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Biochemical characteristics and *in vitro* inhibition of chitinase and acetylcholinesterase in *Bemisia tabaci* Gennadius (Hom: Aleyrodidae)

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ABSTRACT

The biochemical interactions of five pesticides (malathion, phenothrin, cyhalothrin, diuron and chloropropham) on the two important biological targets, chitinase and acetylcholinesterase (AChE), were investigated in *Bemisia tabaci* adults. Enzymes were isolated from *B. tabaci* adults and the optimal conditions for the enzymatic activity were identified. The optimal conditions for chitinase activity were at 37 °C, 120 min incubation time, 1µg protein concentration and pH 4.5, while they were at 35 °C, 20 min incubation time, 2.2µg protein concentration and pH 8.0 for AChE. In addition, the data proved that the activity of either chitinase or AChE was not dependant on the presence of $CaCl_2$, $MgCl_2$ and $MnCl_2$ cations or EDTA and EGTA exogenous chemicals. Effects of tested pesticide on the *in vitro* enzymatic activity of chitinase and AChE isolated from female and male adults of the subject insect were evaluated. The results revealed that in case of chitinase, the significant reduction in the enzymatic activity was recorded for diuron and cholopropham as the percentages of enzymatic inhibition were 80.4, 76.9 % for females and 82.2, 78.6 % for males, respectively. On the other hand, in case of AChE, the significant reduction in its activity was recorded for malathion only where the percentages of inhibition were 81.0, 86.0% for females and males, respectively. Besides, the I_{50} values for diuron and chloropropham as the strongest inhibitors of chitinase activity were determined. I_{50} values for diuron were 0.27, 0.12 µM, while for chloropropham they were 0.45, 0.15µM for females and males, respectively. Moreover, the I_{50} values for malathion as the strongest inhibitor of AChE activity were 0.52, 0.25 µM for females and males of *B. tabaaci* adults, respectively. Furthermore, the inhibition constant (Ki) values were determined for chitinase and AChE inhibitors. Values of Ki in the case of

diuron were 40.0 and 15.0μM whereas in case of chloropropham, they were 52.0 and 18.0μM for females and males, respectively. On the other hand, Ki values were 61.0 and 56.0μM for *B. tabaci* females and males, respectively, in case of malathion. The results of the present study may add some forward steps to chitinase as a target for non conventional pesticides, especially those of phenylurea derivatives, in the IPM programs of the subject insect.

INTRODUCTION

The whitefly *Bemisia tabaci* Genn. is still one of most important pests on vegetables and field crops allover the world. It has a host range of more than 500 plant species (Greathead, 1986). When present in sufficient numbers, it may cause extensive damage through direct feeding and production of large quantities of honeydew. Besides, it is the most important vector of up to 60 geminiviruses among all other whitefly species (Bedford *et al.*, 1992; Byrne *et al.*, 1994). One of the most important factors contributing the recent severe outbreaks of this pest has been the appearance of high levels of resistance to many of the insecticides currently used in whitefly control programs especially AChE target the OP insecticides (Dittrich and Ernst, 1990). The efficacy of OPs at this target site is primarily governed by their affinity for the active site on AChE (Byrne and Devonshire, 1997). Therefore, it was thought profitable in present study to investigate the interaction of some major groups of pesticides on the involved enzyme systems (i.e. chitinase and AChE). This was done hoping better understanding of such interactions in order to find some better control measures for such injurious pest.

The plan of this research was to 1) optimize conditions for assay chitinase and AChE activities in *B. tabaci* adults, 2) investigate the *in vitro* inhibition of chitinase and AChE by five tested pesticides belong to three different groups, 3) determination of some kinetic parameters of the enzymatic activity by the most potent inhibitors, 4) looking for a new aspect can be used in IPM programs for such deleterious subject pest.

MATERIALS AND METHODS

1.The whitefly, *Bemisia tabaci* Genn. laboratory strain:

The subject insect has been taken from a susceptible laboratory culture of the cotton whitefly *Bemisia tabaci* Gennadius, which has been reared on tomato plants since 1999 in greenhouses at 25±7 °C, 65±5 RH and under natural light conditions in Economic Entomology Department, Faculty of Agriculture, Alexandria University.

2-a- Chitinase preparation and activity assay:

Chitinase was prepared from *B.tabaci* adults according to the method of Deul *et al.* (1978). Homogenate of adults was prepared in 10^{-3}M DTT (dithiotheritol) (v/w=2) using glass homoginizer. The homogenate was centrifuged for 15 min. at 12,000g. An equal volume of saturated ammonium sulfate solution was slowly added to the supernatant. After stirring for 1hr, the suspension was centrifuged for 10 min at 10,000g. The precipitate was washed with half-saturated amonium sulfate solution and then recentrifuged, after which it was suspended in a small volume of water, followed by dialysis 20hr .Any occasional precipitate was removed by centrifugation and was discarded as it proved to be enzymatically inactive. After dialysis, water was added to the original ratio (v/w=2).All manipulations were carried out at 0-2 °C.

Chitinase activity was determined according to the method of Reissig *et al.* (1955), which modified by Andrew *et al.* (1982). The assay mixture (350 µl) contained chitin as a substrate (20mg/ml), prepared enzyme and sodium acetate buffer (pH 4.5) with ratio of 1:4:9. The enzyme substrate mixture was incubated at 35°C for 60 min .The reaction was stopped by adding 100 µl of 0.8mM borate buffer (PH10.0) followed by determination of N-acetylglucoseamine according to the method of Reissig *et al.* (1955) by adding P-dimethyl amino benzaldhyde (DMAB) reagent (1:1.5 of total volume). Samples were placed on a shaker water bath at 35°C for 20 min The optical densities were measured spectrophotometrically at 416 nm.

2-b- AChE preparation and activity assay:

Adults were homogenized in tris-HCl buffer (pH 7.4), 80 adults /30ml buffer, with polytron mixer (at 50% power for 50 sec.), then subjected to low speed centrifuged at 5,000 rpm for 15 min at 4°C. The resulting supernatant was centrifuged at 15,000 rpm for 20 min at 4°C. The

supernatant centrifuged at 25.000 rpm for 1hr at 4°C. Pellets were resuspended in 1ml of tris-HCl buffer (pH 7.4) and used as enzyme source. All the centrifugation preparations were accomplished by using Beckman J2-21 rotor.

AChE activity was determined according to the method of Ellman *et al.* (1961). This method is based on the hydrolysis of acetylthiocholine iodide (ATChI) as substrate by the enzyme to produce thiocholine and acetic acid. Thiocholine reacts with 5,5-dithio bis-(2-nitrobenzoic acid), "DTNB" to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production as a function of enzyme activity is measured spectrophotometrically at 412nm. Enzyme specific activity was computed as mg protein/hr.

