Frequency distribution of non-specific esterase in susceptible, field and cypermethrin resistant strains of *Culex pipiens* mosquito

By

Elghareeb, T.A.; Abdel-Aal, Y.A.I.; Ezzeldin, H.A. and Abdu-Allah, G.A.M

Abstract

The frequency distribution of the non-specific esterase activity using α-naphthyl acetate as substrate in susceptible (S-), field parent and cypermethrin resistant (CYP-R) strains were measured in the homogenate of individual larva (about 260 larvae) from each strain. The activity was calculated as *n*mol α-naphthyl acetate hydrolyzing/min/larva. The results indicated that the differences in esterase activity among the individual larvae were about 3.5, 7.0 and 15-fold in CYP-R-strain, field parent and susceptible strains, respectively. The mean values of α-naphthyl acetate hydrolyzed by esterases from susceptible, field parent, and cypermethrin resistant strains were 17.66, 61.31 and 216.36 *n*mol substrate hydrolyzing/min/larva, respectively. Based on the mean value of the esterase activity in resistant strain (216.36), it can be calculate the percentage of resistant genotype in the field parent strain which was up to 9.34%. The frequency distribution of non-specific esterase activity in individual larvae of the three tested strain revealed that the majority of the individuals exhibited a distinguishable activity pattern, consistent with the susceptibility to the pyrethroid cypermethrin. The results suggest that the metabolic detoxification processes via the non-specific esterases play an important role as resistance mechanism against cypermethrin insecticide in the larvae of *C. pipiens* mosquito.

Introduction

Over 135 pests and vector *Anopheline* and *Culicine* mosquito species found in association with habitats and their medical importance are reported. Malaria and Japanese encephalitis are two most serious human diseases transmitted by

mosquitoes but they have been incriminated as vectors of dozens of arboviruses and other parasites and pathogens including the causal agents of West Nile and Rift valley.

The control of mosquito populations depends heavily on chemical applications. To date, major classes of insecticides used to control mosuqiotes are mainly belong to pyrethroid, organophosphate, carbamate (Nauen, 2007). Over-dependence on chemical control has induced insecticide resistance (Hemingway and Ranson, 2000; Rodriguez *et al*., 2001; Casida and Quistad, 2004) and subsequently caused control failures. Using chemical pesticides has generated several problems including insecticide resistance, safety risks for humans and domestic animals and other environmental concerns. These problems and the high cost and sustainability of programs based predominantly on conventional insecticides have stimulated increased interest in integrated control measures (Lacey and Lacey, 1990).

Resistant population of pests including mosquitoes has been reported in many corners of the world (Daaboub *et al*., 2008; Loke *et al*., 2010; Ranson *et al*., 2010 and Lima *et al*., 2011). The emergence of mosquito strains resistant to insecticides widely used in malaria and dengue control has the potential impact severely on the control of these disease vectors. To date, four major types of insecticide resistance mechanisms have been documented: point mutations in insecticide-targeted site genes, over-expression or mutatiosn in the regions of detoxification enzymes, over-expression or mutations in genes involved in cuticle formation and behavioural changes (Whalon *et al*., 2008 and Ranson *et al.*, 2011). The main mechanisms described to date involved either point mutation in insecticide-targeted sites and/or more efficient detoxification mechanisms. The latter, also known as metabolic resistance mainly occurs due to an increase in the expression or activity of three major enzyme families: esterases, glutathione-S-transferases and the cytochrome P450 superfamily of enzymes (Hemingway *et al*., 2004; Li *et al*., 2007 and Russell *et al*., 2011).

Measuring the activity of these enzymes in natural populations is an important step in nontoving insecticides mechanisms worldwide and should be conducted together with the surveillance of control efficacy to prevent significant changes in susceptibility to insecticides being used (Coleman and Hemingway, 2007; Polson *et al*., 2011 and Mentella *et al*., 2012).

The objectives of this study are to determine frequency distribution of non-specific esterases activity in three strains of *Culex pipiens* larval, susceptible, field parent and cypermethrin resistant (CYP-R) strains. The non-specific esterases activity measured in the homogenate of individual larvae of each strain. The correlation between the frequency distribution of non-specific esterase activity toward α-naphthyl acetate as substrate and the susceptibility of the three tested strains to cypermethrin was discussed.

Materials and Methods

1- Chemical used for enzyme assay:

Anhydrous mono- and dibasic sodium phosphate, α-naphthyl acetate (α-NA), 4-aminoantipyrin, potassium ferric cyanide, phenylthiourea. All the chemical used in the esterase activities were purchased from Sigma Chemical Co.

