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Insecticides Resistance Detection in Field-collected Populations of *Pectinophora gossypiella* (Saunders)

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ABSTRACT

In Egypt pink bollworm *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) has recently developed resistance to various kinds of insecticide in the field. The resistance status of pink bollworm to several classes of insecticides currently used in cotton fields was evaluated in the Nile Delta (Kafr-Elsheikh and Menoufia) Egypt. Two field-collected *P. gossypiella* populations have developed resistance to several classes of insecticides. Toxicological bioassays were conducted in the laboratory, LC_{50}'s estimated by probit analysis and Resistance Ratio (RR) were calculated at the LC_{50} level. Of the seven insecticides tested four insecticides (chlorpyrifos, profenofos, thiodicarb and carbaryl) showed the Resistance Ratio (RR) values above 20 folds, against two field-collected populations whereas the other three insecticides (fenpropathrin, spinosad and abamectin) had RR values less than 10 folds. Also, the patterns of selection pressure by abamectin at the LC_{30} level to Kafr-Elsheikh (KFR)-and Menoufia (MNF)-field strains were determined by continuous insecticide application under laboratory conditions for eight successive generations. On the other hand, biochemical assays indicated that both KFR-and MNF-field strains expressed higher levels of Acetylcholinesterase (AChE) activity by 4.1 and 2.8-times, respectively, compared to that of susceptible strain. There were significant differences in esterase activities among the field-collected populations and susceptible strain. Analysis of protein patterns by (SDS-PAGE) showed some differences between resistant strains to abamectin, MNF-and KFR-field strains and the bands differed in intensity and molecular weights between all strains. These findings indicate that rapid biochemical assays coupled with biological assays are potential tools for estimating both the intensity of resistance in the field.

Key words: *Pectinophora gossypiella,* acetylcholinesterase, esterase, insecticide resistance, insecticide pressure, SDS-PAGE1

INTRODUCTION

Cotton is attacked by various pests during the different stages of its development (Abd El-Mageed *et al.*, 2007). The pink bollworm *Pectinophora gossypiella* (Saunders), is the key pest in cotton fields, particularly in middle and late season under dry conditions (Korejo *et al.*, 2000; Unlu, 2004). At present, it has been recorded in nearly all cotton-growing countries of the world. Newly hatched larvae are exposed to insecticides for a very short time before they enter flower buds or bolls while fully grown larvae emerging from bolls for pupation are difficult to control with

chemicals (Attique *et al*., 2001a, b). Traditionally, chemical control is the major method for protecting cotton from pink bollworm damage but the efficacy of these insecticides has declined quickly due to the evolution of resistance to insecticides in pink bollworm (Yang *et al*., 2000). Susceptibility to pesticides in target agricultural pest populations is a valuable natural resource in agriculture that must be managed carefully sustains efficacy of pesticides as long as possible, especially if the pesticide is environmentally friendly. Bioassays have been the basic used for documenting levels of insecticides resistance in field populations on which management decisions are based (Shah *et al*., 2002). Given the history of resistance to various insecticides of insect pests of cotton, a need continues to develop baseline data, to monitor for changes in tolerance, is essential for detecting shifts in susceptibility (Margaritopoulos *et al*., 2008). Therefore, resistance monitoring is fundamentally important to every resistance management program. Continuous monitoring of the populations for changes in resistance frequencies allows informed decision making decisions regarding effective management strategies (Prabhaker *et al*., 1996). Provided that a monitoring survey is sufficiently extensive, patterns of resistance can be detected within a region or between regions. Ideally, current resistance monitoring data should be communicated to growers and pest control advisors who are interested in participating in a resistance management program. With this information, they could alternate to a different insecticide class or alternative control strategy to avoid increasing selection pressure to populations (Prabhaker *et al*., 1996). Biochemical techniques are advantageous because they test for activity that is directly linked to a resistance mechanism (Byrne *et al*., 2000). On the other hand, biochemical assays may also be used to detect and monitor resistance. Biochemical assays measure changes in sensitivity at the pesticide's target site or measure metabolic activities of the pest that are responsible for, or at least linked to, resistance (Roditakis *et al*., 2009). Chemical insecticides are known to be able to act as inducers for the biosynthesis of cellular proteins or as inhibitors of enzymes; thus, the molecular biochemical explanation of variations among the tested field strains can be attributed to either variation or similarity in the background exposure to the chemical control programs. However, early resistance detection is very difficult using conventional bioassay-based monitoring methods, particularly when resistance is recessive (Clark, 2010). In addition, it is difficult collecting large numbers of some insects. A biochemical assay used by Soleno *et al*. (2008) showed different degrees of tolerance associated with increased esterase activities in codling moth populations. Bioassays on adult moths are successfully utilised for monitoring resistance in codling moth (Dunley and Welter, 2000). More recently, it has been demonstrated that larvae can also be used for detection of resistance in field collections (Soleno *et al*., 2008).

