Resistance Status and Associated Resistance Mechanisms to Certain Insecticides in Rice Weevil *Sitophilus oryzae* (Coleoptera: Curculionidae)

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ABSTRACT

The rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae) was collected from El-Behera governorate (EB). The first generation showed reduced susceptibility to deltamethrin and malathion compared with the laboratory susceptible strain (LS). Susceptibility test results (using LS colony as a reference strain) indicated that EB strain has relative toxicity ratio, RR_{50} and RR_{90} of 16.64 and 9.08 for malathion. Although, EB strain was tolerant to deltamethrin with RR_{50} of 5.2, it was marginally resistant to cypermethrin and entirely susceptible to permethrin. The biochemical results showed elevation in the activity of total esterase (ES), carboxylesterase (CE) and glutathione *S*- transferase (GST) which suggested that the metabolic resistance may has a key function in this population. Furthermore, acetylycholinesterase (AChE) activity in EB strain was 3.52 times higher compared with LS strain which pointed the insensitivity of AChE in EB strain. Moreover, esterase analysis indicated genetic polymorphism between EB and LS populations which might be attributed to selection pressure due to continuous exposure to insecticides. This study suggested the existence of malathion and deltamethrin resistance in EB strain which might be a consequence of biochemical alterations. The results of current study indicate the importance of continuous monitoring of resistance of stored product insect pests to plan successful management strategies.

Keywords: insecticide resistance, metabolic resistance, esterase, *Sitophilus oryzae,* malathion.

INTRODUCTION

The rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is one of the most destructive primary pests which can easily infest intact cereal seeds, i.e, rice, wheat, maize and barley or non-cereals like split peas and pasta. It may attack cereal plants in the fields. The existence of this insect causes weight loss, fungi and mites infestation thus decrease the grain quantity and quality through increasing free fatty acids contents (CABI, 2017). The weight loss interrelated to *S. oryzae* and *S. granarius*, under natural conditions estimated in rice, ranged (56 - 74%) (Koura and El-Halfawy, 1972). Furthermore, wheat storage in Egypt for significant assorted periods is crucial because the national production provides only 55% of the total requirements (FAO, 2006). The majority of the Egyptian storage system is conventional as 1.6 million tonnes stored in "Shona" (FAO, 2015) and around 20-30% of wheat lost during storage (Wally, 2014) which demand grain protection measures that rely markedly on chemical pesticides. Chemical pesticides were employed in stored grain protection since 1945 started with lindane followed by malathion in 1958 and then other organophosphate and pyrethroids (Kljajić and Perić, 2007). The continuous use of insecticides to afford lasting grain protection led to development of insecticide resistance in stored product insects (Kljajić and Perić, 2009) and (Hagstrum and Phillips, 2017). Insecticide resistance in stored product insects is a growing problem which was first reported near the beginning of 1960's once resistance to malathion detected in the red flour beetle, *Tribolium castaneum* (Herbst) in Nigeria, the United States, Egypt, Australia (Zettler, 1974) and Kenya (Beeman and Nanis, 1986). Moreover, *S. oryzae* developed resistance to organophosphate pesticides (Julio *et al*., 2017) as well pyrethroid insecticides (Heather, 1986 and Athanassiou *et al.,* 2004). According to Arthropod Pesticide Resistance Database, up to date, there are nine cases of malathion resistance were reported in *S. orayze* populations among 38 cases (APRD, 2017). Susceptibility shift in an insect populations is a demanding limitation factor in chemical control that directly leads to increase application rates and treatments frequency which have harmful impact on economic, environment and human health (Hagstrum and Phillips, 2017). Therefore, the regular evaluation of insect population susceptibility

is considered the foremost step in a successful control program. The present work aimed to evaluate the susceptibility of *S. oryzae* population collected from Elbehera, Egypt to malathion and certain pyrethroids as well to investigate the possible associated mechanism (s) of resistance in this strain.

MATERIALS AND METHODS

***Insect strains**:* A laboratory susceptible strain (LS) of *Sitophilus oryzae* has been continuously reared in the laboratory for more than eight years at Faculty of Agriculture, Alexandria University. Adult beetles (2-3 weeks old) reared on whole wheat under stander conditions of 28 °C ±1, RH of 70 ±5 and photoperiod L/D of 12:12hr (Strong *et al*, 1967).