2- Insect strains:

Three strains of *Culex pipiens* were used in this study as follows:

A – Susceptible strain (S-strain)

The susceptible strain used in the present study was brought from the Institute of Veterinary and Medical Insect in Cairo, which reared in lab for 3 years away from any insecticidal exposure. This strain characterized by the LC_{50} to cypermethrin equal 0.17 ng a.i/L, the esterase inhibitor synergist triphenylphosphate (TPP) synergized the toxicity of cypermethrin by 1.01-fold and the non-specific esterases activity based on $t_{0.5}$ value was 12.06 Min (mg protein/ml).

B – Field parent strains:

The parent field strain was wild larvae (4th instar larvae) of *C. pipiens* which was collected from Arab El-Madabegh area (Wester-most of Assiut City). The non-specific esterase was carried out in 4th instar larvae of field parent strain during 48 hr after the wild larvae transferred to our laboratory. The field parent strain characterized by: the resistance ratio at LC_{50} value (RR_{50}) against the pyrethroid cypermethrin was 4.38-fold, and the activity of the non-specific esterases based on $t_{0.5}$ value was 9.1 Min (mg protein/ml).

C – Cypermethrin resistant strain (CYP-R-strain):

This strain was obtained by selected a part of the field parent strain (≃ 5000 4th instar larvae) with the pyrethroid insecticide cypermethrin solution. Selection pressure was carried out using dipping method to 4th instar larvae for ten successive generations. Each generation was selected by cypermethrin concentration equals the LC_{50} value. The cypermethrin resistant strain was characterized by LC_{50} value 303.548 ng a.i. against cypermethrin.

The cypermethrin resistant strain (CYP-R-strain) exhibit 1,785.58-fold resistance compared to S-strain. The esterase inhibitor synergist (TPP). Synergized the toxicity of cypermethrin by 13.43-fold. TPP reduced the resistance level toward cypermethrin in CYP-R-strain from about 1786-fold to 135-fold resistance. The percentage reduction in resistance level to cypermethrin up to 92.5%. The non-specific esterases activity based on $t_{0.5}$ value was 0.62 Min (mg protein/ml).

3- Rearing procedure:

All strains transferred to laboratory of Plant Protection Department, Fac. Agric., Assuit University under laboratory conditions of 25±2 and 60±5 R.H. during the study (Chander *et al*., 1997). Mosquito (S-strain and cypermethrin resistant strain) rearing was maintained in enamel trays. Transformed pupae were collected from the formentioned trays by means of a wide mouth glass

dropper, then pipette into Petri dishes that were placed in the adult cages (30 cm dimensions). The emerging males and females were fed on 10% sucrose solution and guinea pig blood males, respectively. Suitable containers for egg-laying were provided to the cages 48 hours after the females for egg-laying were provided to the cages 48 hours after the females had their blood meal. Receptacles containing egg rafts were daily collected from the cages, then the newly hatched larvae were then transferred to the breading trays, each containing 2 inch high tap water and such breeding trays bar each a maximum 500 larvae of the same age. After twenty four hours, the hatch larvae were fed on fresh yeast and protein which was evenly sprinkled on the water surface twice daily. They left over yeast, that was not ingested, was carefully removed with a medicinal dropper. Mass-rearing colony of all stages was always maintained at 25±2°C and 60±5% R.H. Temperature and relative humidity reading were daily measured using a thermograph and hydrograph, respectively.

4- Frequency distribution of non-specific esterase activity in the three tested strains of *C. pipiens* mosquito:

The non-specific esterase activity toward α-naphthyl acetate as substrate was examined in individual larvae of susceptible (S-) field parent and cypermethrin resistant (CYP-R) strain of *C. pipiens* mosquito. The hydrolysis product of α-naphthyl acetate was determined colorimetrically according to the method of Bracha and Bonard (1966) using seqoia-turner Model 340 spectrophotometer. A total of 260, 262 and 264 4^{th} instar larvae of approximately the same size were randomly collected from S-, field parent and CYP-R-strains were separately homogenized in phosphate buffer (0.05 M; pH 7.2) then the homogenate solution was centrifuged at 10.00 r.p.m. for 5 mint and supernatant was used immediately after preparation. Then 4.7 ml of the homogenate solution was transferred to the colorimeter tab. A hundred microliters of α-naphthyl acetate (final concentration 0.5 mM) in sodium phosphate buffer (0.05 M, pH 7.2) was added and the reaction was incubated at