3- Determination of optimal conditions for the two tested enzymes:

3- a- Optimal conditions for chitinase activity assay:

To investigate the effects of temperature on chitinase activity, enzyme activity determinations were conducted from 30 to 45°C in 5 °C increments. Incubation times were examined from 60 to 180min. in 30 min. increments after adding the substrate solution. The effect of protein concentration was measured from 0.4 to 1.2ug/ reaction. Sodium acetate buffers from pH 3 to 5 were also tested. The amount of produced N-acetylglucoseamine was determined according to Andrew *et al.* (1982).

3-b- Optimal conditions for AChE activity assay:

Effect of temperature on AChE activity was carried out from 30 to 45°C in 5°C increments. The enzymatic reaction was carried out from 12 to 28min. in 4min. increments. The effect of protein concentration was measured from 1.1 to 2.7ug/reaction. Effect of phosphate buffer pH was also tested from pH 5 to 9.

To evaluate effect of some chemicals on the activity of chitinase and AChE, two exogenous chemicals, ethylenediamine tetra acetate (EDTA) and ethylene glycol bis (2-amin-ethylether)-N,N,N-,N-tetra acetic acid (EGTA) and three salts $CaCl_2$, $MgCl_2$ and $MnCl_2$ were tested.

4- Tested pesticides:

The tested pesticides are:

Two pyrethroide insecticides, cyhalothrin ((S)-α-cyano-3-phenoxybenzyl)

and phenothrin (3-phenoxy benzyl (1RS, 3RS, 1RS, 3RS) -2,2-dimethyl-3-(2-methylprop-1-enyl); organophosphorus insecticide, malathion (Diethyl(dimethoxyphosphinothioylthio) succinate); two phenylurea derivatives, diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea), and chloropropham (Isopropyl-m-chloro-carbanilate).

The above-mentioned pesticides were produce by U.S.A. Environmental Protection Agency (EPA), USA. Stock solutions of these compounds were prepared in pure acetone. All pesticides used were of technical grade, with purities greater than 95%.

5- *In vitro* inhibition and kinetics of chitinase and AChE:

The inhibition of chitinase and AChE activity were determined in newly emerged male and female adults using the LC_{50} values of each of the five tested pesticides as inhibitors. The strongest inhibitors for each of chitinase and AChE were evaluated to determine enzyme kinetic parameters at the optimal conditions. The method of Dixon and Webb (1964) was adopted to draw the Dixon-plots by plotting 1/V versus concentrations of the inhibitor at two concentrations of the substrate.

Estimation of I_{50} value (the concentration of the inhibitor which inhibits 50% of the enzyme activity) was carried out by preincubating the enzyme (insect homogenate) with the inhibitor for 30 min. at the optimal conditions of chitinase and AChE. Also, Ki (the inhibition constant) values for each inhibitor were estimated from Dixon-plot.

The protein content in prepared homogenates of *B. tabaci* adults was assayed spectrophotometrically by the method of Lowry *et al.* (1951) at 750 nm using bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

1. Optimal conditions for chitinase and AChE activity assay:

The optimal conditions for measuring chitinase and AChE activities in whole body homogenates of *B. tabaci* are represented in Fig. 1(a and b). The obtained results showed that the optimum temperature at which the maximum velocity occurs was 37 and 35 °C for chitinase and AChE, respectively. In addition, the maximum velocity of chitinase activity was at 120 min., 1.0 µg protein and pH 4.5; while the maximum velocity of AChE was recorded at 20 min., 2.2 µg protein and pH 8.

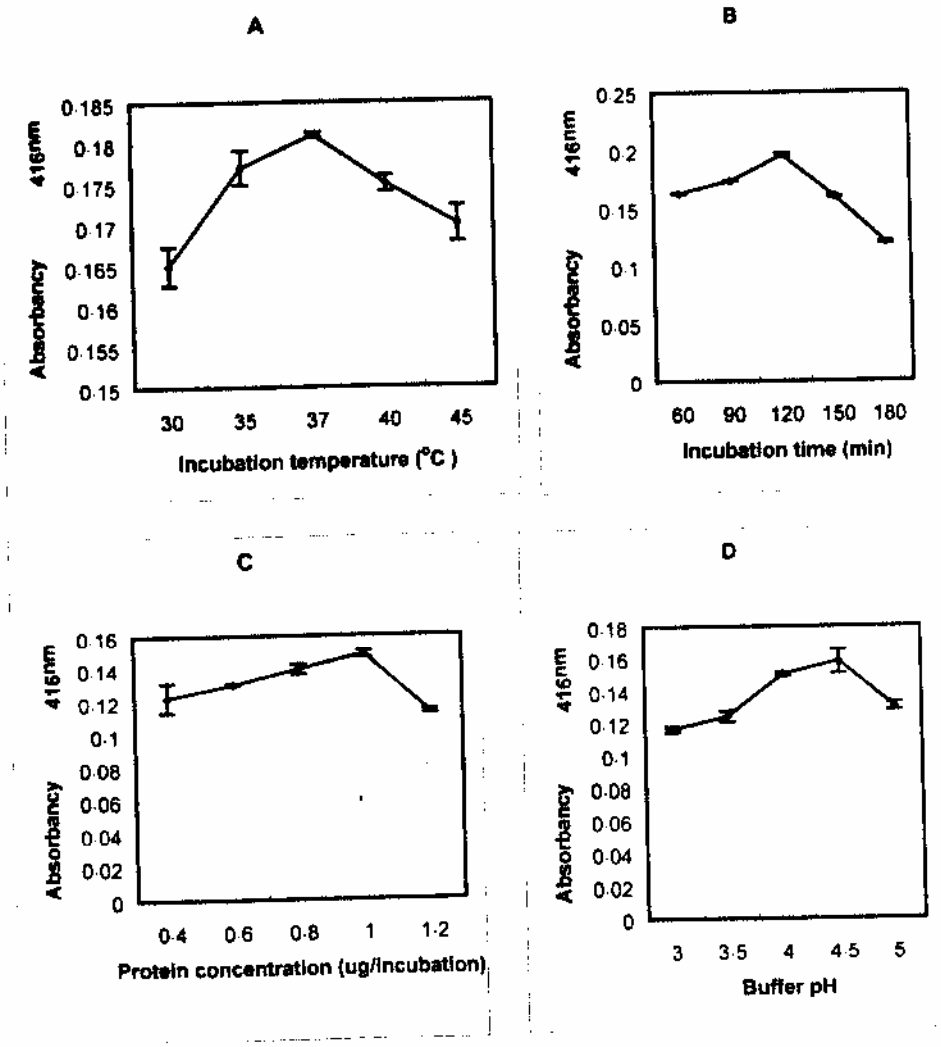


Fig. (1,a): Optimal conditions for B. tabaci chitinase activity assay as a function of Incubation tempreature (A), incubation time (B), protein concentration (C) and buffer pH (D). Each point represents the mean ±.S.E of at least three determinations.