El-Behera strain (EB): samples of rice were purchased and collected from markets located in El-Behara Governorate, Egypt during summer season of 2016. Samples were mixed then the adults of *S. oryzae* were separated and transferred to sterilized whole wheat for eggs lying. After 7 days, the adults were removed and the media were stored at the standard rearing conditions until adult's emergency. Adults of 2-3 weeks old of the first generation were used for the present study.

***Insecticides**:* Deltamethrin (98%), permethrin, cypermethrin (96.62%) and malathion (95%) as technical grad samples of pesticides were obtained from the National Company for Agrochemicals & Investment (Agrochem.), Egypt.

Chemicals: All chemicals used were purchased from Sigma/Aldrich Company

Susceptibility test

Pesticide residual film technique was preformed according to (FAO, 1974) to determine the resistance level in EB colony comparing with the LS strain as a reference strain. A series of insecticide concentrations dissolved in acetone (1 ml) were applied onto Whatman paper No. 1 which placed in Petri dishes (9 cm) and set aside for 15 min to allow solvent evaporation. Twenty adults (2-3 weeks old) from both colonies were transferred to each Petri-dish. Each concentration was replicated three times and kept at 28 °C ±1, 70% ±5RH and 12:12hr photoperiod]. Mortality percentages were recorded after 24 hr of treatment and LC_{50} and LC_{90} values and their confidence limits were calculated according to (Finney, 1971) using Ld-p Line® (a software program).

Crude Enzyme preparation

Adult beetles (2-3 weeks old) from each colony were homogenized in ice cold 0.1M sodium phosphate buffer (pH 7.4) contains 0.5 mM EDTA (1:10 w/v) for 20 sec two times. Then, the homogenates were centrifuged at 1000 rpm for 15 min. After centrifugation, the resultant supernatants were filtrated through glass wool and re-centerifugated at 10,000 rpm for 30 min. The resultant supernatants were used for the biochemical assays.

Total esterase activity: The assay was performed according to (Van Asperen 1962). The reaction mixture (2.5ml) contains: 30 µl crude enzyme, sodium phosphate buffer (0.1M pH 7.4) and fast blue dye solution [2.15 mM α-naphthayl-acetate dissolved in acetone and mixed with 1.2 mM fast blue B slate (O-dianisidine) in phosphate buffer]. Absorbance was measured at wave length of λ_{450} nm after 15 minutes incubation period. The specific activity was expressed as Δ Optical density (OD) at λ_{450}/mg protein/min.

Carboxylesterase* (CE) *activity: CE was determined according to (Mendoza *et al.,* 1971). A reaction was initiated by adding 0.02 M of indophenyl acetate dissolved immediately in acetone to solution of 0.05 M tris-HCl and 30 µl of the crude enzyme. The absorbance was measured at λ_{412} nm after incubation at 37°C for 15 min. The specific activity was calculated as Δ Optical density (OD) at λ_{412}/mg protein/min.

Glutathion-S- transferease (GST) activity: GST was determined according to the method of (Vessey and Boyer, 1984) The reaction mixture consists of 1.45 ml 5 mM glutathione in 0.1 M sodium phosphate buffer pH 7.5, 20 µl of 1-chloro-2, 4-dinitrobenzene (CDNB 75 mM) in absolute ethanol and 30 µl of the crude enzyme. The changes of absorbance were measured continuously at λ_{345}nm. The activity was expressed as Δ Optical density (OD) at λ_{345}/mg protein/min.

Acetylcholinesterase (AChE) activity: AChE was determined according to (Ellman *et al.* 1961). A three ml total volume reaction solution was containing: 10 mM dithiobis-(2 nitrobenzoic acid dissolved in 100 mM phosphate buffer pH 7.4, 75 mM acetylthiocholine iodide and 50 µl of crude enzyme. After 30 min incubation at 37°C the optical density was measured at λ_{412} nm. The activity was calculated as ΔOD at λ_{412}/min/mg protein.

Protein concentration: Protein was measured according to the method of (Lowry *et al*., 1951) using bovine serum albumin (BSA) as a standard.