37°C for 10 min. At the end of the incubated period, 100 *u*l of 4-aminantipyrin (0.5% in distilled water) followed by 100 *u*/potassium ferricyanide (0.7% in distilled water) were added. Absorbance at 500 nm was read and recorded exactly 5 min after the addition of potassium fericyanide. A control tube containing every thing except the enzyme solution was used to correct for nonenzymatic hydrolysis of substrate. The absorbance data were converted from a calibration curve prepared using several concentrations of 1-naphthol under the corresponding conditions.

Results and Discussion

Figure (1) shows the magnitude of larval esterase activity toward α-naphthyl acetate as substrate within and between samples of the 4th instar larvae of susceptible (S-), field parent strain (G_0) and CYP-R-strain (G_{10}) of *C. pipiens* mosquito. The esterase activity was measured in homogenate of individual larva from each strain. It is clear from the frequency distribution of this figure that the difference between the highest and lowest activity of esterases from individual larva was 8.0, 16 and 3.5-fold for susceptible, parent and CYP-R-strain, respectively. These results indicated that the larvae of resistant strain exhibited highest level of homogeneity for the non-specific esteases activity, where larval activity varied by only 3.5-fold. This homogeneity was also observed in the susceptible strain but to a lower extent, whereas the maximum differences in esterase activity between the individual larva was about 7.0-fold. In contrast, the parent field strain (G_0) exhibited highest degree of heterogeneity with respect to esterase activity between the individual larva whereas the difference between the maximum and the lowest activity was 16-fold. The mean value of non-specific esterases among the tested individual representing the three tested strains was calculated. This value in susceptible, field parent and cypermethrin resistant strains was 17.66, 61.31 and 216.36 *n*mol substrate hydrolyzing/min/larva, respectively.

