# Malathion resistance and acetylcholinesterase enzyme changes in field population of the peach fruit fly, *Bactrocera zonata* (Saunders)

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**Abstract:** The peach fruit fly, *Bactrocera zonata* (Saunders) is the most destructive pest of fruits in Egypt. The management of *B. zonata* has been based on the use of malathion (organophosphate insecticide), a practice that induced resistance. The high resistance ratio (RR=30.47 fold) and resistance coefficient (RC=75.33) to malathion were detected in a field population of *B. zonata* compared with the laboratory susceptible strain. There is no cross-resistance between imidacloprid, spinosad lambda-cyhalothrin and malathion resistance in the field flies. The rotation of insecticides with different modes of action is a desirable in insect resistance management programs. The activity of acetylcholinesterase enzyme extracted from heads of laboratory flies was 1.7fold more than that of field flies selected for three generations with malathion (RR=116.4). AChE of malathion resistant insects shows lower catalytic efficiency for substrate and 33.50, 41.14 and 835.58 fold less sensitive to inhibition by paraoxon, Chlorpyrifos-oxon and malaoxon, respectively, compared to that of the laboratory susceptible insects. Direct sequencing of cDNA fragment (264bp) produced from RT-PCR (based on *B. dorsalis* acetylcholinesterase gene (Ace) mRNA partial coding sequence from 1771 to 2034 (Hsu *et al*., 2008) of lab and resistant *B. zonata* total RNA gave 88.3 and 86.4% identical between them on the level of nucleotide and deduced protein, respectively. Twelve amino acid substitutions (I561L, C562S, M563D, S564A, F565V, L566N, I567D, L584F, Q585T, R627S, K630E and S631I) were detected in partial protein (551-638) of AChE from malathion-resistant flies compared with lab flies. Alterations of; aspartic acid at 563&567 positions with hydrophobic methionine & isoleucine, glutamic acid at 630 with basic lysine, hydrophilic serine at 562&627 with hydrophilic cysteine & basic arginine and hydrophobic alanine & isoleucine at 564 & 631 with hydrophilic serine in *ace* gene- C-terminal peptide may be caused resistance of the field *B. zonata* flies to malathion.

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**Key words**: *Bactrocera zonata*, malathion resistance, acetylcholinesterase enzyme, AChE gene, amino acid susbstitutions.

## 1. Introduction

The peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) is a serious pest of fruits in many parts of the world, which originates in South and South-East Asia. Four hundred species belonging to the genus *Bactrocera* are widely distributed in tropical Asia, South pacific and Australia, with very few species in Africa and Europe (Drew, 1989). Where it attacks many fruit species (more than 50 host plants), including guava, mango, peach, apricot, fig and citrus. It has spread to other parts of the world, in particular to several countries in the Near East and to Egypt. It is considered that *B. zonata* threatens countries in the Near East and North Africa, and to a lesser extent in Southern Europe (EPPO, 2002). *B. zonata* was recorded in Egypt in 1999, where it caused a severe damage to a wide range of fruits including guava, peach, apricot and mango (El-Minshawy *et al*., 1999).

Organophosphate insecticides have been used to control this pest for many contrast with its low activity in years. Malathion is the most commonly used in both aerial and ground treatments. This insecticide was the first example of a wide spectrum organophosphorus insecticide combined with a very low mammalian toxicity. The molecule contains two carboxylesters bonds whose hydrolysis led to the detoxification of the insecticide. Its high selective toxicity is attributed to high carboxylesterase activity in mammals and insusceptible insects (Eto, 1974). However, the development of even subtle resistance has been shown to be capable of causing a loss of effectiveness of such control agents. Resistance to malathion and other organophosphates was identified in field populations of the oriental fruit fly, *Bactrocera dorsalis* (Hsu and Feng, 2000), in the olive fruit fly, *Bactrocera oleae* (Hawkes *et al*.,2005), in *Drosophila melanogaster* (Harel *et al*.,2000), in the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Magaña *et al*.,2007). Similar cases of the development of resistance and subsequent reductions in effectiveness to this and other insect species in different localities (Hama, 1983; Konno and Shishido, 1989; Kozaki *et al*., 2001). Because of this,

improved understanding of the actual or potential mechanisms of resistance can be very important for preventing even greater loss of the tools available for pest control.

Acetylcholinesterase plays an essential role in neurotransmission at cholinergic synapses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Organophosphorus insecticides (OPs) bind to the active site and inhibit the enzyme, causing an accumulation of acetylcholine in the synapses. Acetylcholine concentration remains at levels which are continuously too high preventing repolarization of the nerve cell, resulting in continuous firing of the nerve and the eventual death of the animal (Eto, 1974; Main, 1979). ). OPs form a phosphorylated enzyme intermediate instead of the acyl-enzyme intermediate that is formed with the choline ester substrate. The phosphoryl enzyme intermediate is far more stable than the acyl-enzyme and the regeneration of the enzyme is extremely low, presumably due to the inappropriate geometry of the phosphate group (O'Brien, 1976; Järv, 1984). Many organophosphorus pesticides are generally poor inhibitors of esterases, unless they are converted into their active form. The activation is the transformation from the "thion" form (P=S, phosphorothionates and phosphorothiolates) to the "oxon" analog (P=O), which is the molecule active at the AChE site. This activation has been recognized to be performed by the cytochrome P450 monooxygenases (Eto, 1974). After intensive use of malathion and other OP insecticides in pest control, resistance mediated by alterations in the AChE has been selected in many insect species (Oakeshott *et al*., 2005). Changes in the gene's regulation to produce more AChE to overcome the effect of the insecticide has been reported for *D. melanogaster* (Charpentier and Fournier, 2001) and *Aonidiella aurantii* (Levitin and Cohen, 1998). However, point mutations in the AChE gene that make the enzyme less sensitive to inhibition by the insecticides, have more often been identified as being responsible for insecticide resistance (Mutero *et al*., 1994; Walsh *et al*., 2001; Vontas *et al*., 2002; Weill *et al*., 2003; Baek *et al*., 2005; Hsu *et al*., 2006). In addition to the clear evidence associating DNA changes with the acquisition of resistance, it is also important to develop a better understanding of how these mutations may exert either quantitative or qualitative effects on specific genes and their products. In some species, for example the aphid *Myzus persicae*, insecticide resistance has been associated with various mechanisms such as the overproduction of detoxifying esterases, qualitative alterations of the AChE enzyme itself and mutations in other genes conferring knockdown resistance (Margaritopoulos *et al*., 2007).

