KSÜ Tarım ve Doğa Derg 23 (3): 634-640, 2020
KSU J. Agric Nat 23 (3): 634-640, 2020
DOI:10.18016/ksutarimdoga.vi.605716

Overexpression of cytochrome P450 CYP6CM1 gene in *Bemisia tabaci* (Gennadius) in the cotton fields of the Çukurova Region, Turkey

Selçuk ULUSOY
Biyolojik Mücadele Arş. Enst. Müdr. Köprülü Mh. Kışla Cd. P.K.:01321 Yüreğir/Adana
https://orcid.org/0000-0002- 2278-8471
✉: selcukby@gmail.com

ABSTRACT

In Cukurova region, neonicotinoid group insecticides are widely used in cotton cultivation areas for controlling *B. tabaci;* as a result of this use, there is a problem of resistance in these areas. In this study, cytochrome P450 CYP6CM1 relative expression levels, which are the indicators of imidacloprid resistance, were analyzed. Five different *B. tabaci* populations were collected from Adana city cotton cultivation areas in 2018 and the indicators were observed. On the other hand, GST gene relative expression profile, which is an efficient factor in metabolic resistance, cytochrome P450, carboxylesterase (AcHE) and Glutathion S transferase (GST) enzyme activities were determined. The highest cyp6cm1 and GST genes relative activities were determined in Meletmez population; the activity level was 18, 2 and 9 times higher than susceptible population. On the other hand, the highest and lowest cytochrome P450 enzyme activity was observed in Meletmez and Solaklı populations, respectively. According to the analyses, it can be concluded that there is extensive imidacloprid and neonicotinoid resistance of *B. tabaci* populations in Çukurova Region cotton cultivation areas. As a result, excessive gene expression and high-level enzyme activities indicate that there is a resistance in the studied area based on enforced monooxygenases activity.

Research Article

Article History
Received : 16.08.2019
Accepted : 21.01.2020

Keywords
Bemisia tabaci
Cotton
Cytochrome P450
Overexpression

Çukurova bölgesi pamuk alanlarında *Bemisia tabaci* (Gennadius)'de aşırı düzeyde sitokrom P450 CYP6CM1 gen ekspresyonu

ÖZET

Çukurova bölgesi polikültür tarımın yapıldığı bir bölgedir. Bu bölgede pamuk ekim alanlarında *B. tabaci* mücadelesinde neonikotinoid grubu insektisitler yoğun bir şekilde kullanılmakta olup, direnç problemi ile karşılaşılmaktadır. Çalışmada 2018 yılı, Adana ili pamuk alanlarından toplanmış beş farklı *B. tabaci* popülasyonunda imidacloprid dayanıklılığı göstergesi olan sitokrom P450 CYP6CM1 geni relatif expresyon düzeyleri incelenmiştir. Ayrıca metabolik dayanıklılıkda etkili GST geni relatif expresyon profili, sitokrom P450, karboksil esteraz(AcHE) ve Glutathion S transferaz (GST) enzim etkinlikleri belirlenmiştir. En yüksek relatif aktivite Meletmez populasyonunda CYP6CM1 geni 18.2 kat ve Gst geni 9 kat daha fazla gen regülasyonu belirlenmiştir. Ayrıca en yüksek ve en düşük sitokrom P450 enzim aktivitesi meletmez ve solaklı popülasyonlarında gözlemlenmiştir. Yapılan analizlerde Çukurova bölgesi pamuk alanlarında *B. tabaci* popülasyonlarında yaygın imidacloprid ve neonikotinoid dayanıklılığı durumundan bahsedilebilir. Sonuç olarak, aşırı gen expresyonu ve yüksek düzeydeki enzim etkinlikleri, bölgede güçlendirilmiş monooksigenaz etkinliğine dayanan direncin varlığını ortaya koymaktadır.