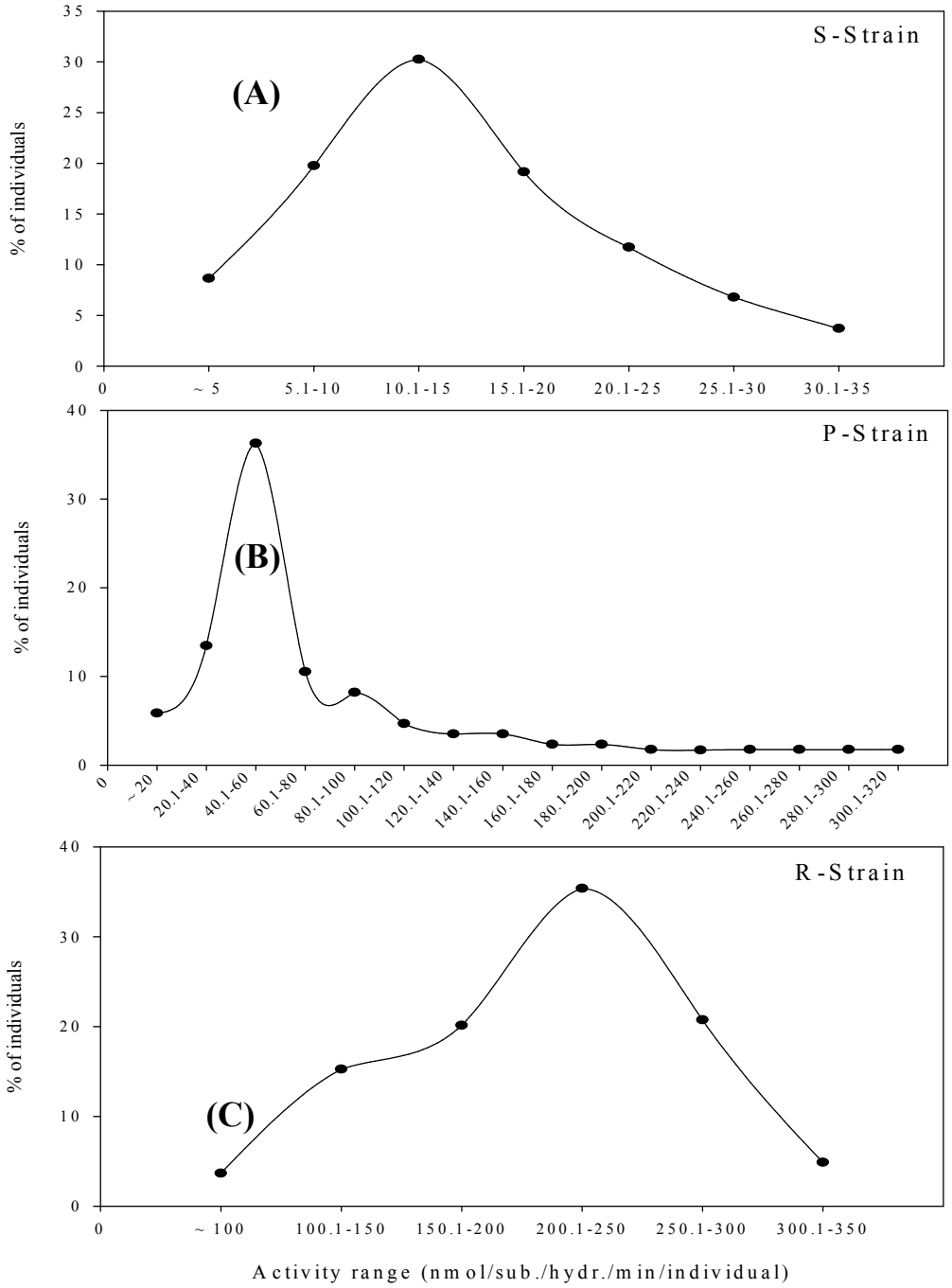

Fig. 1: Frequency distribution of larval esterase activity (expressed as *n*mol α-naphthyl acetate hydrolyzing 1 min/individual) measured in homogenate of individual larvae from (A) susceptible strain (B) parent field strain (G0) and (C) cypermethrin resistant strain (CYP-R). The sample size for each of the tested population was about 260 larvae, obtained from the same sources of mentioned in toxicity bioassay.

The difference in frequency distribution as well as the mean activity between both the parent, cypermethrin-resistant and the susceptible strain explains other results in the present study. Firstly, the potential of the parent strain to develop esterase-mediated resistance was obvious. Secondly, the high extent of cypermethrin resistance after only10 generations of cypermethrin pressure is explained from the exceptional high mean esterase activity in the frequency distribution curve of individual larvae from the 10^{th} generation. Thirdly, the mean activity of esterase in individual larvae of the susceptible, field and cypermethrin resistant strains (respectively, 17.66, 61.31 and 216.36 nmol/min/individual) is qualitatively correlated with the LC_{50} values of cypermethrin against these strains (respectively, 0.17, 0.745, 303.548 ppb). Additional evidence to esterase-mediated resistance in *C. pipiens* is concurrenly obtained not only at the average activity level, but also at the population level. The results in the present study are in agreement with that of Yebakima *et al*. (2004) and Liu *et. al*. (2011) on *C. pipiens*; of Sahgal *et al*. (1994) on other three species of mosqitoes viz., *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* and of Etang *et. al*. (2007) on *Anopheles gambiae* S.L. Esterase over production achieved predominantly by gene up-regulation, is a frequent mechanism of resistance to OP- and pyrethroid insecticides in *C. pipiens* complex (Rooker *et al*., 1996; Raymond *et al*., 1998 and Liu *et al*., 2006). Two gene loci on chromosome α, *Est*-3 (coding estase A) and *Est*-2 (coding esterases β), encode non-specific esteraess through gene amplification or up-regulation in *C. pipiens* complex, thereby expressing a higher amount of non-specific esterase and providing a substantial of insecticide resistance (Rooker *et al*., 1996; Raymond *et al*., 1998; Cui *et al*., 2007 and Liu *et al*., 2006).

From the results of this study, it could be conclude that the metabolic detoxification processes via non-specific esterases may be play a role in

conferring pyrethroid resistance to the larvae of resistant strain (CYP-R-strain) of *C. pipiens* mosquito.

References

Bracha, P. and Bonard, J.P. (1966). Determination of Bromophos residues. J. Agric. Chem. 14: 609-612.

Casida, J.E. and Quistad, G.B. (2004). Organophosphate toxicology: safety aspects of non-acetylcholinesterase secondary targets. Chemical Research in Toxicology, 17: 983-998.

Chandre, F.; Harriet, F.; Hoannio, J.; Riviere, F.; Pasteur, V. and Guillet, P. (1997). Distribution of organophosphate and carbamate resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) in West Africa. J. Medical Entomology., 34 (6): 664-671.