This study interest to evaluate the susceptibility of the field peach fruit fly, *Bactrocera zonata* to four insecticides; imidacloprid, lambda-cyhalothrin, malathion and spinosad. The changes in activity and sensitivity of acetylcholinesterase enzyme correlated to malathion- resistance in *B. zonata* were investigated also.

## 2. Materials and Methods

### 1. Insects

The laboratory susceptible colony of the peach fruit fly, *Bactrocera zonata* was established from collecting infested guava fruits of Ismailia Governorate in July 2006. The fruits were putted on sand in plastic trays; the full grown larvae naturally jump to the sand where they pupate. The emerged adults were placed in two liters plastic cages and fed water, enzymatied yeast, protein hydrolyzed and sugar, until the mating took place then females started oviposition on fresh guava fruits for three generations, then rearing continued on artificial bran diet for larvae. Insects were kept in constant conditions ((25 ± 2°C, 65 ± 5% R.H. and a photoperiod of 14 L: 10 D) in the Central Agricultural Pesticides Laboratory, Egyptian Agricultural Research Center, away from any pesticides exposure. Field insects were collected from infested guava fruits of Ismailia Governorate in June 2011 and kept under laboratory conditions for two generations.

### 2- Toxicological assays

Susceptibility of laboratory and field *B. zonata* adults to four formulated insecticides from different groups: the biological control agent; Spinosad (tracer 24%SC, biochemical product of Actinomycetes- Dow Agro Science), the pyrethroid; Lambda-cyhalothrin (ictone 2.5% EC –Parijat Agencies), the neonicotinoid; Imidacloprid (admire 20%SC- Bayer Crop Science),) and the organophosphate; Malathion (malason 57% EC- Ficom Organics) were tested in the laboratory. A stock solution was prepared as 0.5ml from each insecticides dissolved in 50ml water. Seven serial concentrations were prepared from this solution by added 5% sugar solution. Groups of twenty adults (3-5 days- old) were putted in clean cages without any food for 12h. Cotton piece wetted by insecticide sugar solution were added to each cage and the control insects were feed on diluted sugar solution only (five replicates for each insecticide concentration and control). Mortality counted after 48 hrs. and the $LC_{50}$, $LC_{95,}$ resistance ratio (RR) and resistance coefficient (RC) were calculated according to SAS probit (1997) program and Wegorek *et al.*(2011). The remaining field insects were treated with $LC_{50}$ of malathion for three generations (RR= 116.4 fold).

### 3. Enzymatic assays

### 3.1. Preparation of tissue homogenates

To measure AChE activity, 100 heads from 3- 5-days-old adults of laboratory susceptible and malathion resistant flies were homogenized in 0.1M sodium phosphate buffer pH7 (one head in 50 µl), containing 1% (v/v) of Triton X-100, with a Teflon glass homogenizer. Solubilized protein was isolated by centrifugation (Mikro 22 R Hettich Zentrifugen-Germany) at 16000 g for 5 min at 4 °C (three replicates of each sample). The supernatant was collected and used as enzyme source. The protein concentration was determined according to the procedure of Bradford (1976).

**3.2. Enzyme activity**

The chemicals were purchased from Sigma Chemical Company (USA), spectrophotometric measurements were made using Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Acetylcholinesterase activity was determined by the spectrophotometric method described by Ellman *et al.* (1961). The reaction mixture consisted of 10 µl of tissue homogenate, 2 µl of 100mM ASChI (acetylthiocholine iodide), 2 µl of 9.2mM DTNB (5`5-dithio-bis (2- nitro benzoic acid) and potassium phosphate buffer (0.1M ,pH 7.2) up to 200 µl. The reaction was started by the addition of the substrate (ASChI) and the reagent (DTNB), the change in absorbance at 405 nm was recorded during 5min. The activity was expressed as nanomoles /min/mg protein. The kinetic parameters (the maximum velocity Vmax and the Michael's constant Km) of AChE were determined using seven different concentrations of ASChI (10, 50,100, 250, 500, 750 and 1000 mM), and a double reciprocal plot was generated (Line weaver –Burk plot). Three replicates were performed at each substrate concentration.