Esterase izozymes profile: Agar-starch-polyvinyl pyrolidine (PVP) poly vinyl gel electrophoresis was carried out according to the procedures described by (Shaw and Prasad, 1970). The gel plates [20*30 cm and thickness of 0.9 mm] consisting of PVP 1g and 1g hydrolyzed starch dissolved in 10 ml electrode buffer [(0.07 M Tris and citric acid 0.007M (pH=8.3)] and 90 ml distilled water. Electrophoresis runs were performed at 4°C and 250 mV constant for 90 minutes. After the full set run, gels were incubated for 30 min at room temperature and complete darkness in the staining buffer [l00 ml phosphate buffer (pH=7.0) containing 50mg fast blue RR salt (bis (4-benzamido-2,5-dimethoxybenzenediazonium) zinc (II) chloride)

and 20 mg α-naphthyl acetate, and 20 mg β-naphthyl acetate dissolved in l ml acetone]. Then, the plates were distained in distilled water until a clear background was appeared. The plates were subjected to analysis using PAST: Paleontological Statistics Software (http://palaeo-electronica.org).

RESULTS

Bioassay: The data of susceptibility tests showed that EB strain was significantly resistant to malathion compared with the LS strain with resistance ratios at LC_{50} (defined as LC_{50} resistant (EB)/LC_{50} susceptible (LS) = RR_{50}) of 9.08 fold (Table1) and it was vigorously tolerant to deltamethrin (RR_{50}=5.02 fold). Furthermore, the confidence limits of LC_{50}'s were not overlapped. As a consequence of the different slopes and intercepts of deltamethrin and malathion regression lines of both strains, the RR ratios were varied at LC_{90} for both insecticides (Fig 1: c, d). The deltamethrin RR_{90} decreased to 2.98 fold compared with RR_{50}, while a distinct increase was recorded with malathon (RR_{90} 16.64 fold). Meanwhile, ES strain was slightly tolerant to cypermethin compared with the LS strain with RR_{50} 1.79 fold. The regression lines of both strains were crossed (Fig 1: a) and both the slopes and intercepts were not equals. The RR_{50} and RR_{90} were greatly changed from 1.79 to 9.9 fold, respectively. On the other hand, permethrin did not show any distinction between both strains because the LC_{50} were 0.778 for LS and 0.720 for EB with overlapped confidence limits at (0.05) significance level and approximately identical regression lines given that both intercepts and slopes were not varied considerably (Fig 1:b).

Enzymes activity: Biochemical data are summarized in Table (2). The detoxification enzymes activity in adults of ES strain compared with LS one showed significant higher activity of carboxylesterase, (CE) ($3.95*10^{-3}$ and $1.16*10^{-3}$) Δ OD at λ_{412}/mg protein/min, respectively. As well, the same trend was recorded with total esterase and GST, since the activity were ($1.2*10^{-2}$ and $5.3*10^{-2}$) Δ OD at λ_{450}/mg protein/min and ($6.44*10^{-3}$ and $2.42*10^{-2}$) Δ OD at λ_{345}/mg protein/min in LS and ES strains, respectively. Moreover, significant difference also was found in activity of the target site enzyme (AChE) between LS ($8.41*10^{-4}$) and ES ($2.96*10^{-3}$) ΔOD at λ_{412}/min/mg protein. Accordingly, the detoxification enzymes activity were 4.27 times for total esterase, 3.4 for CE and 3.75 for GST higher in ES population compared with LS strain.

Esterase analysis Among seven identified esterase loci, five loci were migrated to cathode and two for anode (Figure 2).

Table 1: Susceptibility of *Sitopilus oryzae* adults to the tested insecticides after 24hr exposure.