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 16.08.2019
Kabul Tarihi : 21.01.2020

Anahtar Kelimeler
Bemisia tabaci
Pamuk
Sitokrom P450
Aşırı ekspresyon

To Cite : Ulusoy S 2020. Overexpression of cytochrome P450 CYP6CM1 gene in *Bemisia tabaci* (Gennadius) in the cotton fields of the Çukurova Region, Turkey. KSU J. Agric Nat 23 (3): 634-640. DOI: 10.18016/ksutarimdoga.vi.605716.

INTRODUCTION

In Turkey, cotton is a highly important plant as it is a resource of economic contribution; the plant is used as a raw material in various textile and industrial products (Şekeroğlu et al., 1998). Approximately a total of 520.000 hectare cotton area was cultivated in 2018 in Turkey. 100.000 hectare cotton area was cultivated in Adana, Mersin and Hatay regions, which shows that the region is a very important cotton resource (Anonymus, 2019). White fly *Bemisia tabaci* is a polyphagous pest that is highly harmful especially for vegetable and cotton fields; the insect was reported for the first time in 1928 (Kaygısız, 1976; Ulusoy et al., 1996). *Bemisia tabaci* especially sucks on the leaves of cotton plants and causes honey-like formations; besides, it causes secondary infections such as fumaginate, which are formed by saprophytic fungus. On the other hand, they are carriers of more than 100 seriously harmful viruses (Moya et al., 2001; Hsieh et al., 2006). All these cause important yield and quality loss in cotton cultivation. The most effective and common *Bemisia tabaci* biotypes are *Bemisia tabaci* B and Q (De Barro et al., 2011).

The use of insecticides has become more common (neonicotinoids) as they are easy to use and economic for controlling *Bemisia tabaci.* Besides, different levels of resistances to insecticides (organophosphate, carbamate, pyrethroid and neonicotinoids) are reported in various countries (Ahmad et al., 2003; Wang et al., 2007; Sparks et al., 2015).Neonicotinoids are efficiently used in controlling coleoptera, diptera and lepidoptera families, especially aphid and white fly; on the other hand, they have high market share. Because of the common and unconscious use, different levels of resistances are reported in the Mediterranean Region and in different parts of the world ((Nauen et al., 2002, Nauen et al., 2008; Jeschke et al., 2011, Ulusoy et. al., 2018, Satar et al., 2018). It is reported that in Adana cotton cultivation areas, there is 100 times higher resistance to imidacloprid in *B. tabaci* at a specific location when compared to the susceptible population (Satar et al., 2018). It is reported that there are 200 times higher imidacloprid and thiametoxam resistance in *Aphis gossypii* in different cotton cultivation areas of the same region (Ulusoy et. al., 2018).Resistance mechanism results from the decrease in sensitivity level of organisms against xenobiotics because of the reinforced metabolic enzyme activities and mutations in target proteins (Nauen, 2007). Various enzymes such as Esterase, Glutathione S Transferase (GSTs) and Cytochrome P450, which are included in multi-gene family, are efficient in the xenobiotic detoxification process of living organisms. This activity is closely related with the transcription level of genes at cellular extent (Field et al., 1999; Bass & Field, 2011). In the previous studies, it is reported that there is an increase in P450 (Cyp6cm1) and glutathione S-transferase (GST) enzyme genes relative activities in *B. tabaci* populations that are resistant to neonicotinoid and organic phosphate insecticides (Puinean et al., 2010; Qiong et al., 2012; Liang et al., 2014; Yang et al., 2014). P450 (encoded as CYP genes) enzyme group is a super family with multi-gene structure; they actively take part in a wide range of processes such as metabolization of internal and external components, growth, development, nutrition and detoxification of xenobiotics (Scott et al., 1999; Feyereisen, 2005).Reinforced p450 activities in imidacloprid resistant *B. tabaci* individuals are observed through different methods. Firstly, it is proven those imidacloprid individuals' resistance decreases when they are exposed to p450 inhibitors (Nauen et al., 2002). Secondly, it is determined that there is a close correlation between resistance and monoxigenase activity; this is proven with 7-ethoxycoumarin-O-deethylase enzyme reactions (Rauch & Nauen, 2003). Finally, it is presented through in-vivo studies, ^{14}C tests that there are 5-hdroxy-imidacloprid end-products as a result of oxidative degradation in imidacloprid resistant species; it is observed that p450 efficiency is important in resistance (Rauch & Nauen, 2003).