The kinetics of the reaction between AChE and the organophosphate inhibitors; chlorpyrifos oxon (Chem. Service, Inc., USA), malaoxon (Fluka Chem. GmbH, Switzerland) and paraoxon-ethyl (Sigma-Aldrich laborchemikalien GmbH, USA) were investigated. Stock solutions (10 mM) of malaoxon and chlorpyrifos oxon were prepared in ethanol and paraoxon was prepared in isopropanol and stored at 4°C. Further dilutions were prepared in buffer immediately before use. Head extracts were incubated (5min) with different concentrations of each inhibitor ($10^{-8}$, $5x10^{-8}$ , $10^{-7}$, $5x10^{-7}$, $10^{-6}$, $5x10^{-6}$, $10^{-5}$) at 25°C and the control samples contained the same alcohol concentration without inhibitor. The substrate (ASChI) and the reagent (DTNB) were added and the enzyme activity was measured as described above. The experiment was performed three times. The plot of the log of residual activity (AChE) against time was linear for a given inhibitor concentration. The bimolecular rate constant (Ki) was calculated by linear regression as described by Main and Iverson (1966). Results are reported as mean ± standard error and statistically analyzed using Excel Microsoft Office and Student's t-test Program. Differences were considered significant at $p< 0.05$ level.

**4. RT-PCR and direct sequencing of partial AChE nucleotides**

Total RNA was extracted from heads of 15 flies of 3- 5-days-old of the laboratory susceptible and malathion resistant insects using a microscale total RNA extraction kit (Analytik-jena kit, extraction from tissue). After treatment with DNase, one microgram of total RNA was used for the first strand synthesis of cDNA in 20 ml of total volume using the Thermo-kit $^{TM}$ RT reverse transcription cDNA synthesis. Primers designed based on a region conserved (264 bp) in ace gene codon sequences were specifically amplify from cDNA. Primers specific to *B. dorsalis* acetylcholinesterase gene (Ace) mRNA (AY155500) were sense: CGGCAAGTTGAACGAGAG and antisense AGAGGAAGCGGATGATGG (Hsu *et al*., 2008) were syntheses (Biobasic Company). Reverse Transcriptase –PCR (Thermal cycler Gene amp. 9700) steps were initiated by 5°C for 15min then thermal program consisting of one cycle of 95°C for 2min, 40 cycles of 95°C for 30 s, 52 °C for 30 s, 72°c for 1min and followed by a final one cycle of 72 °C for 10 min was used. The assay was repeated three times with total RNA extracted separately for flies from both laboratory and malathion- resistant strains, and three replicates were carried out for each reaction to minimize intra-experiment variation. PCR products were separated by electrophoresis on 1.5% agarose gels. Direct DNA sequencing was performed in Applied Biosystems-Lab Technology. The BLASTp algorithm was used to search the NCBI database for sequences similar to *B. dorsalis* (Hsu *et al*., 2006). Sequences were aligned using Bioedit version 3.1 and phylogenated by Mega 4 software.

**3. Results**

**Susceptibility of laboratory and field *B. zonata* to tested insecticides**

Susceptibility of laboratory and field *B.zonata* to imidacloprid, lambda-cyhalothrin, malathion and spinosad was evaluated in laboratory (Table 1). Imidacloprid was the superior insecticide against lab and field insects ($LC_{50}$ values =0.75 (0.56-1.13) & 1.44 (0.81-2.63) ppm at 95%CL, respectively) followed by spinosad (0.98 (0.63-1.74) & 2.52 (1.60-4.37) ppm) and lambda cyhalothrin (2.68 (1.59-3.82) & 7.47 (2.83-11.22) ppm), respectively. Malathion was the lowest one which had very high $LC_{50}$ (2264.56 (1435.78-5726.14) ppm), resistance ratio (30.47 fold) and resistance coefficient (75.33) for field flies compared with lab flies.

**Table 1: Susceptibility of the Lab and field peach fruit fly, *Bactrocera zonata* adults to tested insecticides**

| Insecticide | Laboratory insects Slope ±S.E | $LC_{50}$ (ppm) (95%CL) | $LC_{95}$ (ppm) (95%CL) | Field insects Slope ±S.E | $LC_{50}$ (ppm) (95%CL) | $LC_{95}$ (ppm) (95%CL) | RR | RC |
|---|---|---|---|---|---|---|---|---|
| Imidacloprid | 2.12 ±0.39 | 0.75 (0.56 -1.13) | 4.37 (2.22 - 6.70) | 0.86 ±0.45 | 1.44 (0.81 -2.43) | 12.83 (9.57 -18.92) | 1.92 | 0.05 |
| Spinosad | 1.86 ±0.56 | 0.98 (0.63 -1.74) | 8.12 (5.94 -12.44) | 0.91 ±0.32 | 2.52 (1.60 -4.37) | 16.45 (11.52 -25.68) | 2.57 | 0.34 |
| Lambda-Cyhalothrin | 1.56 ±0.12 | 2.68 (1.59- 3.82) | 21.42 (15.33- 33.94) | 0.67 ±0.25 | 7.47 (2.83- 11.22) | 62.01 (32.78- 111.64) | 2.79 | 1.32 |
| Malathion | 1.72 ±0.45 | 74.33 (57.47-83.62) | 582.96 (408.14-752.63) | 1.45 ±0.21 | 2264.56 (1435.78-5726.14) | 214679.28 (35613.06- 4485129.89) | 30.47 | 75.33 |

S.E =standard error. CL = confidence limits.
Resistance ratio (RR) = $LC_{50}$ of field insects /$LC_{50}$ of laboratory insects
Resistance coefficient (RC) =$LC_{95}$ of field insects /Field recommended concentration of Insecticide

**Enzymatic activity, kinetics and inhibition of AChE in the laboratory and malathion-resistant flies**

The AChE activity of laboratory susceptible and field malathion resistant *B. zonata* flies were 355±48 and 207±69 nmoles/min/mg protein. The hydrolyzing efficiencies (Vmax) for the substrate were 167.66±23.34 and 75.28±12.50 (nM/min/mg) for lab and resistant flies**.** These values were differed significantly, the Vmax of lab strain about 2.2 fold than that of resistant strain. The substrate affinities (Km) also differed significantly between two strains (Table 2).