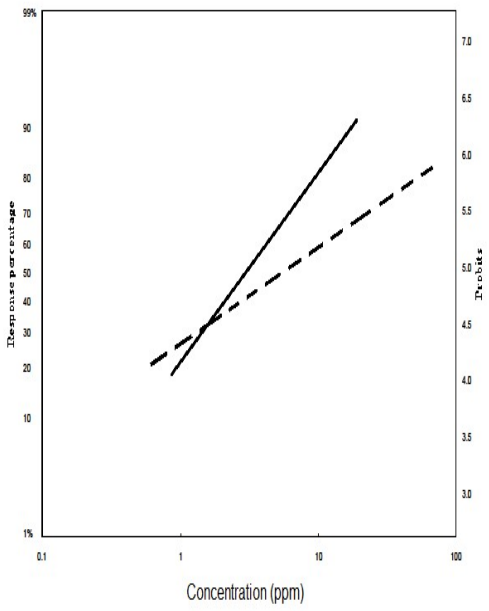
Insecticide	Strain	LC_{50} mg\l	Confidence limits	*RR_{50}	LC_{90} mg/l	Confidence limits	**RR_{90}	Slope ± variance	Chi^2
Cypermethrin	LS	2.960	2.40 - 3.69	--	17.43	10.91 - 36.96	--	1.68 ± 0.25	1.897
	EB	5.030	3.61 - 8.09	1.79	172.58	61.66 - 1187.63	9.90	0.85 ± 0.14	1.72
Deltamethrin	LS	0.230	0.15 - 0.33	--	7.68	5.22 - 12.5	--	0.84 ±0.07	0.33
	EB	1.197	0.82 - 1.50	5.20	22.86	13.85 - 48.67	2.98	1.00 ± 0 .11	7.29
Permethrin	LS	0.778	0.58 - 0.99	--	9.35	6.59 - 15.18	--	1.19 ± 0.12	3.12
	EB	0.720	0.56 - 0.89	0.93	8.71	6.20 - 13.81	0.93	1.18 ± 0.11	3.33
Malathion	LS	0.250	0.16 - 0.33	--	1.79	1.33 -3.01	--	0.15 ± 0.23	5.23
	EB	2.270	1.79 - 3.08	9.08	29.79	15.53 - 87.82	16.64	1.15 ± 0.15	4.69

LS: Laboratory-susceptible strain
EB: El-Behera strain
*RR_{50}=Resistance ratio (LC_{50} of the EB strain/LC_{50} of LS strain)
**RR_{90}=Resistance ratio (LC_{90} of the EB strain /LC_{90} of LS strain)

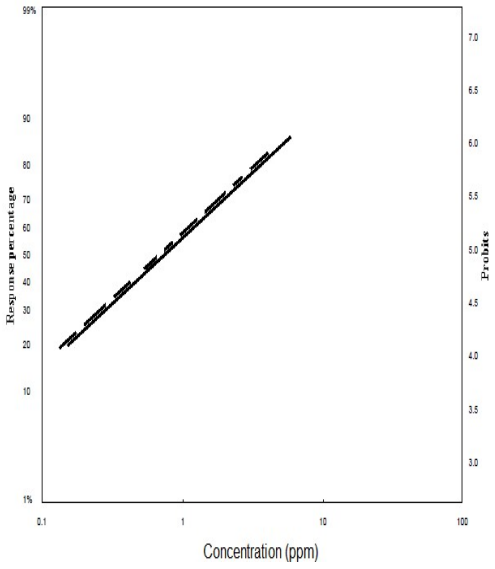
Table 2: Total esterase, acetylcholinesterase, carboxylesterse and Glutathion *S*-tranferase activity in *Sitopilus oryzae* adults of both laboratory- susceptible (LS) and El-Behera field strains(EB).

Emzyme	Strain		AR**
	LS	EB	
	Specific activity* (± SE)	Specific activity* (± SE)	
Total esterase	1.23×10^{-2} (0.1×10^{-3})	5.30×10^{-2} (5.1×10^{-4})	4.23
Acetyle- cholinesterase	8.41×10^{-4} (4.1×10^{-5})	2.96×10^{-3} (6.8×10^{-5})	3.52
Carboxylesterase	1.16×10^{-3} (2.2×10^{-5})	3.95×10^{-3} (1.1×10^{-4})	3.40
Glutathion *S*- transferase	6.44×10^{-3} (1.0×10^{-3})	2.42×10^{-2} (5.0×10^{-4})	3.75

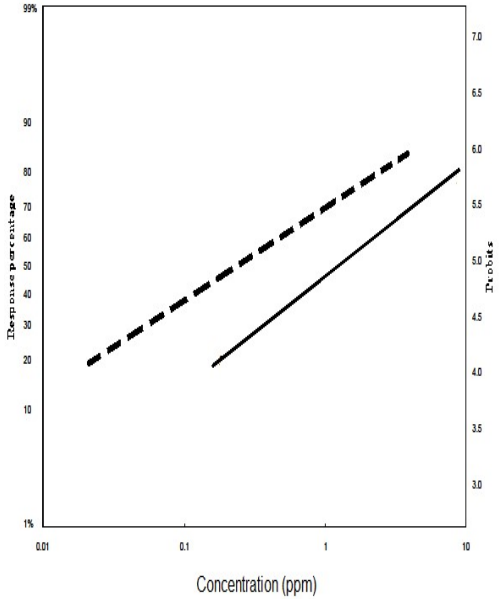
*Specific activity = ΔOD/mg protein/min
**AR=Specific activity of EB strain/Specific activity of LS strain



