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# Real-time PCR analysis of pyrethroid resistance in *Helicoverpa armigera* from Turkey

## [Türkiye'deki *Helicoverpa armigera'*da piretroid dayanıklılığının gerçek zamanlı PCR ile analizi]

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**ABSTRACT**

**Aim:** *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a polyphagous pest of a wide range of crops such as cotton, tomato and soybean. Pyrethroid insecticides have commonly used against it in agricultural areas, but excess amount applications of them result in resistance development in the field populations of *H. armigera*. Resistance development usually occurs with increased metabolism of certain enzymatic systems such as CYP450, GST and esterases. Therefore, expressions of selected CYP450, GST and esterase genes of *H. armigera* field populations (Adana and Mardin) were compared to those of a susceptible strain by real-time PCR method for analyzing role of these systems in pyrethroid resistance development of *H. armigera*.

**Material and Methods:** Real-Time PCR Method

**Results:** It was found that *H. armigera* reacts to pyrethroids mainly by increasing expressions of CYP9A14 gene together with CYP4S1 and CYP9A12 genes. However, analyzed GST and esterase genes expression were not significantly changed in field populations.

**Conclusion:** Consequently, while CYP450 enzyme system is actively involved in pyrethroid resistance, GSTs and esterases enzyme systems don't seem to be actively involved in resistance development against pyrethroid insecticides in *H. armigera* field populations from Turkey.

**Key Words:** *Helicoverpa armigera*, resistance, *q*RT-PCR, CYP450, pyrethroid insecticides

**Conflict of Interest:** The authors do not have a conflict of interest.

**ÖZET**

**Amaç:** *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) polifag bir zararlı olup pamuk, domates ve soyafasuyesi gibi geniş bir ürün grubunun zararlısıdır. Tarımsal alanlarda bu zararlıya karşı yaygın olarak piretroid insektisitler kullanılmaktadır fakat bu insektisitlerin aşırı miktarda kullanımı *H. armigera*'nın tarla populasyonlarında direnç oluşumuna neden olmaktadır. Dayanıklılık oluşumu genellikle CYP450, GST ve esteraz gibi enzim sistemlerinin metabolik hızının artmasıyla olmaktadır. Bu nedenle, Adana ve Mardin tarla populasyonlarındaki CYP450, GST ve esteraz enzim sistemlerinden seçilen genlerin ekspresyonu hassas populasyonla gerçek zamanlı PCR yöntemiyle karşılaştırılarak bu sistemlerin piretroid dayanıklılıktaki rolleri analiz edildi.

**Gereç ve Yöntemler:** Gerçek zamanlı PCR yöntemi

**Bulgular:** *H. armigera*'nın tarla populasyonun'nun piretroidleri başta CYP9A14 olmak üzere CYP9A12 ve CYP4S1 ile birlikte metabolize ettiği bulundu. Bununla birlikte, analiz edilen GST ve esteraz genleri tarla populasyonlarında anlamlı bir değişiklik göstermedi.

**Sonuç:** Sonuç olarak, CYP450 enzim sistemi piretroid dayanıklılığına aktif olarak katılırken, GST ve esteraz enzim sistemlerinin *H. armigera*'nın tarla populasyonlarında dayanıklılık oluşumuna aktif olarak katılmadıkları saptandı.

**Anahtar Kelimeler:** *Helicoverpa armigera*, dayanıklılık, gerçek zamanlı PCR, CYP450, piretroid insektisitler

**Çıkar Çatışması:** Yazarların çıkar çatışması bulunmamaktadır.

## Introduction

*Helicoverpa armigera* (Hübner, 1805) is a pest of wide range of economically important vegetables such as cotton, maize, sorghum, tomato, chickpea and sunflower. Since *Helicoverpa armigera* (*H. armigera*) has a polyphagous nature, it could be adapted to diverse cropping systems, so it spread out easily all over the world. Thus, it appears Africa, Asia, India, Indonesia, Australia and Turkey [1]. Furthermore, it has been seen in some Europe countries such as France [2] and Spain [3]. For controlling *H. armigera* damage on agriculture fields, firstly, organochlorines and organophosphate type insecticides were used until 1980. After that, pyrethroid type insecticides were started to use against it. However, common usage of pyrethroid insecticides results in resistance development in the field populations of *H. armigera*. Pyrethroid resistance developments in *H. armigera* are reported in several countries, including Turkey [4-11].