In their study, Karunker et al., 2008 determined that in DNA based resistance scanning methods, CYP6CM1 gene regulation level is one of the main indicators of imidacloprid resistant *B. tabaci* individuals. On the other hand, during imidacloprid resistant *B. tabaci* individuals RNA interference studies, it is observed that as a result of the P450 CYP6CM1 gene regulation decrease or suspension processes, *B.tabaci* B and Q biotypes detoxification processes declines and death rate increases (Li et al, 2014). In addition to these, it is proven that *E. coli* bacteria recombinant enzymes with *B. tabaci* CYP6CM1 gene clones quickly detoxify imidacloprid molecules (Karunker et al., 2009; Roditakis et al., 2011). Çukurova is one of the most important areas in Mediterranean Region, Turkey as it is a land of polyculture; unfortunately, insecticides are unconsciously and commonly used, which is an important problem for the region. It is reported that there is neonicotinoid resistance in *A. gossypii* and *Bemisia tabaci* in cotton cultivation areas of the region (Ulusoy et al., 2018, Satar et al., 2018). The imidacloprid resistance in *Bemisia tabaci* is carried out only on the basis of the samples collected from cotton cultivation areas, which is why it is limited and there is no other study about the resistance.

For this purpose, DNA based P450 CYP6CM1 gene relative activity and Glutatton S transferase (GST) gene relative activity levels, which are strong indicators of neonikotinoid resistance of *Bemisia tabaci* on different cotton cultivation locations in Adana, are determined. On the other hand, carboxylesterase(CE), Glutatton S transferase (GST),

p450 enzyme activities which have roles in metabolic resistance to insecticides, are analyzed. In the study, five different *Bemisia tabaci* populations were collected from Adana cotton cultivation areas (Eastern Mediterranean) in 2018. They were compared to susceptible *Bemisia tabaci* individuals which had been cultivated without any pesticides for ten years in Adana Biological Control Research Institute.

MATERIAL METHOD

Bemisia tabaci individuals were collected from Adana, Turkey, during July-August 2018; five different locations with intense cotton production were selected for collection (Solaklı, Meletmez, Sirkeli, Yeniköynazımbey, Çevretepe) (Figure 1). Population was used directly for analysis. Susceptible *Bemisia tabaci* populations cultured in Adana Biological Control Research Institute for ten years were chosen as reference. One-way ANOVA and Duncan's multiple range test have been done by IBM SPSS Statistics 23.

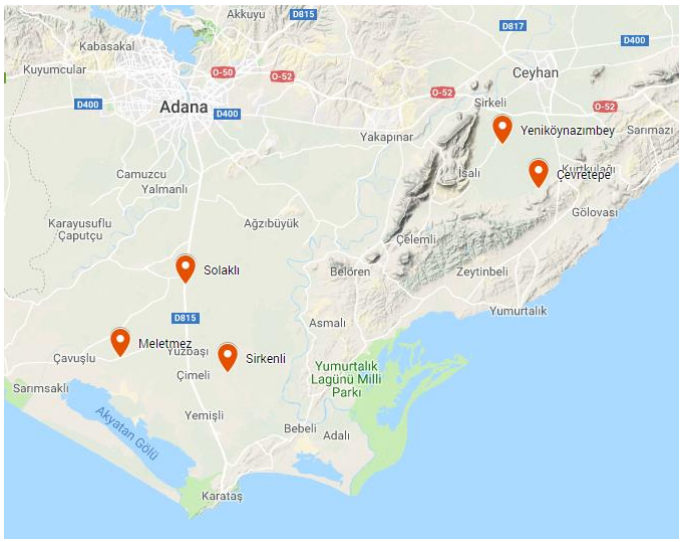

Figure 1. Locations of *Bemicia tabaci* collected in Cukurova Region, Adana, Turkey.