Coleman, M. and Hemingway, J. (2007). Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. J. Pest. Scien., 32 (2):76-69.

Daaboub, J.; Cheikh, R.B.; Lanmari, A.; Ibtissem, B.J.; Feriani, C.B. and Cheikh, H.B. (2008). Resistance to pyrethroid insecticides in *Culex pipiens* pipiens (Diptera: Culicidae) from Tunisia. Acta, Tropica, 107: 30-36.

Etang, J.; Manga, L.; Toto J.C.; Guillet, P.; Fondjo, E. and Chandre, F. (2007). Spectrum of metabolic-based resistance to DDT and pyrethroids in Anopheles gambiae s.l. populations from Cameroon.J Vector Ecol. 32(1):123-33.

Hemingway, I. and Ranson, H. (2000). Insecticide resistance in insect vectors of human diseases. Ann. Rev. Entomol. 45: 371-391

Hemingway, J.; Hawkes, N.; McCarroll, L. and Raason, H. (2004). The molecular basis of insecticide resistance in mosquitoes. Insect. Biochem. Mol. Biol. 34: 653-665.

Lacey, L.A. and Lacey, C.M. (1990). The medical importance of Riceland mosquitoes and their control using alternatives to chemical insecticides. J. The Amerca. Mosq. Cont. Assco. 2: 1-93.

Li, X.; Schuler, M.A. and Berenbaum, M.R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol. 52:231-53.

Lima, E.; Paiva, M.; de Araujo, A.; da Silva; E.; Da Silva U.; de Oliveira, L.; Santanna, A.E.; Barbosa, C.; de Paiva Nete, C.; Goulart, M.; wilding, C.; Ayres, C. and de Melo Santos, M.A. (2011). Insecticide resistance in *Aedes aegypti* populations from Ceara, Brazil. Parasites and Vectors, 4: 5-20.

Liu, N.; Xu, Q.; Zhu, F. and Zhang, L. (2006). Pyrethroid resistance in mosquitoes. Insect Science, 13: 159-166.

Liu, Y.; Zhang, H.; Qiao, C.; Lu, X. and Cui, F. (2011). Correlation between carboxylesterase alleles and insecticide resistance in *Culex pipiens* complex from China. Parasit Vectors. 19 (4): 236-242.

Loke, S.R.; Andy-Tan, W.A.; Benjamin, S.; Lee, H.L. and Sofian-Azirun, M. (2010). Susceptibility of field-collected *Aedes aegypti* (L.) (Diptera-Culicidae) to *Bacillus thuringiensis* israelensis and temephos. Tropical Biomedicine, 27: 134-139.

Montella, I.R.; Schama, R. and Valle, D. (2012).The classification of esterases: an important gene family involved in insecticide resistance a review. Mem Inst Oswaldo Cruz.107:437-49.

Nauen, R. (2007). Insecticide resistance in disease vectors of public health importance. Pest Manag. Sci., 63: 628-633.

Polson, K.A.; Brogdon, W.G.; Rawlins, S.C. and Chadee, D.D. (2011). Characterization of insecticide resistance in Trinidadian strains of *Aedes aegypti* mosquitoes. Acta.Trop.117(1):31-8.

Ranson, H.; Burhani, J.; Lumjuan, N. and Black, W.C. (2010). Insecticides resistance in dengue vectors. Trop IKA. Net J. 1.

Ranson, H.; Nguessan, R.; Lines, J.; Moiroux, N.; Nkuni, Z. and Corbel, V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control.Trends Parasitol., 27(2): 91-8.

Raymond, M.; Cheuillon, C; Guillemaud, T.; Lenormand, T.; Pasteur, N.; Denho, I.A., Pickett, I. and Devonshir, A.L. (1998). An overview of the evolution of over produced esterases, in the Mosquito *Culex pipiens.* Biological Sciences, 353, 1376: 1707-1711.

Rodriguez, M.M.; Bisset, J.; Fernandez, D.M.; Lauzan, L. and Soca, A. (2001). Detection of insecticide resistance in *Aedes aegypti* (Diptera: Culicidae) from Cuba and Venezuela. J. Med. Entomol, 39: 623-628.

Rooker, S.: Guillemaud, T.; Berge, J.; Pasreur, N. and Raymond (1996). Coamplification of esterase A and B genes as a single unit in mosquito *Culex pipiens*.Heredity, 77:555-561.