Three selected OP oxons were used to compare the sensitivity levels of AChE from lab and resistant *B. zonata* flies (Table 3& Figure1). Median inhibition values ($I_{50}$) show that Paraoxon was the most potent inhibitor to AChE of lab (0.482 ±0.024nM) and resistant flies (16.149±1.615 nM) followed by chlorpyrifos oxon (0.794±0.145 and 32.663±3.423 nM) and malaoxon (0.935±0.271 and 781.265 ±26.518 nM), respectively. AChE enzyme from the resistant strain was much less sensitive to inhibition compared to the AChE from the lab flies. These inhibitors also showed higher bimolecular rate constants (Ki) values for the lab flies (27.322- 55.160 $x10^7M^{-1}$ $min^{-1}$) compared to the malathion-resistant flies (10.452-29.676 $x10^7M^{-1}$ $min^{-1}$) and malaoxon showed the least ki with the resistant flies.

**Table 2: Kinetic parameter of acetycholinesterase enzyme from laboratory and field malathion resistant *Bactrocera zonata* flies**

| Insect strain | Vmax ±S.E. (nmole/min/mg) | Km ±S.E. (µM) |
|---|---|---|
| Laboratory | 167.66 ±23.34 | 42.53 ±6.82 |
| Field | 75.28 ± 12.50 | 63.68±3.17 |

Mean of three experimental replicates ± standard error (S.E.).
Significant differences between the laboratory and malathion resistant strains by Student t-test ($P< 0.05$)

**Table 3: Median inhibition values ($I_{50}$) and bimolecular rate constants (Ki) for organophosphate inhibitors to AChE from laboratory and field malathion resistant *Bactrocera zonata* flies**

| Inhibitor | $I_{50}$ (nM) | | | Ki ($x10^7M^{-1}$ $min^{-1}$±S.E.) | | |
|---|---|---|---|---|---|---|
| | Lab | Field | Field/Lab | Lab | Field | Lab/Field |
| Paraoxon | 0.482±0.024 | 16.149±1.615 | 33.50 | 27.322±4.236 | 19.892±2.783 | 1.37 |
| Chlorpyrifos- oxon | 0.794±0.145 | 32.663±3.423 | 41.14 | 55.160±8.411 | 29.676±4.799 | 1.86 |
| Malaoxon | 0.935±0.271 | 781.265±26.518 | 835.58 | 34.836±5.403 | 10.452±3.874 | 3.33 |

Mean of three experimental replicates ± standard error (S.E.)
Significant differences between the laboratory and malathion resistant strains by Student t-test ($P< 0.05$)

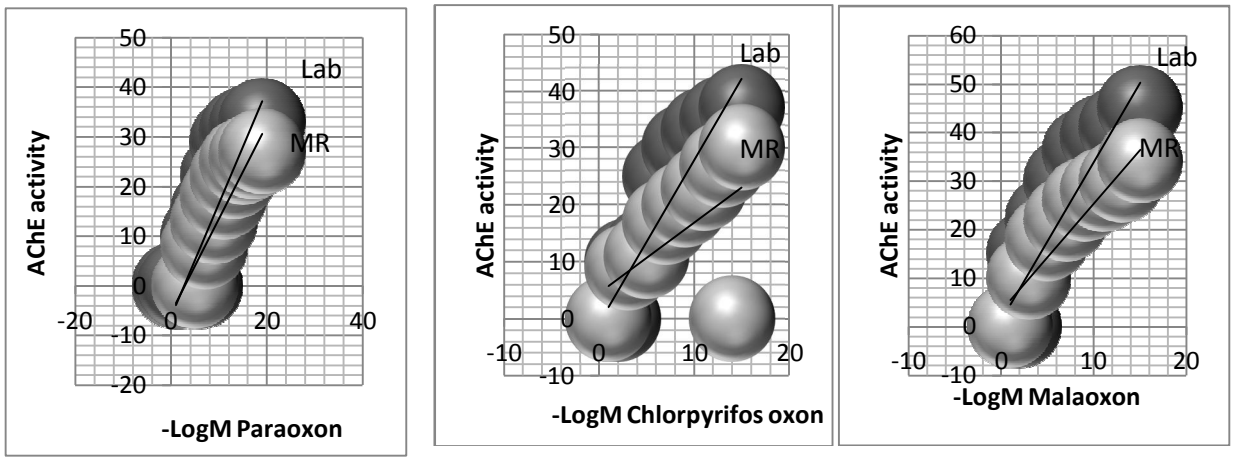


**Fig.1: Effect of OP inhibitors; Paraoxon, Chlorpyrifos-oxon and Malaoxon on activity of AChE (µmol/mg/min) from laboratory (Lab) and field malathion-resistant (MR) *Bactrocera zonata* flies**

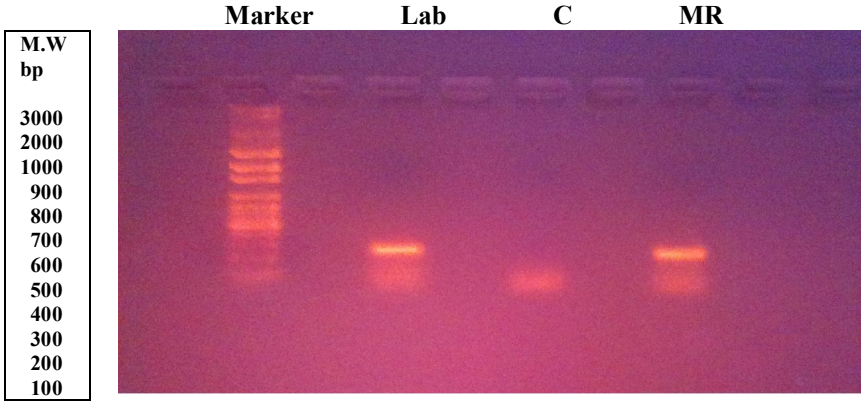


**Fig.2: RT-PCR analysis of total RNA prepared from laboratory susceptible (Lab) and field malathion resistant (MR) *Bactrocera zonata* flies. Lane PCR control (C) without sample. PCR product as cDNA fragment of 264 bp from AChE gene.**