Şekil 1. Türkiye, Adana ili Çukurova bölgesinden toplanmış Bemicia tabaci örnekleri lokasyonları

Determining Bemicia tabaci biotypes

B. tabaci populations total nucleic acid were extracted by using a Thermo Scientific purification kit. After that, the total DNA quality and quantities were measured by nano-drops. Acording to Bel-Kadhi, 2008; Bt-23F, 5'-CGGAGCTTGCGCCTTAGTC-3' and Bt-23R, 5'-GGCTTTATCATAGCTCTCGT-3' RAPD primers were used for determining *B. tabaci* biotypes. In the classical PCR process, there were one cycle of 5 min at 94 ^{0}C, 40 cycles of 1 min at 94 ^{0}C, anneling 1 min at 55 ^{0}C, 1 min at 72 ^{0}C and final extension 10 min 72 ^{0}C.

Determining Carboxylesterase activity

30 individuals of *B. tabaci* were homogenized in Tris HCl buffer (0.05 M, pH 7.5), the homogenate was centrifuged as 10000 g at 4 °C for 25 min, and the supernatant was used as an enzyme source. 125 µl subtrate mixture (4 mg Fast Blue RR salt ve 10 mg α-NA (α-naftil asetat), pH 6.0 Na-P buffer) and 75 µl enzyme were added to the microplate cells and the reaction was started. Reading was carried out in a microplate reader at 450 nm and 27 °C for 7 min(Multiscan Go). The control cells were read without adding the homogenate. The enzyme readings were made with 3 replications and the activity levels were determined by comparison to the controls (Wang et al., 2009).

Determining glutathione S transferase activity

The method by Ulusoy et al., 2018 was modified and used to determine activity. 50 *B. tabaci* individuals were homogenized in a 500 µl Tris HCL buffer (0.05 M, pH: 7.5) by a homogenizer, the homogenate was centrifuged at 11000 g at 4 ^{0}C for 7 min, and the supernatant was used as an enzyme source. 100 µl 0.4 mM 1-chloro-2,4-dinitrobenzene (CDNB), 100 µl 4 mM reduced glutathione (GSH) and 100 µl enzyme were added to the microplate cells and the reaction was started. Reading was carried out in a microplate reader at 340 nm and 25 ^{o}C for 10 min. The control cells were read without adding the homogenate. The enzyme readings were made with 3 replications and the activity levels were determined by comparison to the controls. Changes in absorbance per minute were converted into nM CDNB conjugated/min/mg protein using the extinction coefficient of the resulting 2.4-dinitrophenyl-glutatione (Habig et al., 1974).

Determining cytochrome p450 monooxygenase activity

Cytochrome p450 activity was measured based on the method describe by Ulusoy et al., 2018. 50 *B. tabaci* individuals were homogenized in a Na - phosphate buffer (0.1 M pH: 7.5), the homogenate was centrifuged as 10000 g at 4 ^{o}C for 10 min, and the supernatant was used as an enzyme source. 90 µl of enzyme and 100 µl 2 mM of p-nitroanisole (substrate) were mixed and left for 3-4 min at 34 ^{0}C. Then, 10 µl 9.6 mM of nicotinamide adenine dinucleotide phosphate (NADPH) was added to this mixture, the resulting mixture was put into the microplate cells, and measurements were made at 40 s intervals for 20 min at 405 nm and 25 ^{0}C. The control cells were read without adding the homogenate. The enzyme readings were made with 3 replications and the activity levels were determined by comparison to the controls. The specific enzyme activity was determined using the extinction coefficient of p-nitrophenol (Kranthi, 2005).

All enzymes protein quantities were calculated according to Bradford (1976) with OD values

determined.

B.tabaci total RNA extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

B. tabaci individuals were frozen at -80 °C, 100 of the individuals were homogenized, and their total RNAs were extracted by using a Thermo Scientific RNA purification kit. After the extraction, the total RNA amounts were measured by nano-drops, and equal concentrations of 75 ng/µl each were diluted by a TE buffer.