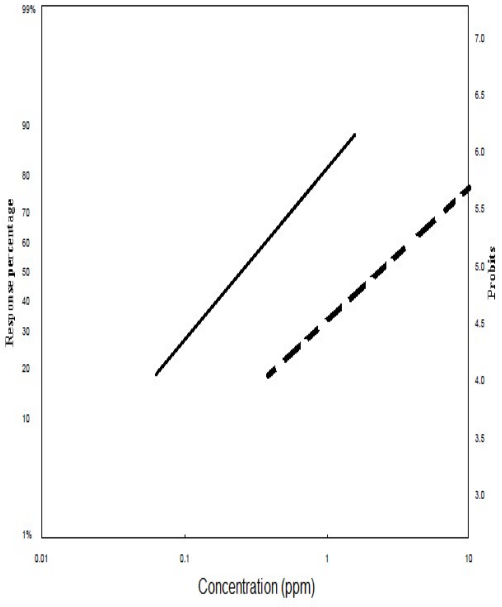
Cypermethrin



Permethrin



Deltamethrin



Malathion

Figure 1: Ld-p lines of *Sitophilus oryzae* adults to the tested insecticides Laboratory- Susceptible strain (___) and El- Behera strain (- - -)

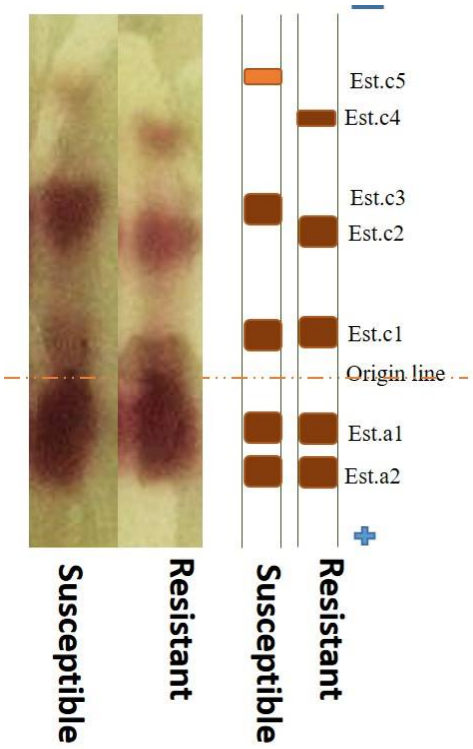


Figure 2: Esterase isozymes profile of *Sitophilus oryzae* adult of the laboratory susceptible (LS) and El-Behera (EB) field strains (Agar-starch-polyvinyl pyrolidine electrophoresis gel stained with α and ß naphthyl acetate).

A common locus (Est1) was detected for all samples. Although, an extra locus (Est5) was detected in the LS weevils' pattern compared with the resistant EB weevils, both strains were sharing four loci (Est.1, Est.2, Est.3 and Est.4) in the cathode direction. On the other hand, in the anode direction, two loci were identified in both strains pattern. In addition, variance in the staining intensity was observed between both strains and the loci size, noticeably, the staining intensity was darker in the resistant strain compared with the susceptible one.