**Sequencing of the partial AChE cDNA from *B.zonata* and point mutations in the malmthion-resistant flies**

A fragment 264 bp of cDNA from AChE gene (Bzace) of laboratory susceptible and malathion-resistant *B. zonata* flies was produced from RT-PCR based on a partial AChE coding sequence from 1771 – 2034 of *B. dorsalis* acetylcholinesterase mRNA (Ace gene -AY155500) (Hsu *et al.,* 2006) (Gen-Bank submitted).The nucleotide sequences of the fragment showed a 95.1% and 93.6% similarity to the corresponding fragment of *B. dorsalis* and *B. oleae*. The deduced protein sequence (88 amino acids from 551-638) of susceptible insects was 92% identical to that of *B. dorsalis* acetycholinesterase (Ace) mRNA complete cds (AY155500.1), *B. dorsalis* fenitrothion-insensitive acetylcholinesterase mRNA complete cds (AY183672.1), *B. dorsalis* mRNA for AChE protein (Ace gene-AJ517503.1), *B. dorsalis* Ace mRNA for fenitrothion insensitive acetylcholinesterase complete cds(AB096610.1), *B. dorsalis* Ace mRNA for carbamates insensitive acetylcholine(AB096609.1), *B. dorsalis* mRNA for fenitrothion insensitive acetylcholinesterase (ache-fenit gene-AJ517506.1) and 89.8% identical to *B. oleae* acetylcholinesterase mRNA, complete cds(AF452052.1)(Vontas *et al.,* 2002) (Figures 2- 4). Seven amino acid substitutions in partial peptide of acetycholinesterase from *B. zonata* corresponding to *B. dorsalis*; L561M, S562L, D563N, N566I, D567E, L568F and I631Lwere detected (Table 4).

The identical of 88.3 & 86.4% between the lab and field malathion-resistant flies on the level of nucleotides & amino acides, respectively. Residues for the malathion-resistant and laboratory susceptible were compared (Table 4) and twelve amino acid changes; I561L, C562S, M563D, S564A, F565V, L566N, I567D, L584F, Q585T, R627S, K630E and S631I were found. Alterations of; aspartic acid (acidic amino acid) at 563 & 567positions with

methionine and isoleucine(hydrophobic amino acids), glutamic acid (acidic amino acid) at 630 with lysine (basic amino acid) ,serine (hydrophilic amino acid) at 562 &627with cysteine (hydrophilic amino acids) and arginine (basic amino acid), alanine and isoleucine (hydrophobic amino acids) at 564&631 with serine (hydrophilic amino acids), asparagine (hydrophilic amino acid) at 566 with leucine (hydrophobic amino acids), respectively. Substitution of hydrophobic amino acids with each others; isoleucine, phenylalanine and leucine altered leucine, valine and phenylalanine at 561, 565&584 positions, respectively. Substitution of hydrophilic amino acids with each other; glutamine altered threonine at 585 position. These substitutions may be produced the insensitivity of AChE from field flies to malathion.

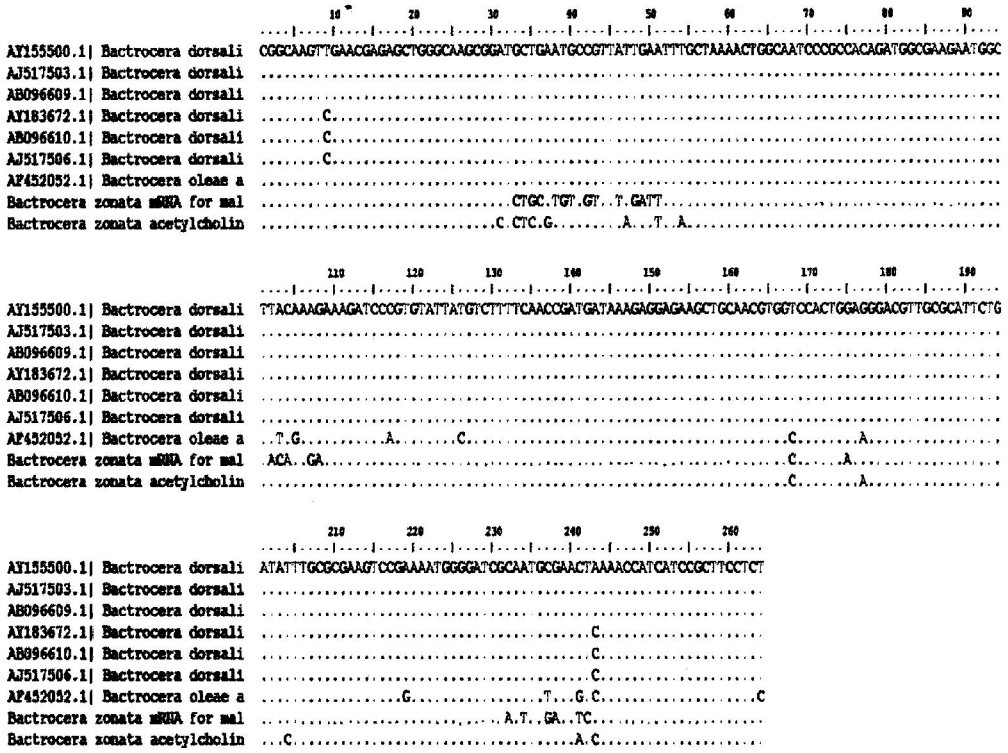


**Fig.2. The multiple alignment of cDNA nucleotide sequence (1771-2034) of acetylcholinesterase gene from susceptible and malathion-resistant *Bactrocera zonata* with the same position sequence of *B. dorsalis* and *B.oleae* acetylcholinesterase (Ace) mRNA.**