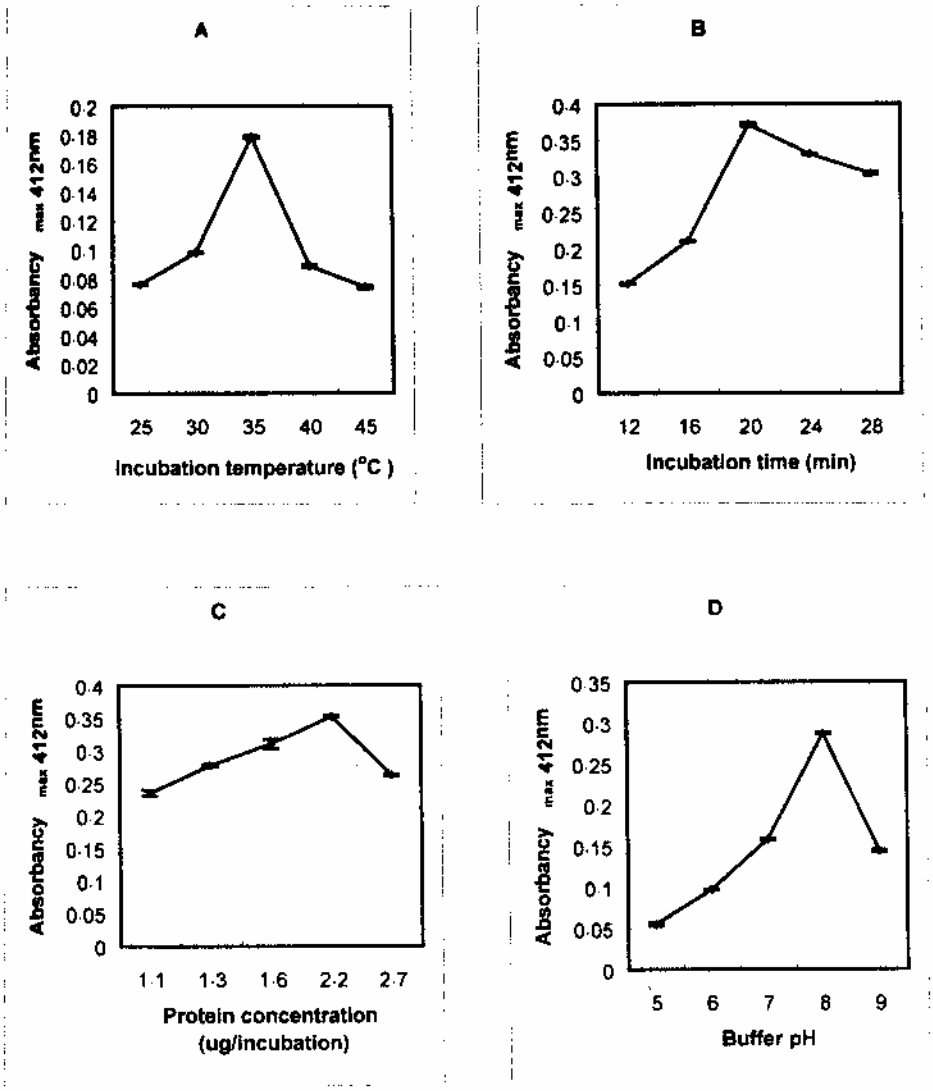


Fig. (1,b): Oplimal condilions for B. tabaci AChE activity assay as a function of Incubation temperature (A), incubation time (B), protein concentration (C) and buffer pH (D).Each point respresents the mean ± S.E. of at least three determinations.

The present findings concerning the chitinase optimum temperature and pH are almost agreeable to those of Ulboa and John (1992) which declared that the optimum conditions for *Trichoderma harzianum* chitinase activity was at 40°C and pH 4.0. Also, Konda *et al.* (2002) found that the purified chitinase from *Pieris rapae* had an optimal pH of 5.0 for the hydrolysis reaction when glycol chitin was used as a substrate.However, Babiker *et al.*(1999) showed that the optimum pH for 54KDa chitinase from *Bombyx mori* was 6.0 toward a short substrate , N-acetylchitopentose.

The previously observed similar optimal pH (7.5-8.0) and temperature (35.0-40.0) of *Lygus hespersus* AChE (Zhu and Brindley, 1992) could be considered supportive to the present results. Similarly, the present results agreed to large extent with those of Devonshire (1975) who found that the optimum temperature and pH for hydrolyzing ATCh in cotton leafworm AChE was 35°C and 7.5.

2. Effect of EDTA, EGTA and some cations on enzymatic activity:

The influence of EDTA, EGTA, $MgCl_2$, $CaCl_2$ and $MnCl_2$ on the enzymatic activity of chitinase and AChE was investigated and the results are shown in Fig. (2). It is quite clear from the obtained results that although some of these chemicals slightly enhanced the enzymatic activity, there were no significant differences in comparison to controls. This proved that the activities of the two tested enzymes were not dependent on any of these chemicals so there were no cation requirements for enzymatic activity. The present results are parallel with that suggested by Andrew *et al.* (1982) on stable fly chitinase. However, Bade and Stinson (1981) found that the presence of Ca^{+2} gives high chitinase activity for *Drosophila melanogaster* larvae. Besides, Machida and Saito (1993) reported that the purified chitinase from housefly showed a requirement for Mg^{+2} as it stimulates the enzyme activity approximately 5-fold.