The objectives of this study were (1) to further describe the expression of resistance to different insecticides of field populations of the pink bollworm collected from cotton fields in the Nile Delata (2) to prolong the useful life of abamectin we selected field strains of the pink bollworm. We herein present the results of our characterization of resistance level of the selected strains and possible mechanisms of resistance and (3) to investigate the activities of esterases, acetylcholinesterase and analysis of protein patterns by (SDS-PAGE) in both resistant and susceptible strains.

MATERIALS AND METHODS

Strain collection and rearing procedures: A susceptible strain of pink bollworm was obtained from the Lab. of Bollworms Department at the Institute of Plant Protection, Dokki, Egypt and maintained in the laboratory under insecticide-free conditions. It was used as the baseline reference strain for the comparisons levels of resistance between the studied strains. Two *P. gossypiella* field

strains were collected from different fields of cotton located in Menoufia (MNF) and Kafr-Elsheikh (KFR), Nile Delta area in season 2009. Pink bollworm larvae were reared on a modified artificial diet as described by El-Hafez *et al.* (1982). All bioassay trials were done on the newly hatched larvae of the pink bollworm.

Chemicals and insecticide bioassays: The commercial formulations of insecticides used in bioassays were: chlorpyrifos (Dursban 48% Emulsifiable Concentrate (EC), DowElanco Co.); profenofos (Curacron 72% EC, Ciba-Geigy AG); thiodicarb (Larvin 80% DF [dust flowable], Union Carbide Agricultural Co., Inc.); carbaryl (Sevin 85% WP [wettable powder], Rhone-Poulenc); fenpropathrin (Danitol 30% EC, Sumitomo Chemical Co. Ltd; spinosad (Success 12% SC [suspension concentrate], Dow AgroSciences); and abamectin (Vertemic 1.8% EC, Syngenta, Basel, Switzerland). All other chemicals used in biochemical assays were purchased from Sigma Chemical Company as analytical standards.

Contact bioassay: A stock solution of each insecticide formulation was prepared using the acetone and then serially diluted concentrations were prepared. One ml of each insecticide concentration was pipetted into a Petri dish (7.5 cm diameter). After accurately distribution of solutions, dishes were left until dryness then ten newly hatched larvae (12 to 24 h-old) were transferred to each dish containing control diet and held at the same conditions (27±1°C and 70-75% RH). Control Petri dishes were precoated with acetone only. Mortality was assessed 48 h after treatment. Control mortality was never >10%; data were corrected by Abbott (1925) formula. The data were analyzed by probit analysis PC (Finney, 1971). Resistance Ratio (RR) was calculated by dividing the LC_{50} of field-collected strain by the LC_{50} of lab-susceptible strain.

Establishment of the resistant strain to abamectin: For selecting the resistant strain of pink bollworm *P. gossypiella*, abamectin (Vertemic 1.8% EC) was dissolved in acetone and applied using the contact bioassay technique. One mL of the abamectin concentration which kills 30% of first-instar larvae LC_{30} (after 48 h) was pipetted into a Petri dish. The surviving larvae were transferred into glass vials containing artificial diet. Toxicity tests were conducted every two generations to calculate its LC_{50}.

Biochemical assays

Acetylcholinesterase (AChE) assay: AChE assays were measured using pink bollworm adult head homogenates following the method of Ellman *et al.* (1961). Data were corrected using a blank test without substrate in the mixture reaction. Protein content was determined using methods described by Bradford (1976). The AChE activity was determined using an extinction coefficient of 13.6 mM^{-1} cm^{-1} for thionitrobenzoic acid.