Pyrethroid resistance of *H. armigera* in Turkey was firstly reported in 1984 [12]. Similar report was followed by Ernst & Dittrich [13]. Then, resistance to lambda-cyhalothrin and esfenvalerate were reported in the Adana and Antalya strains of *H. armigera* [8]. Recently, Adana and Mardin field populations, used in this study, displayed higher resistance ratios especially for pyrethroid insecticides. Although, resistance ratios through tested pyrethroids (esfenvalerate, bifenthrin, beta-cyfluthrin and lambda-cyhalothrin) was in range of 6.0-67.0 fold, carbamates (methomyl), oxadiazine (indoxacarb) and organophosphates (azinphosmethyl) were in the range of 0.45-8.6 fold [11]. Resistance development to applied insecticides generally occurs with different mechanisms. One of these mechanisms is increasing metabolism of that insecticide by certain enzymatic systems like esterases (EST), cytochrome P-450 monooxygenases (CYP450) and glutathione S-transferases (GST). However, GSTs have not proved that they are participating in direct metabolism of pyrethroid insecticides. Instead, they might have role in mediating oxidative stress responses. They may catalyse conjugating reactive species and activated compounds, detoxifying lipid peroxidation products and oxidized DNA bases, formed during metabolism of pyrethroid insecticides [14]. Therefore, there are controversy mainly between monooxygenases and esterases related with relative roles of pyrethroid metabolizing enzyme systems in *H. armigera*,

In this study, relative roles of CYP450, EST and GST enzyme systems in pyrethroid resistant midgut tissue of *H. armigera*, important detoxification site in *H. armigera* larvae [15], were analyzed with real-time PCR (*q*RT-PCR) method.

## Material and Methods

### *Midgut Tissue Isolation from Helicoverpa armigera Larvae*

*H. armigera* larvae samples were obtained from cotton fields in Adana and Mardin provinces during the years 2008-2009. *H. armigera* susceptible strain was obtained from Germany. Larvae were fed on artificial diets in laboratory and 1 or 2 day old sixth instar larvae were used in real-time PCR experiments (Figure 1). In order to dissect midgut tissues from 1 or 2 day 6th instar larvae, firstly, larvae were paralyzed by keeping on ice. Then, they were cut along their length by razor blade. Finally, these midguts were cleaned in 1.15 M KCl, dried on filter paper and stored in deep freezer at -80 ˚C until RNA isolation

**Figure 1.** Preparation steps of *Helicoverpa armigera* midguts from larvae

### *RNA isolation and cDNA preparation*

Total ribonucleic acid (RNA) was isolated from single larvae midgut according to the method of Chomczynski and Sacchi [16]. After isolation, quantity and quality of these isolated total RNAs were checked by spectrophotometrically measurements at 260 nm and 280 nm wavelengths. In order to calculate concentration of RNA, OD260 value was used. By the way OD280 value was used to calculate OD260/OD280 ratio, used as a quality marker of extracted RNAs. If it was between 1.8 and 2.0, it was considered as a good quality RNA. Finally, template cDNA was prepared with First Strand cDNA Synthesis Kit (Fermentas) by using 1µg qualified total RNA to use it in real-time PCR (*q*RT-PCR) analysis.