Russell, R.J.; Scott, C.; Jackson, J.C.; Pandey, G.P.; Taylor, M.C.; Coppin, W.C.; Liu, Jian-Wei and Oakeshott, J.G. (2011).The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects. Evol. Appl. 4(2): 225–248.

Sahgal, A.; Kumar, S. and Pillai, M.K.K. (1994). Microplate Assay of elevated esterase activity in individual pyrethroid resistant mosquitoes. Journal of Biosciences, 19: 193-199.

Whalon, M.E.; Mota-Sanchez, D. and Hollingworth, R.M. (2008). Analysis of global pesticide resistance in arthropods," in Global Pesticide Resistance in Arthropods, M. E. Whalon, D. Mota-Sanchez, and R. M. Hollingworth, Eds., pp. 5–31, CABI, Oxfordshire, UK. 305 pp.

Yebakima, A.; Marquine, M.; Rosine. J.; Yp-Tcha, M.M. and Pasteur, N. (2004). Evaluation of resistance under insecticide selection pressure in *Culex pipiens quinquefascialus* (Diptra: Culieidae) from Martinique. J. Med. Entomol. 41: 718-725.

التوزيع التكراري لنشاط الإنزيمات المحللة للأسترات في ثلاث سلالة حساسة وحقلية ومقاومة لمبيد السيبرمثرين

تسنيم عبدالرءوف الغريب ، يحي عبد الحميد عبد العال ، حسام عز الدين ، جمال عبد الله

قسم وقاية النبات – كلية الزراعة – جامعة أسيوط

أجري تقدير التوزيع التكراري لنشاط الإنزيمات المحللة للأسترات في ثلاث سلالات هي سلالة حساسة وأخري حقلية وسلالة مقاومة لمبيد السيبرمثرين (1786 مقاومة قدر السلالة الحساسة) وتم تقدير النشاط باستخدام مادة الألفانافثيل أستيت كمادة للتفاعل وقدر النشاط علي أساس التحلل الأنزيمي لمادة التفاعل مقدراً بالنافومول لكل دقيقة لكل يرقة – حيث استخدمت عدد لا يقل عن 260 يرقة من كل سلالة وكان مصدر الإنزيم هي يرقات العمر الرابع من كل سلالة عن طريقة الطحن والطرد المركزي لكل يرقة علي حدة. بعد تقدير النشاط في رسم التوزيع التكراري لكل سلالة علي حدة وقد أظهرت النتائج ما يأتي:

أولاً: إن اليرقات في السلالة المقاومة أظهرت درجة عالية من التجانس حيث أن الفرق بين أعلي وأقل نشاط لكل يرقة لا يزيد عن 3.5 مرة بينما كانت درجة التجانس أقل في السلالة الحساسة حيث كان الفرق بين أعلي واقل نشاط لكل يرقة 8 مرات بينما أظهرت السلالة الحقلية (هي سلالة الآباء التي تكونت منها السلالة المقاومة للسيبرمثرين) أعلي درجة من عدم التجانس وكان الفرق بين أعلي وأقل نشاط لكل يرقة هو 16 مرة.

ثانياً: بحساب متوسط النشاط لكل اليرقات المستخدمة لكل سلالة وجد أن متوسط النشاط كان للسلالة الحساسة والسلالة الحقلية والسلالة المقاومة علي الترتيب 17.66 و 61.33 و 216.36 نانومول تحلل مادة التفاعل لكل دقيقة لكل يرقة ومن منحني التوزيع الطبيعي أمكن حساب عدد الأفراد المقاومة المحتمل وجودها في السلالة الحقلية والذي تحمل عوامل وراثية خاصة بالمقاومة عن طريق زيادة نشاط الإنزيمات المحللة حيث تصل نسبة هذه الأفراد إلي 9.34% من جملة العينة المستخدمة.

ثالثاً: أن التوزيع التكراري لنشاط الإنزيمات المحللة لكل يرقة في السلالات الثلاثة يتفق مع درجة التحمل لكل سلالة تجاه السيبرمثرين.

رابعاً: الدراسة تعتبر هامة جداً حسب الاتجاهات الحديثة لدراسة التركيب الجيني لكل مجتمع حشري قبل إجراء عملية المكافحة عن طريق الكيمياء الحيوية أو التكنولوجيا الحيوية لمعرفة نوع وعدد وتوزيع الأفراد المقاومة لكل مجتمع حشري وهذا يساعد علي اختيار المبيدات المناسبة ويقل فرصة تكوين السلالات المقاومة.