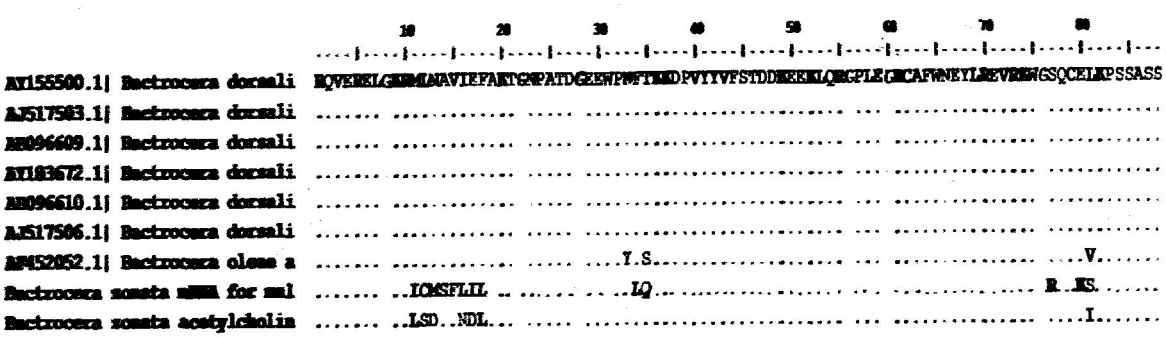


**Fig.3. The multiple sequence alignments of amino acids (551-638)of acetylcholinesterase gene from susceptible and malathion-resistant *Bactrocera zonata* with *B. dorsalis* acetylcholinesterase (Ace) mRNA complete cds (AY155500.1), *B. dorsalis* fenitrothion-insensitive acetylcholinesterase mRNA complete cds (AY183672.1), *B. dorsalis* mRNA for AChE protein (Ace gene-AJ517503.1), *B. dorsalis* Ace mRNA for fenitrothion insensitive acetylcholinesterase complete cds (AB096610.1) , *B. dorsalis* Ace mRNA for carbamates insensitive acetylcholine(AB096609.1), *B. dorsalis* mRNA for fenitrothion insensitive acetylcholinesterase (ache-fenit gene-AJ517506.1) and *B. oleae* acetylcholinesterase mRNA, complete cds(AF452052.1).**

**Table 4: Amino acids substitution (551-638) in acetylcholinesterase of *B.zonata* compared with *B. dorsalis* and malathion-resistant strain compared with laboratory susceptible strain of *B. zonata***

| ***B.zonata* / *B. dorsalis*** | | **Malathion resistant/ susceptible strains of *B.zonat*** | | | |
|---|---|---|---|---|---|
| **Position** | **substitution** | **Position** | **substitution** | **Position** | **substitution** |
| **561** | **L/M** | **561** | **I/L** | **584** | **L/F** |
| **562** | **S/L** | **562** | **C/S** | **585** | **Q/T** |
| **563** | **D/N** | **563** | **M/D** | **627** | **R/S** |
| **566** | **N/I** | **564** | **S/A** | **630** | **K/E** |
| **567** | **D/E** | **565** | **F/V** | **631** | **S/I** |
| **568** | **L/F** | **566** | **L/N** | | |
| **631** | **I/L** | **567** | **I/D** | | |

**The amino acids are indicated with one-letter code**

## 4. Discussion

The toxicological bioassay showed that the wild peach fruit fly, *B.zonata* had a very high level of resistance to organophosphate insecticide (malathion) when compared with laboratory susceptible insects. Malathion-bait sprays, which was introduced for the control of fruit flies in1960 and continue being used today, has been the most successful and widely used insecticide for the control of these pests throughout the world (Viñuela, 1998; Raga and Sato, 2005). The high level of resistance is related with the frequency of field treatments and the concentration of malathion in the protein baits, used in aerial treatments. Magaña *et al*. (2007) found that field populations of *C. capitata* from citrus and other fruit crops from different geographical areas in Spain showed lower susceptibility to malathion (6- to 201-fold) compared with laboratory populations.

In insect resistance management (IRM) programs, the rotation of insecticides with different modes of action is a desirable, it is important to understand the resistance potential and cross-resistance spectrum of these toxins to each other (Elbert *et al*., 2007). In this study; low resistance ratio and resistance coefficient of the field flies to lambda cyhalothrin (synthetic pyrethroid), spinosad (biochemical insecticide) and imidacloprid (neonicotinoid) treatments were observed. All tested insecticides had effect on insect nervous system in different sites of action. Alpha-cyano pyrethroid (lambda cyhalothrin) disrupts the normal functioning of the nervous system in an organism by modifying the kinetics of voltage sensitive sodium channels which mediate the transient increase in the sodium permeability and reduced open chloride channel probability of the nerve membrane that underlies the nerve action potential (Soderlund *et al.,* 2002; Breckenridge *et al*., 2009). Spinosad is a biochemical insecticide consists a mixture of spinosyns A and D, which are fermentation products of the soil actinomycete *Saccharopolyspora spinosa*. Spinosad act on the insect nervous system (Salgado, 1998; Salgado and Sparks, 2005), in a manner that appears to be distinct from that of all other insecticides including pyrethroids, neonicotinoids, avermectins, carbamates, organophosphates, fiproles, and cyclodienes (Crouse *et al*.,2007; Orr *et al*., 2009 ; Watson *et al.,* 2010; Dripps *et al*., 2011). Available evidence indicates that spinosad acts primarily through a novel interaction with the insect nicotinic acetylcholine receptor (nAChR) at a site distinct from that of the neonicotinoids. Recent studies in *D. melanogaster* suggest this target site is associated with an α7-like nAChR subunit; the Dm α6- nAChR in *D. melanogaster* (Watson *et al.,* 2010).and Px α6-nAChR