DISCUSSIONS

According to bioassay data, ES strain developed resistant to malathion and vigor tolerance to deltamethrin but not to permethrin and only marginally to cypermethrin. Similarly, previous studies demonstrated that, *S. granaries* from Aptain, Yugoslavia was resistant to malathion and deltamethrin with RR_{50} 4.2 and 3.4 , respectively, while the same strain was entirely susceptible to cypermethrin (Kljajić and Perić, 2006). Also, resistant to malathion and deltamethrin were observed in *R. dominica* from India (Babu *et al*, 2017). Distinctly malathion still the most available and acceptable pesticide for grain protection in Egypt (APC, 2017). Although pyrethroids are not incorporated in stored grain protection programs in Egypt, EB strain showed tolerance to deltamethrin and cypermethrin which underline the consequence of wheat importing. As researchers referred the unpredictable resistant distribution of *S. zeamais* Brazilian populations to the trading and local selection (Guedes *et al*, 1995 and Fragoso et al, 2005). Apparently, the log dosage probit lines (Ldp lines) of EB and LS strains for malathion and deltamethrin were neither parallel nor possessed equal intercepts which suggest an alternation of detoxification enzymes qualitatively, and/or quantitatively or may be dissimilar enzymes involved in the detoxification process in both strains (Hardman et al , 1959 and Kuperman *et al* , 1961). Furthermore, these Ldp lines showed heterogeneity in EB population which proposes that, resistance is in a developing stage and alerts supposed increase in resistance level on the horizon. Additionally, these data is totally in harmony with the biochemical data, as the relatively high resistance ratios recorded with malathion and deltamethrin could be a consequence of biochemical transformations in EB strain that were observed as elevation in activity of total esterase, CE and GST as well AChE. Insecticide resistance in insects could be referring to three major mechanisms. First, the behavioral mechanism; which occurs as result of conversion in

insect habits towards the minimum exposure (Hemingway and Ranson, 2000) and (Lee and Lees, 2001). Second, The physiological mechanism that includes the changes in penetration and transportation of the pesticide into the insect body (Scott, 1999). Third, the biochemical mechanism which is representing in metabolic resistance and the target site insensitivity (Gao *et al*, 2006), (Konuş, 2015). Metabolic resistance includes the elevation or alternation in activity of some enzyme families; mixed function oxidase P450, esterase and glutathione *S*- transferase that lead to accelerate pesticide detoxification (Paine and Brooke, 2016). The resistance mechanism of elevation or alternation of detoxifying enzymes has contributed to a reduce of the available dose at the target site that is crucial to cause the lethal effect in an insect, while alternation of target site contributes to lose the binding between the insecticide molecule and its target (Panini *et al*, 2016). Esterase based resistance could be achieved as a result of qualitative and/or quantitative alternation causing enzyme overproduction or modifications of enzyme structure (Oppenoorth and van Asperen, 1960). Both malathion and deltamethrin insecticides could be subjected for hydrolysis by esterase (Konus, 2015) and thus the high activity of esterase which was detected in EB adults suggest that, esterase are involved in malathion and deltamethrin resistance mechanisms in this population. CE was found to attack the carboxylester moiety of malathion molecule (Matsumura and Brown, 1960) thus organophosphate resistance, which refers to CE is known as specific esterase mechanism. This mechanism was confirmed in most cases of malathion resistance in *T. castaneum* strains (Julio *et al*, 2017). Moreover, Pyrethroid resistance have been reported to be correlated to esterase in *Tribolium castaneum* (Dyte and Rowlands 1968) and (Wool and Front 2002). Accordingly, in our case of study we may consider deltamethrin resistance and cypermethrin resistance partly lay on esterase as pyrethroids has been found to be sequestered in the insect's haemolymph as consequence of a high affinity binding site on carboxylesterase (Lee and Clark 1996) and (Boyer *et al*, 2012). Moreover, esterase analysis indicated high degree of genetic polymorphism between EB and SL populations of *S. oryzae*. The results in line with that reported by (Coelho-Bortolo *et al*, 2016) in which genetic variance might be attributed to wide geographical distribution and importing of stored product commodities. Esterase isozymes in insects often show large polymorphism that resultant in strong selection pressure such as what was recorded in *T. castaneum* (Price, 1984) and *O. surinamensis* (Lee and Lees,2001) and (Silva and Lapenta 2011). Our data suggested that esterase(s) are obviously involved in resistance to malation and deltamethin in EB strain. However, other studies revealed a negative relation between pyrethroid resistance and esterase activity patterns (Han *et al*, 1998) and(Ali and Turner 2001) accordingly other mechanisms in particularly target site insensitivity may be involved in decreasing susceptibility to deltamethrin and cypermethrin in EB strain (Collins, 1990). The development of resistance in EB strain to cypermethrin and deltamethrin but not to permethrin might be due to their distinct mode of action which also correlated to absence (permethrin) or presence of cyano group (cypermethin and deltamethrin). Furthermore, AChE as a non metabolic resistance known as a target site insensitivity mechanism found to be related to malathion resistance (Guedes and Kun Yan Zhu 1998) and (Lee and Lees 2001). Malathion molecule is a competitive inhibitor analogous to the molecule of Ach, the substrate of AChE and the resistance mechanism occurs due to mutation in the target protein (Fournier and Mutero, 1994). Also, GST is involved in organophosphoates resistance through O- dealkylation or O-dearylation (Hayes and Wolf 1990) and (Huang *et al*, 1998) detoxification of metabolites as a minor mechanism (Hemingway *et al*, 1991) and dehydrochlorination (Clark and Shamaan, 1984). While dissimilar mechanisms found to be associated with pyrethroid resistance since GST eliminate lipid peroxidation products which is initiated by pyrethroids via preventing oxidation which is the reason of insect damage (Vontas *et al.,* 2001) and/or through a sequestration mechanism by binding pyrethroid molecules (Kostaropoulos *et al*, 2001). Moreover, GST may have a role as binding proteins increasing the activity of other detoxifying enzymes as esterase (Grant and Matsumura 1989) and (Kostaropoulos *et al*, 2001). In conclusion, this study results indicated that EB population developed resistance to malathion, deltamithrin and slightly to cypermethin but not to permetrin. There was genetic polymorphism between EB and SL populations. These changes and the resistance appear to be directly related to selection pressure due to continuous application of malathion and ultimately related to wheat importing. The mechanism of resistance suggested being biochemical alteration. These results suggest that EB population became resistant but the resistance is still in its developing stages which underline the importance of periodic monitoring as the foremost step in pest control. However, further studies using synergists and molecular techniques are required to determine the specific resistance mechanism and the possible countermeasures precisely.