3. *In vitro* inhibition of *B. tabaci* chitinase and AChE:

Effects of the tested concentrations of each tested pesticides on the *in vitro* inhibition of chitinase and AChE extracted from newly emerged female and male adults of the subject insect were investigated. The obtained results illustrated in Fig. (3, A) revealed that diuron and chloropropham exhibited significant reduction in chitinase activity. Percentages of chitinase inhibition were 80.4, 85.5% for females and 76.9, 79.3% for males by diuron and chloropropham, respectively. On the other hand, in the case of AChE, the significant reduction in its activity was recorded for malathion

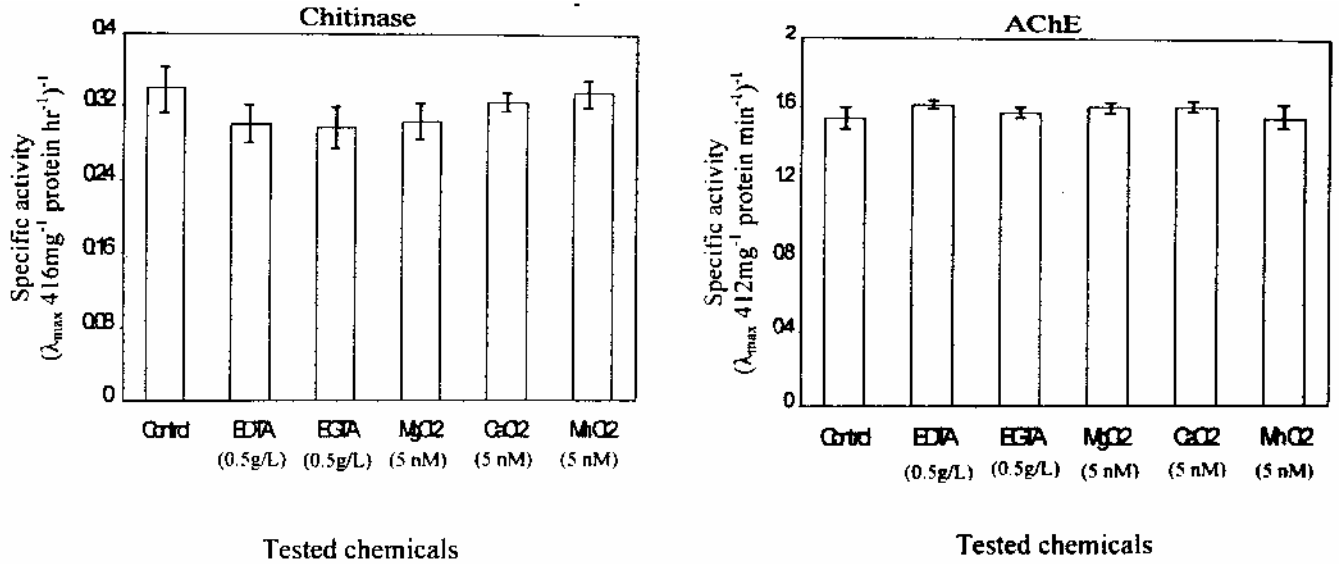


Fig. 2: Effect of some chemicals on the specific activity of chitinase and AChE in *B. tabaci* adults. Values are means ± S.E. of three determinations. Data are not significantly different (P>0.01; Duncan's multible range test by using SAS program, 1986).

only (Fig. 3, B). The percentages of AChE inhibition were 81.0, 86.0 for *B. tabaci* females and males, respectively.

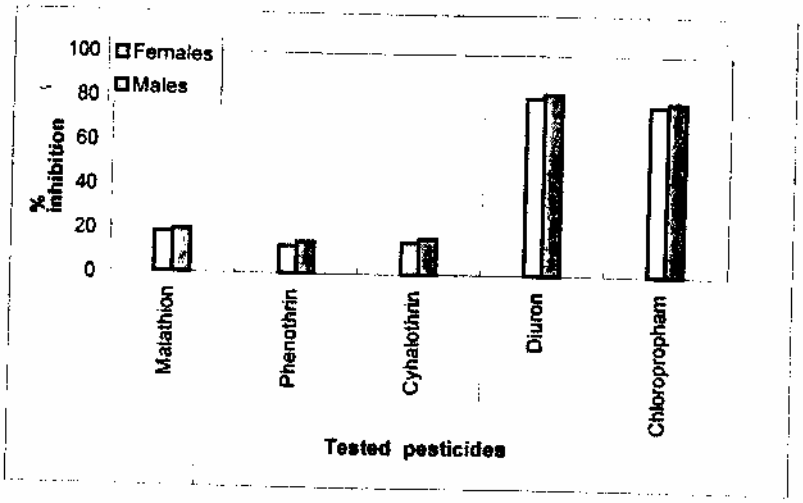
According to the above-mentioned results, diuron and chloropropham showed to be the strongest inhibitors for chitinase activity while malathion was the strongest inhibitor for AChE activity. Therefore, the following experiment was conducted to evaluate the inhibitory effect of different concentrations of each inhibitor on the enzymatic activity. The present results indicate that the inhibitory effects of diuron and chlorobropham on chitinase activity are relatively most obvious in males as percentages of inhibition in males exceeded those of females (Fig. 4). The same trend was recorded in the case of the *in vitro* inhibition of AChE by malathion concentrations (Fig. 5). Therefore, it could be concluded that the response of each tested enzyme to inhibitors was found to be different in either male or female adults. These latter findings look acceptable in consideration to the probable differences in the reproductive biotic potentials of either sex.

The increased sensitivity of *B.tabaci* males when compared to females may be contributed to the increase sensitivity of male chitinase and AChE to inhibition rather than those of females. Such latter comment looks acceptable on the light of Gao *et al.* (2005) results on *Pediculus capitis*, which declared that the percentages of AChE inhibition in males increased 2-fold than those of females. Taking into consideration the suggestion of Wang *et al.* (2004), that the insecticidal tolerance is largely due to metabolic activities, which either detoxify or limit the intoxicating ability of the insecticide.

4. Kinetic parameters of chitinase and AChE inhibition:

To characterize more details about the *in vitro* inhibition of chitinase and AChE by the strongest inhibitors, Ki value of each inhibitor was estimated from the graphical method of Dixon and Webb, 1964 (Figs. 6, 7 and Table 1). The Ki values were 40.0 and 15.0μM for *B. tabaci* females and males respectively in the case of diuron. While these values were 52.0 and 18.0 μM for females and males respectively in the case of chloropropham. On the other hand, Ki values were 61.0and 56.0 μM for female and male adults, respectively, in case of malathion.

The present results are in accordance with those reported by Zhu and Brindley (1992) who reported competitive inhibition of AChE purified from *Lygus hesperus* by six OP compounds.