### *Real-time PCR Measurements*

In this study, it was planned to analyze individual expression levels of five suspected CYP450 genes from 3 main CYP450 gene families, CYP4, CYP6 and CYP9. These family members showed increased gene expressions in several insecticide resistant strains of insects [17]. Furthermore, one esterase and two different GST gene expressions levels were also analyzed. In order to

normalize *q*RT-PCR data, elongation factor-1α (EF-1α) gene was selected as an internal standard in these experiments. The forward and reverse primer sequences of analyzed genes were indicated at table 1 with annealing temperature and amplicon size of that gene. Each sample was measured triplicate. Melting curve analysis was done for checking reaction specificity. Serial dilutions up to 1000X of cDNA template were used for the standard curve development for each gene. Real-time PCR amplification conditions for EF-1α gene, CYP9A14, CYP9A12 and CYP6B7 genes as follows: 5 min at 95 °C for enzyme activation, then 30 sec at 95 °C; then 30 sec at 58 °C for EF-1α gene, CYP9A14, 55 °C for CYP9A12, 52 °C for CYP6B7; 45 sec at 72 °C with 40 cycles except activation step [18]. CYP4S1, ESTX018 and GSTX01 genes cycling conditions were 95 °C for 15 min, then 95 °C for 20 sec, then 60 °C for 30 sec and 72 °C for 30 sec with 40 cycles of last three steps [19]. GST gene cycling condition 95 °C for 5 min, then 24 cycles of 95 °C for 30 sec, then 5 °C for 30 sec and 72 °C for 30 sec with 72 °C for 10 min final extension [20]. Furthermore, while CYP6B6 gene cycling conditions were 95 °C for 15 min, then 94 °C for 15 sec, then 60 °C for 50 sec and 72 °C for 30 sec with 40 cycles of last three steps [7]. In addition, reverse transcription polymerase reaction of CYP6B6 gene was done under following conditions; 95 °C for 5 min, then 28 cycles of 95 °C for 30 sec, 48.8 °C for 30 sec and 72 °C for 30 sec with 72 °C for 10 min final extension [21].

## Statistical analysis

Analysing gene expression using *q*RT-PCR amplification data was done with Relative Expression Software Tool 2008 (REST2008) (http://www.genequantification.de/rest-2008.html) [22].

## Results

Because of huge amount of individual *H. armigera* sample requirements (approximately, about 50 individual midguts for one assay duplicate measurement) for each assay, CYP450 enzyme systems role could not be analyzed with biochemical assays in our previous work [23]. Moreover, pooling midgut samples may cause for masking the ones, showing increase in enzyme activity. Therefore, instead of analyzing protein level of midgut sample groups, we decided to study this enzyme system together with GST and EST system at mRNA level for individual midgut samples with *q*RT-PCR technique.

**Table 1.** Primer sequences and annealing temperature for real-time PCR experiments

| Gene | Forward and Reverse Primer Sequences | Amplicon Size(bp) | Annealing Temperature (°C) | PCR[f] Efficiency |
|---|---|---|---|---|
| EF-1α[a] | F 5''-GACAAACGTACCATCGAGAAG-3''<br>R 5''-GATACCAGCCTCGAACTCAC-3'' | 279[a] | 58 | -3.319 |
| CYP4S1[b] | F 5''-AGCGTGCCTTTTATTGCGAGAG-3''<br>R 5''-CGGCGGTGCAGGTCATAGAT-3'' | 100-120[b] | 60 | -3.287 |
| CYP6B6[c] | F 5''-TTGAAGAAAGGCGTATGAAA-3''<br>R 5''-ACACGCAAGATACACAAAGG-3'' | 60-150[c] | 60 | ----- |
| CYP6B6[e*] | F 5''-GTTGATATCTCCTCAAAATG-3''<br>R 5''-CATAGTGAATGCCTCTTGG-3'' | 1219[e] | 48.8 | ----- |
| CYP6B7[a] | F 5''-TCTTGTGGACAACATTATTAGC-3''<br>R 5''-AAGTGATGTTACTTCATCAAGA-3'' | 130[a] | 52 | -3.248 |
| CYP9A12[a] | F 5''-ATCACCTCATAGAAGATATCC-3''<br>R 5''-CATGTCTTTCCATTCTTGACC -3'' | 234[a] | 55 | -3.242 |
| CYP9A14[a] | F 5''-ACCCTGAGGTACAGGAGA-3''<br>R 5''-TAGACCACACCGGGATCA-3'' | 258[a] | 58 | -3.265 |
| GSTX01[b] | F 5''-TAAACAGTCTTCGCGTATATAGC-3''<br>R 5''-ATCAGATAGTTGACTTGATTGATG-3'' | 100-120[b] | 60 | -3.317 |
| GST[d] | F 5''- CTGTGCTAGAGGATGGGGA-3''<br>R 5''-AGCGATGTAGGTGGTGCGA -3'' | 289[d] | 55 | -3.249 |
| ESTX018[b] | F 5''-TCCCATATGAACATCCCAAACAG-3''<br>R 5''-TTGAGATCCTCATTGTTGGGTAG -3'' | 100-120[b] | 60 | -3.484 |