in diamondback moth, *Plutella xylostella* (Rinkevich *et al*., 2010). Neonicotinoids are important excitatory, neurotransmitter-gated ion channels in both vertebrates and invertebrates; the selective toxicity of neonicotinoids for insects has been attributed, at least in part, to their high affinity for insect receptors. Imidacloprid is a nicotinic acetylcholine receptor (nAChR) agonist with potent insecticidal activity. Nicotinic receptors are pentameric transmembrane complexes assembled from a diverse family of subunit subtypes 10 nAChR subunits have been identified by molecular cloning in *D. melanogaster* (Zewen *et al*., 2005). Urbaneja *et al*. (2009) used spinosad, phosmet and lambda-cyhalothrin as alternatives to malathion for controlling *C. capitata* in Spain. Also, spinosad, spinosad bait, and imidacloprid seem to be acceptable substitutes for organophosphate and carbamate insecticides for controlling adults and larvae of western cherry fruit fly, *Rhagoletis indifferens* (Yee and Alston, 2006 ;Yee, 2008). Liburd *et al*. (2005) reported that the potential for using sphere design treated with spinosad and imidacloprid in field applications for controlling caribbean fruit fly *Anastrepha suspense* and medfly *C. capitata* in Florida. Chloronicotinyl (neonicotinoid) class insecticides were used to control larvae of all instars of cherry fruit fly inside infested fruit. Imidacloprid provided post infestation control to high degree offer as part of a pre-harvest control program in Washington (Smith and Gutierrez, 2007).

In this study the acetylcholinesterase activity of head homogenate from laboratory susceptible *B.zonata* flies was 1.7fold of that from field malathion resistant flies. Changes in the AChE levels may also play a role in the resistance to OPs. The reduction in activity of AChE from resistant individuals is also similar to results seen in studies of resistance in the Colorado potato beetle (Zhu and Clark, 1995). However, in studies of organophosphate-resistance for insects such as the green rice leafhopper (Hama, 1983, 1984) and lesser grain borer (Guedes *et al*., 1998) no similar reductions were seen. Charpentier and Fournier (2001) showed that there was a correlation in natural populations of *D. melanogaster* between the amount of AChE in the central nervous system and their resistance to insecticides. The hydrolyzing efficiency (Vmax) and substrate affinity (Km) of lab *B.zonata* flies enzyme were 2.2 and 0.7fold than those of resistant flies. Magaña *et al*.(2008) mentioned that the AChE of *C. capitata* individuals with the WR phenotype showed higher km (lower affinity) and lower Vmax, resulting in a 2.4-fold reduction in the efficiency (Vmax/km) of the altered enzyme when compared to the C phenotype. Similar reductions in the affinity for the substrate have been obtained in resistant strains of *M. domestica* and *P. xylostella* containing this mutation (Walsh *et al*., 2001; Lee *et al*., 2007).

AChE from the lab and resistant *B.zonata* flies examined of inhibition by three OPs inhibitors (as measured using $I_{50}$ values). The malathion-resistant strain was insensitive to inhibition even under high concentrations of malaoxon ($10^{-8}$ -$10^{-5}$), and was 835.58, 41.14 and 33.50 fold more insensitive to inhibition by malaoxon, chlorpyrifos oxon and paraoxon, respectively, compared with the lab strain. This range of effects using different inhibitors is to some extent also consistent with cross resistance to other organophosphate insecticides seen previously in *B. dorsalis* (Hsu *et al*., 2004). The correlation observed between reduced AChE activity and reduced sensitivity to malaoxon in the W strain, may be a very important fact indicating possible fitness costs associated with AChE insensitivity (Magaña *et al*., 2008) .The resistance to malathion and other organophosphorous insecticides can be due to mutations on the target site, the acetylcholinesterase (Mutero *et al*., 1994; Walsh *et al*., 2001; Oakeshott *et al*., 2005), or to the detoxification of the insecticides by metabolic mediated mechanism (Ranson *et al.,* 2002; Feyereisen,2005). Acetylcholinesterase that is less sensitive to malathion may confer cross resistance to other organophosphorous and carbamates insecticides, whereas metabolic resistance may result in even a wider range of cross-resistance by the inactivation of insecticides with different modes of action (Oakeshott *et al*., 2005). In addition to the association of mutations with the acquisition of insecticide resistance it is important to examine whether such mutations are associated primarily with either quantitative or qualitative effects on the production and/or activity of specific enzymes (Hsu *et al*., 2008). In *B. oleae*, it is clear that the mutations were associated with reductions of the catalytic efficiency of the AChE enzyme on the order of 35– 40% (Vontas *et al.,* 2002). The development of resistance to OP insecticides has been associated with point mutations in the gene (ace) encoding the AChE enzyme especially in light of the work in the congeneric species *B. dorsalis* and *B. oleae* that shows a decreased sensitivity to the inhibitors and a qualitative reduction of the catalytic activity of the AChE enzyme as the basis for resistance in these species (Hsu *et al.*, 2008, Kakani *et al*.,2008; 2011). However, in non-dipterans species such as those in the Hemiptera (Aphididae) at least three distinct mechanisms have been associated with the acquisition of resistance. These include alterations exhibiting both quantitative and

qualitative effects on the structure and function of the AChE enzyme and on distinct genes involved in sodium channeling (Margaritopoulos *et al*., 2007).