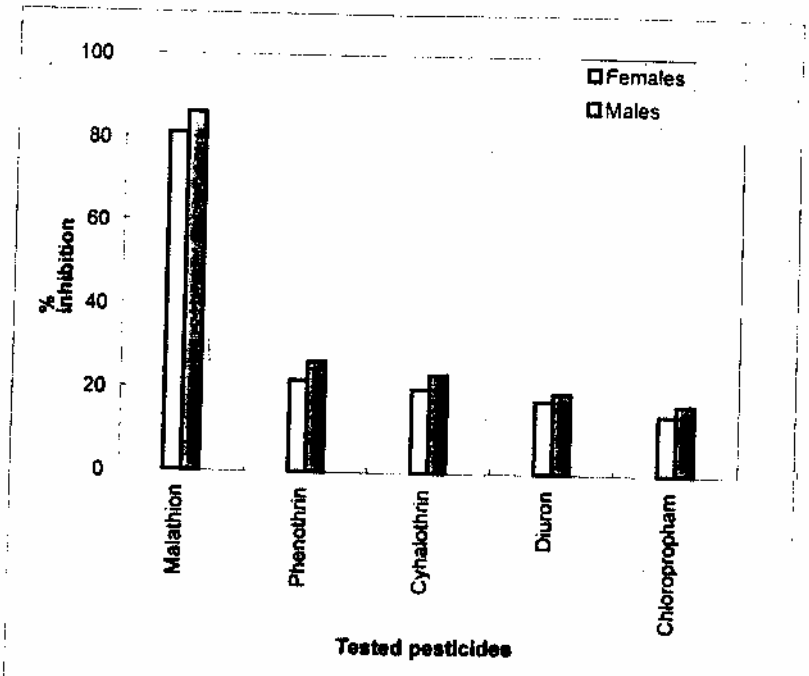


Fig.(3): *In vitro* inhibiton of *B.tabaci* chitinase(A) and AChE(B) activities by tested pesticides.(concentrations of 1.88, 0.44 µM for malathion; 4.62, 1.83µM for phenothrin; 0.16, 0.09µM for cyhalothrin; 0.99, 0.37µM for diuron; 5.96, 2.03µM for chloropropham were tested against females and males, respectively.

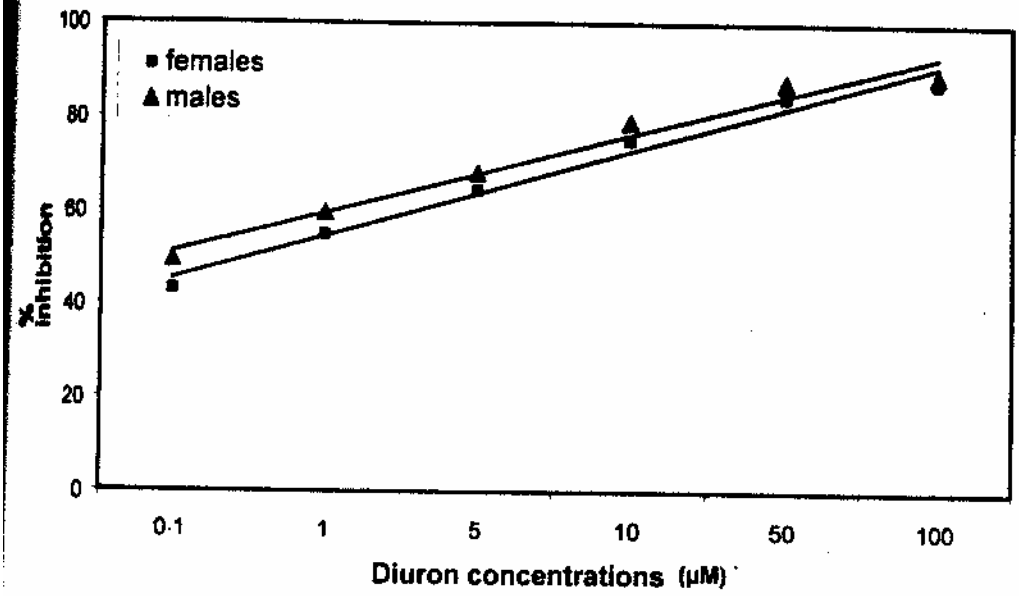
Table1: Kinetic constants of chitinase for diuron and chloropropham, and of acetylcholinesterase for malathion.

Tested pesticides	Chitinase				AChE			
	I_{50}		Ki		I_{50}		Ki	
	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
Diuron	0.27	0.12	40.0	15.0	---	---	---	---
Chloropropham	0.45	0.15	52.0	18.0	---	---	---	---
Malathion	---	---	---	---	0.52	0.25	61.0	56.0

In addition, the I_{50} values for the potential inhibitors; diuron and chloropropham; for chitinase and malathion for AChE activity; were determined (Table1). The recorded I_{50} for diuron were 0.27, 0.12 µM for females and males, respectively. While, I_{50} values for chloropropham were 0.45, 0.15 µM for females and males, respectively. Moreover, I_{50} values for malathion against AChE were 0.52 and 0.25 µM for females and males, respectively.

In comparing the inhibition potency of diuron and chloropropham against chitinase activity within the same sex, it is clear that diuron showed to be the strongest inhibitors for both *B. tabaci* sexes. On the other hand, the I_{50} values of each of diuron and chloropropham in females in comparison with males indicate that male chitinase is more succeptible than that of females to these two inhibitors. The same trend was found in the inhibition potency of malathion against AChE extracted from female and male adults as I_{50} value for female represents about 2 times of that of males (0.52 & 0.25 µM, respectively). Such latter findings supported the above-mentioned results discussed from Fig. (4 &5).

Chitinase plays an essential role during ecdysis chitin. This enzyme is vital to moulting in insects, and may also affect gut physiology through their involvement in peritrophic membrane turnover (Fitches *et al.*, 2004). The exoskeleton of insects might constitute a useful target site for insecticidal chemicals (Gunnar *et al.*, 1985). Most of the chemicals that act to kill insects by affecting the cuticle do so by compromising the insect's ability to moult. It might be a good target for the development of selective insecticides (Nitoda *et al.*, 1999). Therefore, it was thought promising to test the effect of two herbicides (diuron and chloroprophan) on the subject