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الملخص العربي

حالة المقاومة فى سوسة الأرز لبعض المبيدات الحشرية والميكانيكيات المرتبطة بها

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تم تجميع سوسة الأرز من محافظه البحيرة. وعند مقارنة سمية بعض المبيدات المختبرة لتلك السلالة مقارنة بالسلالة المعملية أظهر الجيل الأول إنخفاض في مستوي الحساسية لكلا من مبيدي الدلتامثرين و الملاثيون. وكان معدل السمية النسبية لمبيد الملاثيون اعتماداً على قيمة الجرعة المسببة لموت ٥٠% من أفراد كل سلالة هي (٩,٠٨) ضعف ومعدل السمية النسبية اعتمادا على قيمة الجرعة المسببة لموت ٩٠% من أفراد كل سلالة هي (١٦,٦٤) ضعف بالمقارنة بالسلالة الحساسة. كما أظهرت سلالة البحيرة تحمل لمبيد الدلتامثرين حيث كان معدل السمية النسبية اعتماداً على قيمة الجرعة المسببة لموت ٥٠% من أفراد كل سلالة (٥,٢) ضعف. كذلك رصدت مقاومه ضئيلة لمبيد السيبرمثرين بيمنا أظهرت سلالة البحيرة حساسية لمبيد البيرمثرين. وأظهرت نتائج الدراسات البيوكميائية ارتفاع نشاط بعض الأنزيمات التي لها دور في إزالة السمية(الاستيريزات الكلية، أنزيم كربوكسيل إستيريز، أنزيم جلوتاثيون اس ترانسفيريز) مما يشير إلى أن المقاومة ربما تكون نتيجة لميكانيكية إزالة السمية. و قد أظهرت سلالة البحيرة ارتفاع ملحوظ في نشاط أنزيم الاستيايل كولين استيريز مقارنة بالسلالة المعملية يعادل ٣,٥٢ ضعف. وبالتالي قد يكون لهذا الانزيم دور في ظهور وتطور صفة المقاومة في هذه السلالة. بالإضافة إلي ذلك أوضح بروفيل الاستيريز وجود بعض التغيرات الوراثية بين سلالة البحيرة والسلالة المعملية وربما يعزي ذلك إلي الضغط الانتخابي نتيجة التعرض المتكرر للمبيدات. وأكدت الدراسات البيوكيميائية أن سوسة الآرز(سلالة البحيرة) طورت مقاومة لكل من مبيدي الملاثيون والدلتامثرين مما يؤكد أهمية الرصد الدوري لوجود المقاومة ومستوياتها حتى يمكن التدخل بالبرامج الفعالة لمكافحة سوسة الأرز وغيرها من الآفات الحشرية التي تهاجم الحبوب المخزونة.