The RT- PCR analysis of total *B.zonata* RNA based on *B. dorsalis* partial coden sequence (1771-2034) of acetylcholinesterase gene produced a fragment of 264 bp. Eighty eight peptide residues corresponding the direct sequencing of this fragment were obtained and 88.3 & 86.4% identical between the lab and field malathion-resistant flies on the level of nucleotides & amino acids, respectively. Twelve substitutions in amino acids of AChE partial protein from field malathion- resistant flies compared with those of laboratory flies. Amino acids changes corresponding to positions; I561L, C562S, M563D, S564A, F565V, L566N, I 567D, L584F, Q585T, R627S, K630E and S631I were detected. Alterations of; aspartic acid in 563&567 positions with hydrophobic methionine & isoleucine, glutamic acid at 630 with basic lysine, hydrophilic serine at 562&627 with hydrophilic cysteine & basic arginine and hydrophobic alanine & isoleucine at 564&631 with hydrophilic serine in AChE protein may be caused resistance of the field *B.zonata* flies to malathion. A total 23 mutations have been found for 15 species of arthropods that involve 15 DmAChE equivalent sites. Different patterns can originate from combination of various point mutations in AChE gene and high levels of AChE insensitivity could come from the combination together of several point mutations (Mutero *et al*., 1994). Fourteen mutations associated to resistance to insecticides have been reported in AChE of higher Diptera (Mutero *et al.,* 1994; Kozaki *et al*., 2001; Vontas *et al.,* 2001& 2002; Walsh *et al.,* 2001; Hsu *et al*., 2006). In tephritids, a total of three mutations have been reported Ile129Val, Gly396Ser and Gln591Arg. The Ile129Val substitution has been observed in a dimethoate resistant strain of *B. dorsalis* and in a fenitrothion resistant strain of *B. oleae* (Hsu *et al.,* 2006; Vontas *et al.,* 2002) and is equivalent to the well characterized Ile129Val resistance associated mutation in *D. melanogaster* (Mutero *et al*., 1994). In *B. oleae*, it was found in combination with the Gly396Ser substitution and in *B. dorsalis* in combination with Gly396Ser and Gln591Arg. The Gly396Ser substitution has been only reported in *B. dorsalis* and *B.oleae* (Vontas *et al*., 2001& 2002; Hsu *et al*., 2006), this mutation may alter the configuration of the adjacent glutamate in the catalytic triad and promote the nucleophilic attack by water on the carbonyl group of the phosphorilated serine (Vontas *et al.,* 2002). The third mutation, Gln591Arg, occurs near the end of the peptide and has been only observed in *B. dorsalis*. It was found in combination with the two others mutations Ile129Val and Gly396Ser (Hsu *et al*., 2006). The extensive study of Walsh *et al*.(2001) and Menozzi *et al*. (2004) on single and multiple mutations in the house fly *M. domestica* and *D. melanogaster* enzymes explains why the single mutation found in *C. capitata* only provides a low level of enzyme insensitivity to malaoxon and insect resistance to malathion (Magaña *et al*.,2008). In the olive fruit fly, *B. oleae*, two mutations (I214V and G488S) localized in the catalytic gorge of AChE were initially shown to confer resistance to organophosphate insecticides (Vontas *et al*., 2002). However, the unexpected discovery that a resistance-associated mutation in the ace gene of the olive fly that did not lie in the catalytic domain of the enzyme but, rather, in its C-terminal peptide (Kakani *et al*., 2008), necessitated further examination of the possible underlying molecular mechanism. The search for additional mutations in the ace gene that encodes AChE revealed a short deletion of three glutamines (Δ3Q) from a stretch of five glutamines, in the C-terminal peptide that is normally cleaved and substituted by a glycophosphatidylinositol (GPI) membrane anchor. We verified that AChEs from *B. oleae* and other Dipterans are actually GPI-anchored, although this is not predicted by the "big-PI" algorithm. The Δ3Q mutation shortens the unusually long hydrophilic spacer that follows the predicted GPI attachment site and may thus improve the efficiency of GPI anchor addition (Kakani *et al*., 2011).

**Conclusion**

The field peach fruit fly, *B. zonata* from Ismailia Governorate was highly resistant to malathion (resistance ratio RR=30.47 fold and resistance coefficient RC=75.33). There is no cross-resistance between imidacloprid, spinosad lambda-cyhalothrin and malathion resistance in the field flies The resistance of malathion in field *B. zonata* results from qualitative effects on the AChE enzyme. Mutations in the ace gene producing twelve predicted amino acid substitutions (I561L, C562S, M563D, S564A, F565V, L566N, I567D, L584F, Q585T, R627S, K630E and S631I) in partial peptide (551-638 of *B. dorsalis* according to Hsu *et al*., 2006) of the AChE enzyme were detected, and significant reductions in the catalytic efficiency of the enzyme and decreased sensitivity to inhibition were observed in association with resistance. As described in this paper, all of these alterations appear to be located in the C-terminal peptide of the AChE enzyme, and this certainly would be expected to

have impact on enzyme activity and sensitivity to various organophosphate based insecticides.

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