In the real-time PCR (RT-PCR) analysis, the relative activity of the cytochrome P450s enzyme CYP6CM1(cytochrome P450 CYP6cm1) and GST (Glutathione S-transferase gene) genes were determined by examining the expression levels. CYP6CM1 gene spesific; CYP6CM1-F-5'-GCC ATC GGT GAT AAA GGA GA-3', CYP6CM1-R-5'-AAC TCG GTT TCC TCA TCG TG-3', (NCBI Accession No: GQ214539) primers and GST gene spesific; GSTs-F-5'-GTG GAG GAA AAA CAC CCT CA-3', GSTs-R-5'-AGT CGG TTT TTG GCC TCT TT-3', (NCBI Accession No: EU723684) primers were used in RT-PCR(Karunker et al, 2008; Li et al., 2014). As the housekeeping gene, this study used the EF1α elongation factor1-alpha gene that was proven to be more stable than others, forward EF1α-F-5'-GAT GGC ACG GAG ACA ATA TG-3' and reverse EF1α-R-5'-TTG TCA GTG GGT CTG CTA GG-3'(Liang, et al., 2014).

In the classical PCR experiment, the primers were tested and proven to work by using a Thermo Scientific One-Step RT-PCR kit. In the classical PCR process, there were one cycle of 15 min at 50 ^{0}C, one cycle of 2 min at 95 ^{0}C, 40 cycles of 20 s at 95 ^{0}C, waiting time of 30 s at 60 ^{0}C and another 1min at 72 ^{0}C.

The real-time PCR process was carried out with 3 replications for each gene in each population by using a Thermo Scientific One-Step qRT-PCR kit, the Ct values were determined, and these values are taken into analysis by taking the average of 3 replications. In order to confirm the specificity of the real-time reactions, a melting curve cycle was applied. The same temperature table was used in the RT-PCR process. The melting curve program included a cycle of 30 s at 95 ^{0}C, a cycle of 10 s at 60 ^{0}C and a waiting time of 10 s at 60 ^{0}C.

The relative activity levels of the CYP6CM1 and GST genes were determined by making ΔΔCt calculations based on the reference population by taking the average of the obtained Ct values (Livak & Schmittgen, 2001).

RESULT and DISCUSSION

Since its discovery in 1991, imidacloprid resistance in *B. tabaci* was firstly observed in Southern Spain and it has rapidly increased since that time (Cahill et al., 1996, Sparks & Nauen, 2015). In Turkey, the first imidacloprid resistances in *B. tabaci* and *A. gossypii* pests were observed in 2009 and 2016 (Satar et al., 2018, Ulusoy et al., 2018)(table 2). At the end of PCR reactions of the study, which are made for *B. tabaci* biotype discrimination, it was determined that the population used in the study is B biotype (figure 2).

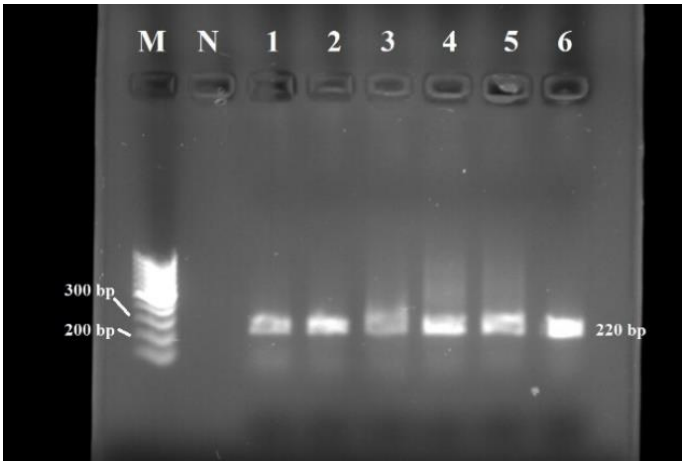

Figure 2. *B. tabaci* RAPD-PCR agarose gel electrophrosesis photograph. M: 100 bp ladder, N.Negative control, 1:Çevretepe, 2:meletmez, 3:sirkenli, 4:Solaklı, 5:Yeniköynazımbey 6:hassas. All PCR products 220 bp.