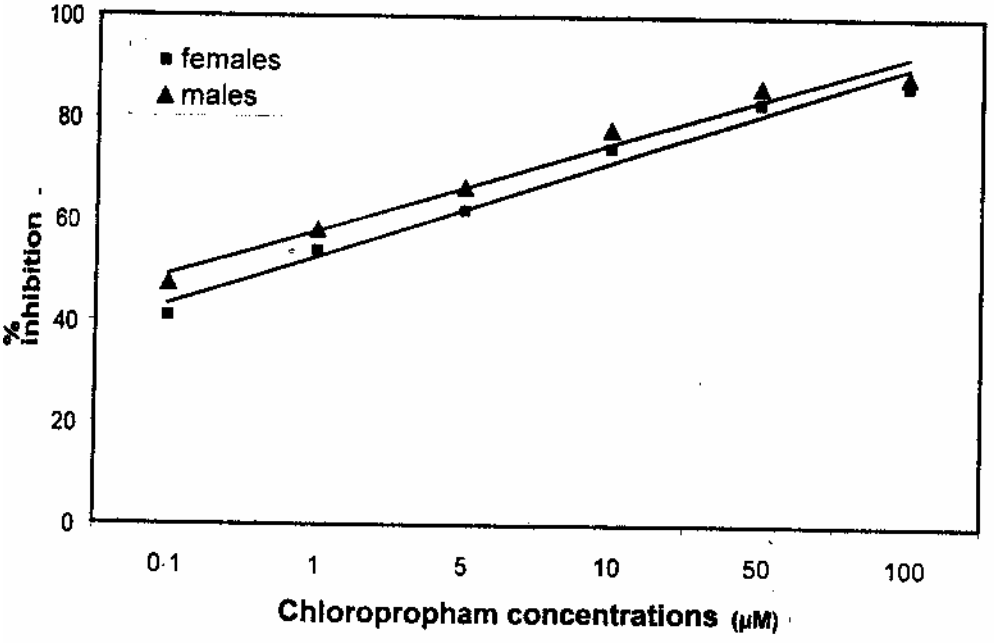


Fig. (4) : *In vitro* inhibition of chitinase activty by diuron (A) and Chloropropham (B) in both female and male adults of *B.tabaci.*

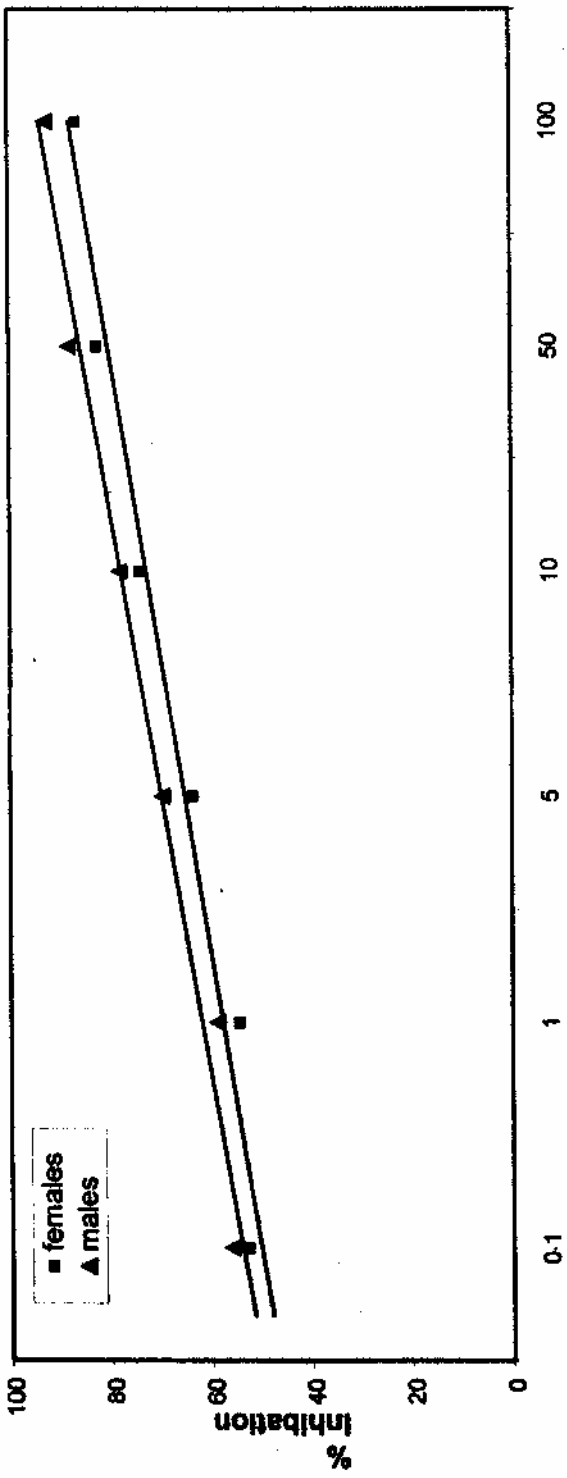
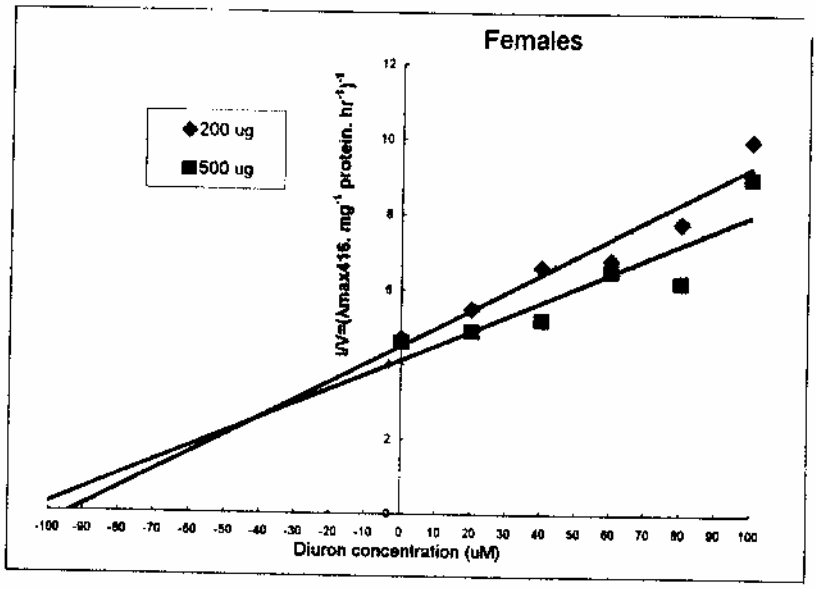


Fig. (5):*In vitro* inhibition of AChE activty by malathion in both female and male adults of *B.tabaci*.



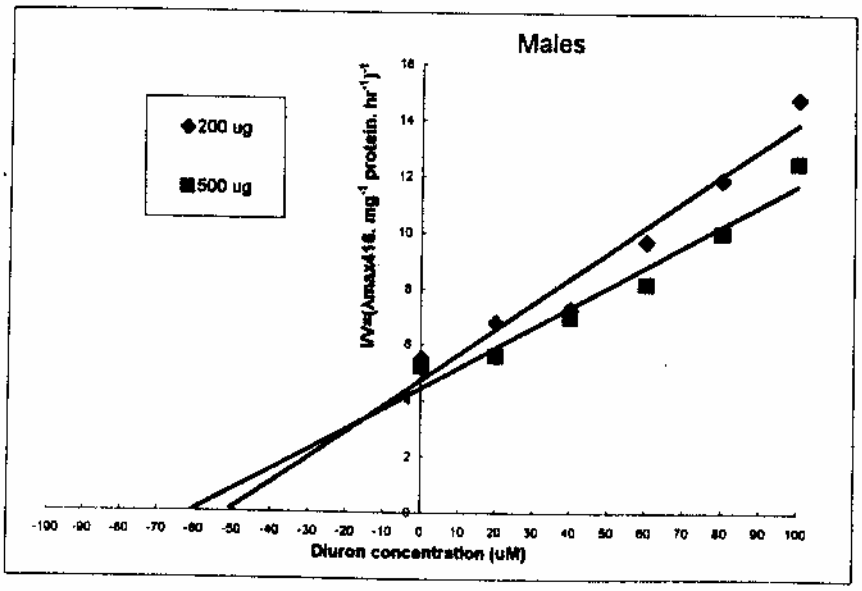


Fig (6-A): Dixon-plot of diuron effects on chitinase activity in *B. tabaci* female and male adults at two substrate concentrations.