Esterase assays: Esterase activity towards α-NA was quantified using the assay method of Van Asperen (1962). An esterase assay was conducted to measure the production of α-naphthol from α-naphthyl acetate substrate. Esterase activity was presented relative to protein concentration which was measured using the assay of Bradford (1976). The esterase activity was expressed as μmol α-naphthol produced/min/mg protein.

Electrophoretic analysis (SDS-PAGE) of protein: SDS-PAGE was carried out according to methods described by Laemmli (1970). The intensities of the bands on each gel were then scanned

with a computing densitometer at absorbance of 6J3 nm and results were graphically depicted with Image Phoretic (IDA) Quantifier software (Phoretix International, London). The peak heights, R_f, intensity and area were used as a relative comparison of band intensity between zygomorphs on the same gel.

Statistical analysis: The data was analyzed using analysis of variance (ANOVA) followed by the Student-Newman Keuls test to determine significance between different groups. These tests were performed using a computer software CoStat system for Windows, CoStat Program, 2006 Berkeley, CA, USA.

RESULTS AND DISCUSSION

Insecticides resistance in field populations: Present studies were carried out to evaluate the toxicity of some selected insecticides bearing novel modes of action against first instars of pink bollworm from the Lab-susceptible and field strains collected from cotton fields.

High level of resistance was detected for both field-collected strains to all of the organophosphate and carbamate insecticides tested (Table 1). Among the field-collected strains, KFR was more resistant to the organophosphates and carbamates than the MNF strain. Tests with pyrethroids revealed low resistance levels to fenpropathrin in both field-collected strains (Table 1).

Table 1: Responses of 1st instar of pink bollworm larvae from field strains to selected insecticides as assayed by contact technique bioassay

			LC_{50}[a]			
Classes	Insecticide	Strain	ppm	(95% CL)	Slope±SE	RR[b]
Organophosph-ates	Chlorpyrifos	L-SUS	7.5[c]	(6.2-8.9)	1.56±0.13	-
		MNF	161.2[k]	(143.6-182.7)	2.31±0.28	21.49
		KFR	213.6[l]	(192.6-240.1)	2.64±0.26	28.48
	Profenofos	L-SUS	12.3[e]	(11.1-13.6)	1.92±0.18	-
		MNF	249.1[m]	(231.2-261.5)	2.68±0.24	20.25
		KFR	296.5[o]	(279.8-314.3)	3.25±0.32	24.11
Carbamates	Thiodicarb	L-SUS	5.2[b]	(4.3-6.3)	1.83±0.17	-
		MNF	167.3[k]	(146.2-188.6)	2.15±0.35	32.17
		KFR	274.6[mn]	(246.5-295.0)	2.69±0.37	52.81
	Carbaryl	L-SUS	9.4[d]	(7.8-10.7)	1.82±0.15	-
		MNF	266.2[mn]	(244.3-290.2)	2.32±0.29	28.22
		KFR	329.6[p]	(300.2-361.4)	2.93±0.30	35.06
Pyrethroids	Fenpropathrin	L-SUS	2.7[a]	(2.3-3.1)	1.58±0.18	-
		MNF	18.4[f]	(14.1-25.4)	2.73±0.36	6.81
		KFR	25.0[fg]	(18.9-31.2)	3.56±0.33	9.26
Biopesticides	Spinosad	L-SUS	28.0[g]	(19.8-49.3)	1.43±0.15	-
		MNF	93.6ij	(62.9-121.3)	2.18±0.29	3.34
		KFR	71.2ij	(56.2-104.8)	2.01±0.33	2.54
	Abamectin	L-SUS	44.2h	(30.5-68.0)	1.14±0.12	-
		MNF	95.6j	(71.2-121.8)	2.63±0.34	2.16
		KFR	64.5i	(46.9-92.2)	3.24±0.37	1.46

[a]Expressed as ppm after 48 h of treatment and LC_{50} values followed by the same letter within each vertical column are not significantly different (95% CL do not overlap). [b]Resistance ratio (RR) calculated by dividing the LC_{50} for the field strain by the LC_{50} for the Lab-susceptible strain