Şekil 2. *B. tabaci RAPD-PCR agaroz jel elektroforez görüntüsü. M: 100 bp işaretçi, N.Negatif kontrol, 1:Çevretepe, 2:meletmez, 3:sirkenli, 4:Solaklı, 5:Yeniköynazımbey 6:hassas. Bütün PCR ürünleri 220 bç(baz çifti) bulunmuştur.*

When CYP6CM1 expression levels of the samples were observed, it was seen that there was a higher change (when compared to the sensitive population) varying from 7 to 18.2 times (Figure 3). It is found that the highest expression level was in Meletmez population (18.2 times higher than the sensitive population) (Figure 3).

Karunker et al., 2008, collected B and Q type B. *tabaci* from different parts of the world. At the end of the analysis process, they reported that only imidacloprid resistant populations have higher CYP6CM1 gene expression when compared to susceptible population (17 times). On the other hand, it is proven that through imidacloprid resistance *B. tabaci* RNA interference studies, it is possible to stop or slow down P450 CYP6CM1 gene regulation, which decreases imidacloprid resistance (Li et al., 2014).

When the previous studies in the same region were analyzed, it was seen that there are reports showing that while there is 230 times imidacloprid resistance in *A. gossypii* (in six different locations) there is up to 95 times imidacloprid resistance in *B. tabaci* (in one location) (Table 1).

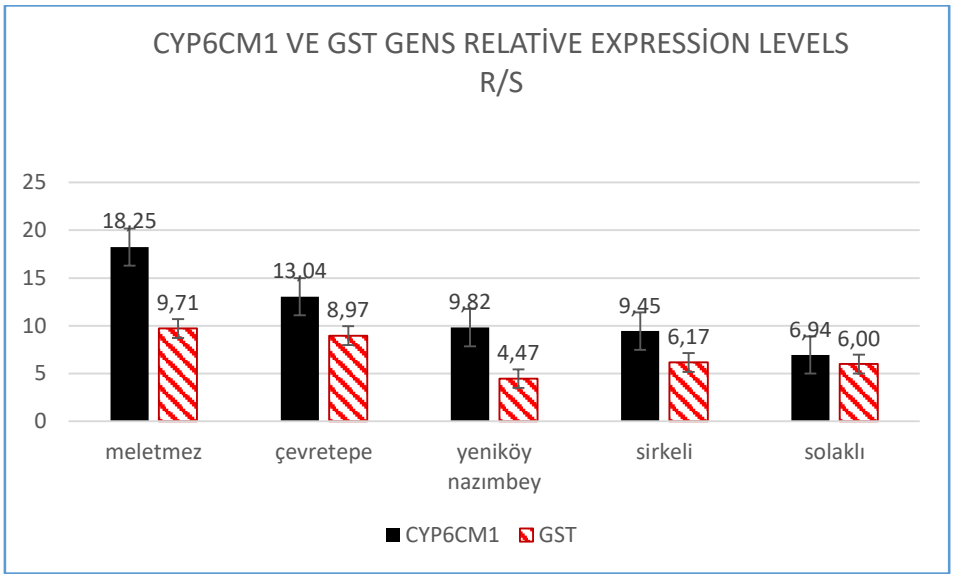

Figure 3. Cytocrome P450 CYP6CM1 and GST gens relative expression levels of *Bemicia tabaci* populations (R/S: Resistant population/susceptiple population)

Şekil 3. Bemicia tabaci sitokrom P450 CYP6CM1 ve GST genleri relatif ekspresyon düzeyleri(R/S: dirençli popuasyon/hassas popülasyon)

Table 1. Imidacloprid resistant insects sp. Cotton fields in Cukurova region in Adana, Turkey. a:Ulusoy et al, 2018; b:Satar et al., 2018

