من قش الارز) تجاه دودة ورق القطن (سبودوبتراليتورالز) وذلك من خلال تعرض يرقات العمراليرقى الرابع لأوراق الخروع التى تم غمرها في تركيزات مختلفة من المركبات المختبرة. وقد تم تحديد التركيزات المميتة ل 25% و50% و 70% من العمر اليرقى الرابع وقد تراوح التركيز المميت ل25% من العمر اليرقى الرابع بين 0.092و0.154% لمركب سيانو أسيتيل يوريا وبين 0.191و 0.225% لمركب بنزيميزاذوليل أسيتيل يوريا وبين0.009و 0.021% لمركب سيانو أسيتيل هيدروسيليت بينما تراوح التركيز المميت ل 70% من العمر اليرقى الرابع بين 1.289و2.009% لمركب سيانو أسيتيل يوريا وبين 1.445و2.159% لمركب بنزداميدوذوليل أسيتيل يوريا وبين0.339و 0.627% لمركب سيانو أسيتيل هيدروليزيت. كما تم تسجيل التشوهات الشكلية الناتجة من المعاملات المختلفه.

وقد كان الهدف الاخر لهذه الدراسة هو تقدير فاعلية التكثير العشوائي للحمض الوراثي المتباين RAPD لتحديد التباين الوراثي بين يرقات العمر السادس لسبودوبترا ليتورالز الغير معاملة و المعاملة كعمريرقى رابع بالمركبات المختبرة عند كلا الجرعتين LC_{25}،LC_{70} . وقد حددت خمس بادئات نووية و هي: OP-01, OP-02, OP-03, OP-04, OP-05 و التي أظهرت بحد أقصى 23 13 20, 24, 26 واسم وراثي على التوالي. أظهرت نتائج الRAPD اختلافات في العدد و الوزن الجزيئي (حجم) للواسمات الوراثية بين العينات المعاملة و الغير معاملة. تراوح التباين الوراثي ما بين 44% و 100%. كان التباين الوراثي أعلى في العينات المعاملة عند LC_{70} (77.8, 84.4, 86.4%) منه لدي العينات المعاملة عند LC_{25} (69.6, 80.3, 79.4) لليرقات المعاملة بسيانو أسيتيل يوريا، بنزداميدوذوليل أسيتيل يوريا، و سيانو أسيتيل هيدروليزيت على التوالي. كما حقق أعلى معدل للتباين الوراثي في العينات المعاملة ب سيانو أسيتيل هيدروليزيت 86.4%) مقارنة بتلك المعاملة ب سيانو أسيتيل يوريا (77.8%)و بنزداميدوذوليل أسيتيل يوريا (84.4%) وذلك بناء على التركيز LC_{70}،. من هذا نستخلص بلا شك أن نتائج ال RAPD قد أكدت نتائج اختبارات السمية و الدراسات الشكلية كما أظهرت أيضا أن تلف الحمض النووي DNA و احتمال حدوث طفرات هو العامل الأساسي وراء ظهور تباين وراثي بهذا الشكل بين اليرقات المعاملة و غير المعامل. ومن النتائج السابقة الذكر نجد ان المركبات الحديثة المختبرة هى مركبات كيميائية فعالة لمكافحة دودة ورق القطن ومع ذلك يجب ان يتم تحسين مواصفاتها لنزيد من سميتها وبقائها لكى نتمكن من استخدامها فى برامج المكافحة المتكامله للأفات.