The field-collected strains did not differ from each other in susceptibility to fenpropathrin. Resistance to the biopesticides, spinosad and abamectin was low or insignificant in the case of KFR's response to abamectin. The field strains showed less than 2.2-fold resistance to abamectin but exhibited variable levels (6.81 to 52.81-fold) of resistance to other covenantal insecticides used. Both field strains use in this study appear to not be homogenous for resistance to the insecticides we tested. Resistance of pink bollworms to biopesticides seems to be correlated with the pattern of insecticide-use prevalent in the localities studied. Therefore, our study is in agreement with the findings of Osman *et al*. (1991) who suggested that monitoring of pink bollworm resistance in field strains reared in the laboratory should be performed in the F_I generation. The spinosad, abamectin and fenpropathrin resistance were generally low. It has been suggested that insects should not be considered resistant until a resistance ratio of 10 is exhibited (Torres-Vila *et al*., 2002; Kranthi *et al*., 2002; Bues *et al*., 2005). Current levels of resistance to selected insecticides, chlorpyrifos, profenofos, thiodicarb and carbaryl, in two field locations were high which is probably due to intensive use of these insecticides that results in high selection pressure for the development of resistance. These developments have had a dramatic effect on management plans for pink bollworm *P. gossypiella* control. For example, management strategies for pyrethroid resistance have relied on organophosphates and carbamates to reduce the frequency of pyrethroid use during recent years (Plapp *et al*., 1990), so exposure of insect to these compounds has increased. The cotton insect management plan recommended by Baldwin and Graves (1991) for Louisiana seems to be reliable to apply in Egypt. In essence, the window strategy, based on the use of a single class of insecticide on discrete generations of an insect pest is still advocated. However, the pyrethroids are recommended from 1 July to 15 August and organophosphates are then recommended during the late season. Based on our current study we are in agreement with recommendations of Baldwin and Graves (1991) which encourage the use of biological insecticides, especially during the early season. The susceptibility of pests to insecticides may change depending on the selection pressure of these compounds on population (Sayyed *et al*., 2005).

Selection of the abamectin-resistant strain from collected field populations: Resistance of the KFR-strain at F_2 was estimated to be about 3.85-fold compared with the susceptible-strain (LC_{50} = 170.2) (Table 2).

Resistance in the selected KFR strain increased steadily from 3.85-fold at F_2 to 21.01-fold at F_8 (Table 2). The development of resistance in the MNF strain was minor until F_4 after which it increased sharply to 15.90-fold at F_8. Exponential increase of resistance was found from the F_8. After 8 generations of selection, 48 h-LC_{50} value of abamectin against the 1st instar larvae increased significantly which was from 95.6 to 702.6 ppm. After 8 generations of selection the resistance level to abamectin in the MNF-selected strain increased more than 15-fold compared with the parental strain. Clarification of the resistant mechanism of *P. gossypiella* to abamectin is important to Integrated Pest Management (IPM) and Insect Resistance Management (IRM). The results showed that the level of resistance increased more than 20-fold compared with the parental strain after 8 generations of selection. Although biopesticides are effective on *P. gossypiella* larvae, its resistance has been documented in other bollworms of some areas (Gunning and Balfe, 2002; Wyss *et al*., 2003; Ahmad *et al*., 2003). In Pakistan, *Spodoptera litura* were investigated for spinosad, resistance ratio was in the range of 7 to 122-fold and 4 to 186-fold for abamectin (Ahmad *et al*., 2008). The selection pressure is responsible for the difference in resistance among regions is the positive correlation found between the number of insecticidal applications and the degree of resistance development (Kakani *et al*., 2010).