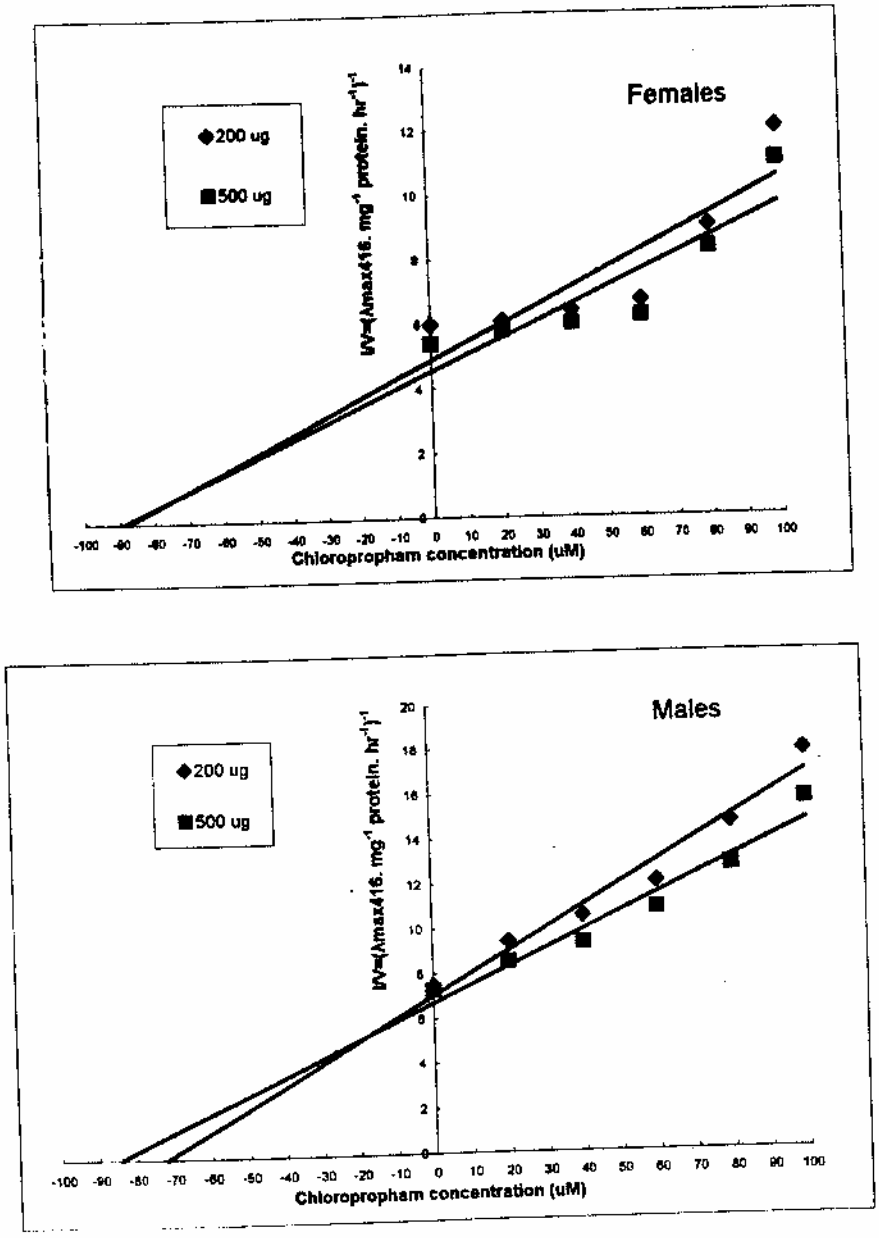


Fig (6-B): Dixon-plot of chloropropham effects on chitinase activity in *B. tabaci* female and male adults at two substrate concentrations.