Tablo 1. Türkiye, Adana iIi Çukurova bölgesinde pamuk alanlarında imidacloprid dirençli böcekler. a:Ulusoy et al, 2018; b:Satar et al., 2018

İnsect spp.	Imidacloprid LC50 (mg L^{-1})	Resistant Factor *(Dirençlilik düzeyi)*	Location *(Lokasyon)*
Aphis gossypii[a] (collected 2016) *(2016 yılında toplanmış)*	17.7	206.55	Six location cotton fields in Cukurova (*Çukurova bölgesinden toplanmış altı lokasyon)*
Bemicia tabaci[b] (collected 2009) *(2009 yılında toplanmış)*	56.9	93.4	One location in Cukurova *(Çukurova bölgesinden toplanmış tek lokasyon)*

In line with these results, the high level of CYP6CM1 expression (18 times) in the study population indicates that imidacloprid resistance in cotton fields is high. Populations in the study have high p450 enzyme activities, which is in parallel with the CYP6CM1 expression levels. The highest level of p450 enzyme activity was observed in Meletmez population (3.24 M min^{-1} mg^{-1} protein) which was in line with the CYP6CM1 gene expression level (Table 2).

Table 2. Carboxyle esterase (CE), glutathione S-transferase (GST), cytochrome P450 monooxygenase enzyme activities of resistant and susceptible populations

Tablo 2. Dirençli ve hassas popülasyonların karboksilesteraz (CE), glutatyon-S-transferaz (GST), sitokrom P450 monooksigenaz enzim aktivitesi düzeyleri

Populations	**CE (M min^{-1} mg^{-1} protein)**	**Gst (M min^{-1} mg^{-1} protein)**	**p450 (M min^{-1} mg^{-1} protein)**
çevretepe	7.50 ± 0.71c	73.64 ± 1.16bc	3.07 ± 0.41bc
meletmez	12.05 ± 0.74cd	87.86 ± 0.86bc	3.24 ± 0.51d
sirkenli	8.01 ± 0.99bc	68.48 ± 1.23b	0.10 ± 0.12ab
solaklı	5.15 ± 0.97b	57.00 ± 1.22b	0.08 ± 0.15cd
yeniköynazımbey	8.47 ± 0.68d	45.00 ± 1.05d	2.02 ± 0.22bcd
susceptible	3.56 ± 0.40a	23.00 ± 0.95a	0.04 ± 0.12a

a, b, c, d,e – Duncan's multiple range test ($p < 0.05$)

a, b, c, d, e – Duncan çoklu karşılaştırma testi ($p < 0.05$)

When carboxyl esterase and GST enzyme activities were analyzed, it was seen that the highest levels of both enzymes were in Meletmez population (CarE:12 and GSt: 87.8 M min-1 mg-1 protein). When compared to the sensitive population, Meletmez had four times higher enzyme activity.

According to the previous studies in the literature, general esterase and GST activities can be high in populations that are resistant to organophosphate, carbamate, pyrethroid group insecticides (Devonshire & Moores, 1982; Hemmingway&Georghiou, 1984; Rauch & Nauen, 2003; Yang et al, 2014). Different types of insecticides are commonly used in Çukurova Region which is a poly-culture agricultural production area (vegetables, fruits, cotton etc.). Although different agricultural products are cultivated in the region, agricultural lands are almost within one another. As *B. tabaci* is a polyphagous species and frequently exposed to insecticides with different mechanisms such as organophosphate, carbamate, pyrethroid and neonicotinoid, it is unavoidable for it to have high carboxylesterase and GST activities.

As a result of the study, it can be said that, there was a common imidacloprid resistance in *B. tabaci* populations in Adana cotton cultivation areas in 2018; the previous studies and CYP6CM1 gene over-expression criteria support this finding. On the other hand, it is possible to claim that there is a metabolic resistance in the populations resulting from the common use of insecticide. According to these findings, it is important to review and manage the insecticide control of *B. tabaci* in Çukurova region cotton cultivation areas; it is also crucial to develop more efficient and different control strategies in order to cope with the resistance in the region.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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