Table 2: Rate of development of resistance at 48 hr in first-instar of pink bollworm larvae field strains (Menoufiya and Kafr-Elsheikh) after exposure to selection at LC_{30} level with abamectin for eight successive generations

	LC_{50}[a]			
Generation	ppm	(95% CL)	Slope±SE	RR[b]
Lab-Susceptible strain	44.2[a]	(30.5-68.0)	1.14±0.12	-
Menoufiya strain				
F0	95.6[c]	(71.2-121.8)	2.63±0.34	2.16
F2	160.2[d]	(114.6-215.0)	2.05±0.33	3.62
F4	375.3[e]	(300.9-416.8)	1.98±0.24	8.49
F6	518.5[f]	(428.1-586.5)	1.87±0.26	11.73
F8	702.6[g]	(649.7-736.9)	1.74±0.18	15.90
Kafr-Elsheikh strain				
F0	64.5[b]	(46.9-92.2)	3.24±0.37	1.46
F2	170.2[d]	(135.5-211.6)	2.95±0.28	3.85
F4	454.3[f]	(421.0-537.1)	1.80±0.24	10.28
F6	710.3[g]	(623.6-768.5)	1.72±0.26	16.07
F8	928.5[h]	(834.9-1065.3)	1.43±0.20	21.01

[a]Expressed as ppm after 48 h of treatment and LC_{50} values followed by the same letter within each vertical column are not significantly different (95% CL do not overlap). [b]Resistance ratio (RR) calculated by dividing the LC_{50} for the field strain by the LC_{50} for the Lab-susceptible strain

AchE and esterase activities: Insects from field populations collected either from MNF (F_0, parents) or KFR (F_0, parents) cotton fields, expressed higher levels of AChE activity than the reference (Lab-susceptible) strain, in which AChE activity was 2.80 and 4.10-fold higher than that from the Lab-susceptible strain, respectively (Table 3).

However, AChE activity increased following laboratory rearing under selection pressure of both MNF and KFR indicating that these field-collected strains appeared to increase their resistance to abamectin. The activity in F_8 generation of MNF strain was similar to that in the KFR (F_0, parents) strain whereas insects from MNF-F_8 generation showed an increase in AChE activity by 3.90-fold higher than that in Lab-susceptible strain. Therefore, present data demonstrated that increase in resistance to abamectin in both field-collected strains seems to be associated with AChE activity. This also may be particularly important for understanding AChE insensitivity is an important insecticide resistance mechanism in a number of insect species (Soderlund and Blopmquist, 1990; Harold and Ottea, 1997). Esterase activities toward α-naphthyl acetate (α-NA) in pink bollworm larvae from both MNF-and KFR field strain were higher than those measured in Lab-susceptible strain (Table 3). Similarly, esterase activities in F0 of both field-collected strains were slightly elevated, 1.31 and 1.51-fold for the MNF and KFR strains, respectively, relative to the Lab-Sus strain. Additionally, esterase activity increased to 1.61 and 2.26-fold for these respective strains. Metabolic resistance to abamectin has also been implicated for the American bollworm, *Helicoverpa armigera* (Christie and Wright, 1990).

Protein levels: Differences in protein level among the various strains were observed in last instars (Table 4). The abamectin resistance selected strains had the highest protein levels followed by the field-collected strains. Similarly, protein level in F_0 of both field-collected strains were slightly elevated, 1.07 and 1.10-fold for the MNF and KFR (F_0) strains, respectively, relative to the Lab-Sus

Table 3: Levels of acetylcholinesterase (AChE) and esterase activities in lab-susceptible strain, field-strains (F_0) and F_8-abamectin resistant strains of pink bollworm

	AChE activity[a]		Esterase activity[b]	
Strain	nmol/min/mg protein	R/S (activity ratio)	nmol/min/mg protein	R/S (activity ratio)
Lab-Sus	5.0×10^{-3} d	1.00	8.20±0.45 c	1.00
F_0-KFR	20.5×10^{-3} ab	4.10	12.40±0.85 b	1.51
F_8-KFR	26.4×10^{-3} a	5.27	18.56±1.02 a	2.26
F_0-MNF	14.0×10^{-3} c	2.80	10.73±0.92 bc	1.31
F_8-MNF	19.5×10^{-3} b	3.90	13.24±0.89 b	1.61

[a]AChE activity was measured in heads of pink bollworm moths toward acetylcholine iodide and expressed as nmol/min/mg protein. [b]Esterases activity were measured in midgut homogenate from pink bollworm larvae toward α-naphthyl acetate (α-NA) and expressed as nmol α-naphthol/min/mg protein. Values by the same letter within each vertical column are not significantly different (p = 0.05)

Table 4: Mean of protein levels in lab-susceptible strain, field-strains (F_0) and F_8-abamictin resistant strains of pink bollworm