[a] Primer sequences were obtained Yang *et al.,* 2006.
[b] Primer sequences were obtained Wee *et al.,* 2008.
[c] Primer sequences were obtained Grubor *et al.,* 2007.
[d] Primer sequences were obtained Tang *et al.,* 2005.
[e] Primer sequences were obtained Liu *et al.,* 2006.
[f] PCR efficiency was indicated as slope of log-linear phase of a set of serials dilutions (up to 1000X).
* This primer couple was used for reverse transcription polymerase reaction.

According to the CYP450 *q*RT-PCR results of Adana population, only CYP9A14 gene expression was 10.1 fold up-regulated ($p<0.05$, REST 2008) compared to susceptible population. Interestingly, other measured CYP4S1, CYP6B7 and CYP9A12 genes expressions showed 0.073, 0.001 and 0.001 fold ($p<0.05$, REST 2008) down regulation, reciprocally (Table 2). In addition, there were not determined any significant changes in tested GST and EST genes expressions, as well (Table 3).

*H. armigera* Mardin population samples showed up-regulation in mRNA levels of CYP4 and CYP9 family genes except for the CYP6 family. CYP4S1, CYP9A12 and CYP9A14 genes showed 4.49, 3.37 and 874.3 fold ($p<0.05$, REST 2008) up regulation, reciprocally (Table 2). Furthermore, CYP6B6 gene products were not detected in this study with primers couples of Grubor et al. [7], used for CYP6B6 in *H. armigera*. Moreover, another primer couple from Lui et al. [21], previously used to analyse expression level of CYP6B6 gene with reverse transcription polymerase reaction, was used to analyse this gene, but it could not be possible to detect CYP6B6 gene products in *H. armigera* field samples from Turkey, as well. In addition, there were not also determined any statistically significant ($p<0.05$, REST 2008) increase in mRNA expression levels of measured GST and esterase genes in all field populations of *H. armigera* (Table 3).

**Table 2.** CYP450s Real-time PCR Results of *Helicoverpa armigera* Strains

| STRAIN | CYP4S1 | CYP6B6 | CYP6B7 | CYP9A12 | CYP9A14 |
|---|---|---|---|---|---|
| Adana | 0.073*↓ | n. d. | 0.001*↓ | 0.001*↓ | 10.1*↑ |
| Mardin | 4.49 *↑ | n.d. | 0.31 | 3.37 *↑ | 874.3*↑ |

Numerical values indicate that expression ratio of that gene in field population compare to the susceptible population
*Value significantly different from the susceptible strain ($p<0.05$) with REST 2008
n.d.: not detected

**Table 3.** EST and GSTs Real-time PCR Results of *Helicoverpa armigera* Strains

| STRAIN | GST | GSTX01 | ESTX018 |
|---|---|---|---|
| Adana | 0.25 | 1.58 | 0.92 |
| Mardin | 1.11 | 1.36 | 0.34 |

Numerical values indicate that expression ratio of that gene in field population compare to the susceptible population

## Discussion

The population of *H. armigera* from Adana field showed similar expression pattern for GST and EST enzyme systems. There were no significant increases determined in tested GST and EST gene expressions. However, it was previously determined that there were significant increases in protein level analysis of this field strain of *H. armigera* [23]. This might be explained with post-translational modifications. This modifications might be induces activation of esterase and glutathione S-transferases isoenzymes in Adana field population. By the way, there would be other GST and EST isoenzymes that would have an additional effect on protein level [23]. Although, there were no significant increases in mRNA levels of tested GST and EST genes, significant increases in their enzymatic activities display that they might have a role in developed pyrethroid resistance in Adana field population.