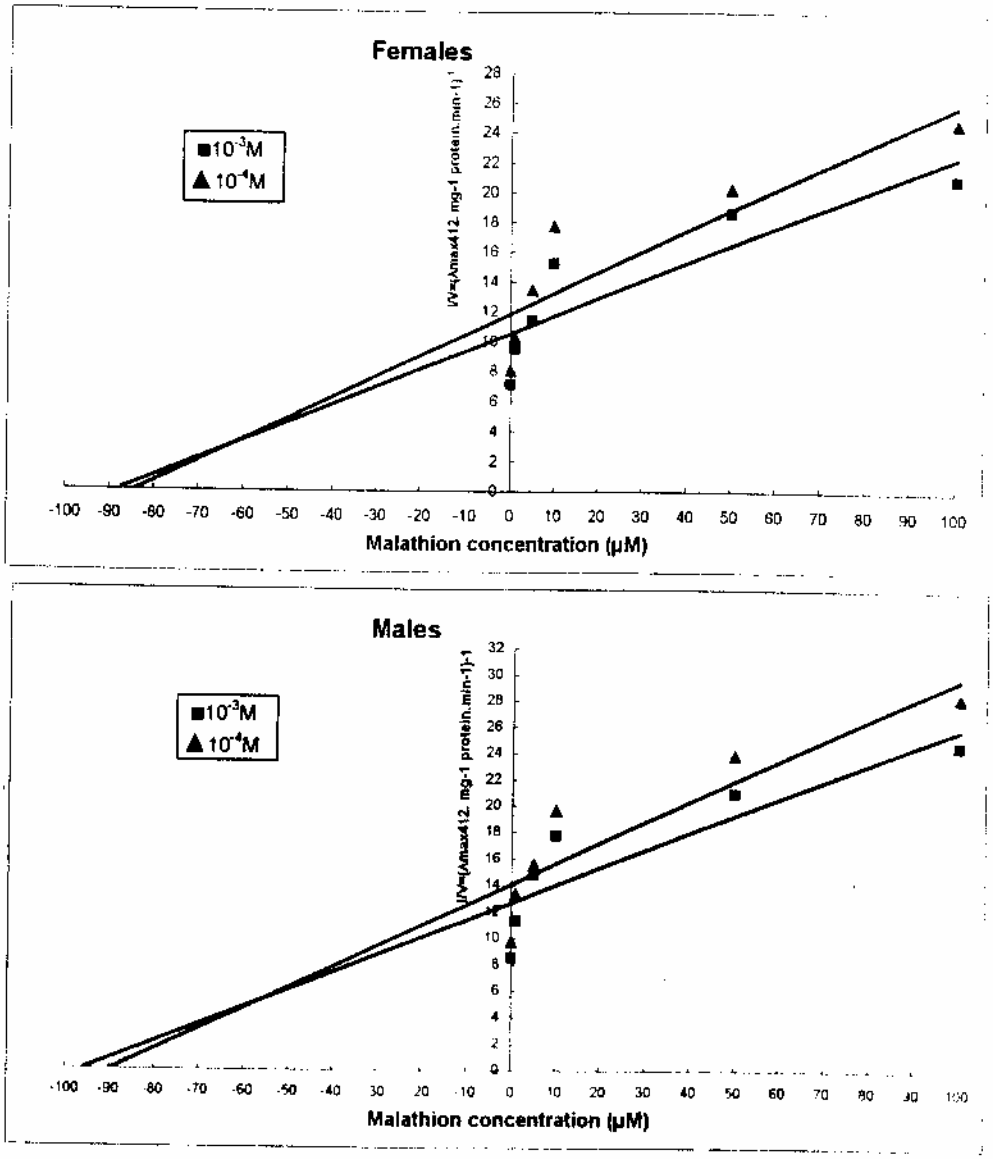


Fig. (7): Dixon-plot of malathion effect on AChE activity from *B.tabaci* female and male adults with two concentrations of ATChI .

insect. These tested herbicides have analogus structures to acylureas which prevent the synthesis of chitin microfibrils and might induce expression of the enzyme chitinase (Reynolds, 1987).

Finally, the present study has provided some basic information of AChE and chitinase that will be useful to understand the mechanisms of insecticide tolerance in the subject whitefly.

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بعض الخصائص البيوكيميائية لإنزيمي الكيتينيز والأسيتايل كولين إستيريز مع تثبيطهما خارجياً فى حشرة ذبابة القطن البيضاء

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تـم دراسـة التداخل البيوكيماني لخمسة من المبيدات (مالاثيون، فينوثرين، سيهالوثرين، ديارون وكلوروبروفام) على إثنان من أهم الأهداف البيولوجية فى الحشرات الكاملة لذبابة القطن البيضاء وهما إنزيم الكيتينيز والإنزيم المحلل لإستر الأسيتايل كولين AChE . تم إستخلاص كلا الإنزيميـن من الحشرات الكاملة وتم تحديد الظروف المثلى لنشاط كل منهما. فوجد أن الظروف المـثلى للـتفاعل الإنزيمي فى حالة إنزيم الكيتينيز كانت عند درجة حرارة 37°م، زمن تحضين لمخلـوط الـتفاعل 120 دقيقة ورقم حموضة 4.5. كما كان أنسب تركيز بروتيني اميكروجرام بروتيـن. بيـنما كانت الظروف المثلى للتفاعل الإنزيمي فى حالة إنزيم الأسيتايل كولين إستيريز هـي عـند درجة حرارة 35°م، رقم حموضة 8، زمن تحضين لمخلوط التفاعل 20دقيقة وكان

أنسب تركيز بروتيني 2.2 ميكروجرام بروتين. كما أثبتت النتائج أن كلا الإنزيمين غير معتمدين في نشاطهما على وجود الكاتيونات.

كذلك فقد تم إختبار تأثير التركيز القاتل لـ 50% من إناث وذكور الحشرة وذلك للخمسة مبيدات المختبرة على النشاط الإنزيمي للكتينيز والأسيتايل كولين إستيريز *in vitro*. ولقد أوضحت النتائج المتحصل عليها أنه في حالة إنزيم الكيتينيز فقد تم تسجيل إنخفاض معنوى في النشاط النوعي للإنزيم في حالة الديارون والكلوروبروفام فقط حيث كانت النسبة المئوية لتثبيط النشاط النوعي هي 80.4، 76.9% للإناث و82.2، 78.6 % للذكور من المبيدين المذكورين على الترتيب. بينما في حالة إنزيم الأسيتايل كولين إستيريز فلقد سجل إنخفاض معنوى للنشاط الإنزيمي في حالة المالاثيون فقط حيث كانت نسبة التثبيط في النشاط الإنزيمي هي 81، 86 % لكل من لإناث وذكور الحشرة على الترتيب.

كما تم تقدير قيم الـ I_{50} (تركيز المبيد اللازم لتثبيط 50% من النشاط الإنزيمي) فوجد أنه بالنسبة لتأثير كل من الديارون والكلوروبروفام، بإعتبارهما أقوى المثبطات لنشاط إنزيم الكيتينيز، أوضحت النتائج أن قيم الـ I_{50} كانت 0.27، 0.45 ميكرومولر للإناث و 0.12، 0.15 ميكرومولر للذكور، على الترتيب. بينما كانت قيم الـ I_{50} لمبيد المالاثيون، بإعتباره أقوى المثبطات للـ AChE في الدراسة الحالية، هي 0.52 ميكرومولر للإناث بينما كانت 0.25 ميكرومولر للذكور.

كذلك تم تقدير بعض الثوابت الإنزيمية مثل ثابت التثبيط Ki وذلك للمركبات المثبطة لكل من الكيتينيز والـ AChE. فوجد أن قيم ثابت التثبيط في حالة الديارون هي 40، 15 ميكرومولر لكل من إناث وذكور الحشرة على الترتيب. بينما في حالة الكلوروبروفام كانت قيم ثابت التثبيط هي 52، 15 ميكرومولر لكل من إناث ذكور الحشرة على الترتيب. أما في حالة المالاثيون كانت قيمة ثابت التثبيط هي 61، 56 ميكرومولر لكل من إناث وذكورذبابة القطن البيضاء على الترتيب.

نتائج الدراسة الحالية تلقى بعض الضوء على إمكانية الإتجاه إلى إنزيم الكيتينيز كهدف لبعض المبيدات الغير معتادة الإستعمال لمكافحة حشرة ذبابة القطن البيضاء خاصة تلك المركبات التي تعتبر مشتقات لمركبات الفينيل يوريا، حتى يمكن إدخالها ضمن برامج المكافحة المتكاملة لهذه الآفة الخطيرة.