Strain	Protein levels (mg g^{-1})	R/S
Lab-Sus	24.8[c]	1.00
F_0-KFR	27.3[b]	1.10
KFR-abamectin resistant (F_8)	33.4[a]	1.35
F_0-MNF	26.5[bc]	1.07
MNF-abamectin resistant (F_8)	32.2[a]	1.30

Values by the same letter within each vertical column are not significantly different (Turkey's p = 0.05)

Table 5: Molecular weight and relative mobility (R_f) values of protein bands in the body tissues of pink bollworm larvae

	MNF-F_0		MNF-F_8		Protein Marker		KFR-F_8		KFR-F_0	
Band No.	MW[α]	R_f	MW[α]	R_f	MW[α]	R_f	MW[α]	R_f	MW[α]	R_f
1							152	0.166		
2									145	0.190
3	136	0.221	130	0.239	128	0.245				
4					100	0.344	106	0.325	102	0.337
5			89	0.370			89	0.370		
6	79	0.393	70	0.417	70	0.417			77	0.399
7	64	0.442					62	0.448	59	0.472
8	58	0.589	55	0.62	58	0.577	58	0.577	57	0.601
9	37	0.690	36	0.699			47	0.656		
10			22	0.792			22	0.792		
11	7	0.890	7	0.890	8	0.883	10	0.850	10	0.850

[a]Molecular weight in kilodaltons. The four protein profile patterens (1,2,4 and 5) which presented by field-strains (F_0) and F_8-abamectin resistant strains of *P. gossypiella*

strain. However, the F0-MNF strain did not significantly differ from the Lab-Sus strain. Additionally, protein level increased significantly to 1.30 and 1.35-fold for the MNF and KFR abamectin resistant (F_8) strains, respectively.

Protein electrophoresis: Gel scanning with respective standard molecular weights revealed variation in protein bands number and molecular weight type among the four strains (Table 5). There were two new bands in the KFR-, MNF-abamectin resistant strains (F_8) which had molecular weight (89 and 22), compared with both field-collected strains (F_0). These specific protein bands