Interestingly, CYP4S1, CYP6B7 and CYP9A12 genes expressions were down-regulated in the range of 0.001-0.073 fold in Adana field population. Although CY4S1, CYP6B7 and CYP9A12 genes are suggested that they are actively involved in pyrethroid resistance in *H. armigera* [7, 19, 24], down-regulation in these genes by pyrethroid insecticides indicating that they might play a role in sensory function during pyrethroid stress in *H. armigera*. However, CYP9A14 gene expression was 10.1 fold up-regulated in Adana field population. This result suggests that CYP9A14 gene might play the main role in pyrethroid metabolism in *H. armigera* in Adana field population.

As real time-PCR and enzyme activity results of Mardin population were evaluated, GST and EST systems results were found similar to Adana population. Likewise, while GST and ESTs enzymatic activities were increasing [23], there was no significant changing in mRNA level of analyzed GST and EST genes. Therefore, GST and ESTs might have a contribution to pyrethroid resistance in Mardin population, too. Nonetheless, *q*RT-PCR analysis results in Mardin population showed up-regulation in mRNA levels of CYP450 genes except for the CYP6 family. CYP4S1, CYP9A12 and CYP9A14 genes showed 4.49, 3.37 and 874.3 fold ($p<0.05$, REST 2008) up regulation, reciprocally. These results demonstrate that CYP4 and CYP9 family member these genes participate actively in pyrethroid metabolism to overcome hazardous effect of these pesticides in Mardin field population. Nonetheless, Wee et al. [19] reported that CYP4S1 up regulated in pyrethroid resistant *H. armigera* strains from Australia, Yang et al. [18, 24] reported that CYP9A12 and CYP9A14 genes over-expressed in pyrethroid resistant *H. armigera* from China, it seems that CYP4S1, CYP9A12 and CYP9A14 genes possibly participate together pyrethroid resistance in Turkey.

According to the overall *q*RT-PCR findings related with cytochrome P450 enzyme systems, CYP6B7 gene expression was not increased significantly in all analysed field populations. Although, Ranasinghe et al. [25] suggested that CYP6B7 was the enzyme mainly responsible for pyrethroid resistance in Australian *H. armigera*, Grubor et al. [7] found that CYP6B2, CYP6B6 and CYP6B7 genes were not responsible for fenvalerate resistance in AN02 strain from Australia. CYP4S1 and CYP9A12 gene expressions increased only in Mardin population Moreover, whereas CYP9A14 gene expression showed increase in all field samples of *H. armigera*. Addition-

ally, an increase in gene expressions of CYP9A14 was dramatically high especially in Mardin population 874.3 fold compared to susceptible population.

As a consequence of overall *q*RT-PCR results for tested GST and EST system members, there were no up regulation in expressions of GST, GSTX01 (similar to sigma class of GSTs) and ESTX018 (similar to insect carboxylesterase) genes in both Adana and Mardin field population of *H. armigera*. In addition, it seems that CYP9A14 have an important role in developing pyrethroid resistance in field population *H. armigera*. Furthermore, CYP9A12 and CYP4S1 also have a role in developing pyrethroid resistance in *H. armigera* from Turkey. In conclusion, it was concluded that cytochrome P450 enzyme systems seemed to be major contributor in pyrethroid resistance development in field populations of *H. armigera*. In addition, GST and ESTs may have a supportive role in resistance development of field populations *H. armigera* from Turkey, as well.

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**Conflict of Interest:** The authors declare that there is no conflict of interest.

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