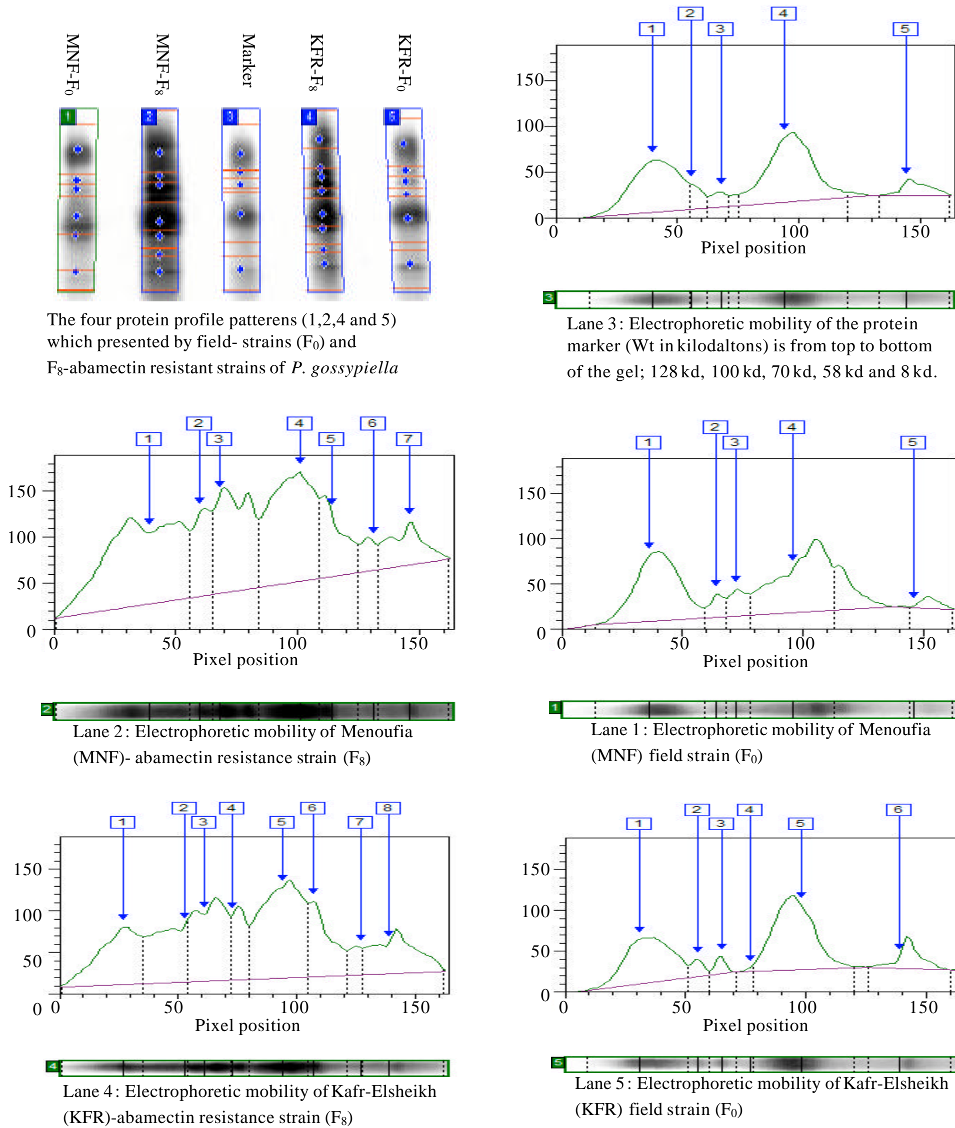

Fig. 1: Electrophoretic analysis of total proteins patterns by (SDS-PAGE) extracted from the last-larval instar of *P. gossypiella* belong to four different strains: Lane 1: Menoufia (MNF) field strain (F_0), Lane 2: Menoufia (MNF) abamectin resistance strain (F_8) Lane 3: Protein Marker, Lane 4: Kafr-Elsheikh (KFR), abamectin resistance strain (F_8) Lane 5: Kafr-Elsheikh (KFR) field strain (F_0)

were absent in field-collected strains, which may indicate a resistance mechanism present in these strains and that the SDS-PAGE technique may be used to screen and detect abamectin resistance. A rapid detection tool such as this may be valuable for determining in-season insecticide selection for resistance management Fig. 1.

Similarly, Abdeen (1985) found that exposure of cotton bollworm, *Helicoverpa zea*, to diflubenzuron resulted in an increase of some major proteins through the first two days post

treatment. Also, Hewady (1990) indicated that when the haemolymph protein of normal 5th instar spiny bollworm *Earias insulana* was separated on acrylamide gel electrophoresis, 12 different proteins were recorded. Their mobility resolution (M.R.) values were 6.49 to 98.7. Twenty days after pyriproxyfen treatments caused appearance or disappearance of some bands with different mobility resolution. Jongsma *et al.* (1996) characterized the proteolytic activity present in the luminal contents of larval gut of *Spodoptera exigua*. Separation of gut proteins by SDS-PAGE and subsequent incubation with proteinase substrates visualized 6 separate activities with apparent molecular masses that ranged from 29 to 110 Kda. Eid *et al.* (1979) reported that no protein bands related to insecticide poisoning were observed in the larval tissue of *S. littoralis*, until 18 h post insecticide treatment when two different bands were detected in haemolymph protein as a result of profenofos and fenitrothion treatments.

CONCLUSION

Early detection of insecticide resistance provides a basis for the management of resistant pest populations (Osorio *et al.*, 2008). It seems that little by little, the industrialized countries adapt new procedures for control of agricultural pests (Mirmoayedi *et al.*, 2010). Rapid biochemical assays coupled with biological assays are potential tools for estimating both the intensity and the frequency of resistance in the field (Rauch and Nauen, 2003; Farghaly, 2010). For example, measurement of AChE sensitivity to inhibition by insecticides or resistance associated esterases with naphthyl acetate esters provides a rapid indication of resistance mechanisms expressed in insecticide-resistant pests (Brown *et al.*, 1996). Additionally, the SDS-PAGE technique can be a biomarker for detection of susceptibility levels or resistance in the field strains which should lead to a change in the currently applied chemicals (Massoud *et al.*, 2006).

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