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# Role of Carboxyamidase in the *in vivo* metabolism of Chlorfluazuron in the Black Cutworm, *Agrotis ipsilon* (Hfn.) (Lepidoptera : Noctuidae)

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## ABSTRACT

Triphenyl phosphate (TPP), a carboxylesterase inhibitor, has synergized chlorfluazuron in the resistant strain of the black cutworm but not in the susceptible strain. *In vivo* metabolism of $^{14}C$ – chlorfluazuron revealed that the amount of radiolabeled major metabolites (2,6 – difluorobenzoic acid and 2,6 – difluorobenzamide) recovered from resistant larvae was four times higher than susceptible larvae. Addition of TPP did not affect chlorfluazuron metabolism pattern in the susceptible insects but it caused a significant reduction of the amount of detected metabolites in the resistant insects. Cleavage of chlorfluazuron molecule took place at one or both of the urea C-NH-C bonds which suggests the responsibility of carboxyamidase enzyme for chlorfluazuron metabolism. *In vitro* assays revealed that carboxyamidase activity was about four times higher in the resistant larvae than the susceptible ones which supports the hypothesis of the involvement of carboxyamidase in chlorfluazuron resistance in this insect pest.

**Key words:** black cutworm – chlorfluazuron – *in vivo* degradation – carboxyamidase

## INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae) is a serious cosmopolitan pest of corn and several agricultural crops. This noctuid is almost polyphagous that attacks a large number of vegetable crops (Hill, 1983). The control of this pest has become a serious challenge facing applied entomologists nowadays regarding the widening circle of resistance and cross resistance to most available conventional insecticides, including organophosphorus and carbamate insecticides.

Chlorfluazuron, a benzoylphenyl urea compound, is a promising insecticide which is highly effective as a chitin synthesis inhibitor especially on lepidopterous and coleopterous larvae. It is characterized by its unique action, safety to mammals and lack of cross resistance with conventional insecticides (Ishaaya, 1992). Chlorfluazuron has a relatively long half-life in insect bodies with a slow metabolism and elimination rate (Fahmy and Miyata, 1992 and Sammour *et al.*, 2008) which render it as a candidate insecticide for better pest control strategies.

Xenobiotic resistance in insects has evolved predominantly by increasing the metabolic capability of detoxication systems. Carboxylesterases, one of these systems, have been reported as the major metabolizing enzyme of benzoylphenyl urea chitin synthesis inhibitors (Xianchun *et al*., 2007; Elbert and Nauen, 2000 and Li et *al.*, 1998).

This study aims at clarifying the role of carboxylesterase metabolic enzymes in the *in vivo* degradation of chlorfluazuron and assessing the *in vitro* activity of the

involved esterases in chlorfluazuron susceptible and resistant strains of the black cutworm.

## MATERIALS AND METHODS

**Insects:**

Chlorfluazuron resistant and susceptible strains, of the same origin, of the black cutworm, *Agrotis ipsilon* (Hfn.) were used in this study. The resistance ratio of the resistant strain was more than 100 folds that of the susceptible strain. Rearing technique adopted by Abdin, (1979) was carried out under incubation at a constant temperature 25°C and 70 % relative humidity.

**Chemicals:**

Chlorfluazuron and $^{14}$C-labeled chlorfluazuron (654 MBq/nmol) which was uniformly labeled at the 2,5-difluorophenyl ring were used for larval treatment. Unlabeled proposed chlorfluazuron metabolites i.e. 2,6-difluorobenzoic acid and 2,6-difluorobenzamide along with the parent compound (chlorfluazuron) were separated by thin layer chromatography (TLC) to assess their RF values. Triphenyl phosphate (TPP) (95% pure) was used as a synergist. Silica gel 60 $F_{254}$ pre-coated plates were used for TLC and X-ray films were used for tracing radioactivity. Acetyl p-nitroanilide was used as a substrate for assaying the *in vitro* activity of carboxyamidase.

**Synergism study**

Chlorfluazuron was mixed with the same concentration of triphenyl phosphate (TPP) at the ratio of 1 : 1 (v/v). Castor oil leaves, *Ricinus communis* were dipped in this mixture or in chlorfluazuron alone, left to air-dry at room temperature then offered to the newly moulted 4$^{th}$ instar larvae.

***In vivo* metabolism of $^{14}$C-chlorfluazuron:**

Early 4$^{th}$ instar larvae were previously starved for 24 hours before experiment to ensure a whole consumption of the treated castor oil leaf. Two µ l of acetone solution containing $10^{-3M}$ of radiolabeled chlorfluazuron, or chlorfluazuron with TPP synergist at 1 : 5 ratio (w/w), was applied to a small castor oil leaf disc (3 mm X 3 mm). They were left to air-dry and then put singly to each glass homogenizer. Larvae were then introduced singly to each glass homogenizer. Five larvae with three replicates were used for each treatment. Each leaf disc was totally consumed by each larva within four hours. The five larvae of each test were then collected in one of the glass homogenizers and incubated for 96 hours at 25$^{o}$C. The glass homogenizers were then rinsed three times with 1 ml of extraction solvent (acetonitrile : ethyl acetate : methanol : water, 1:1:1:1 v/v) and the rinsing solution was added to the glass homogenizer containing the larvae. The larvae were then homogenized in the same solution for one minute at 4$^{o}$C (El-Saidy *et al*., 1989). The products were extracted three successive times by addition of one ml of the extraction solvent then centrifuged at 15000 rpm for 5 minutes. The combined extracts were concentrated under vacuum at 35$^{o}$C using a rotary evaporator.

The extract was analyzed by TLC using a developing solvent system (ethyl acetate: toluene: acetic acid, 50:45:5, v/v). The plate was then exposed to X-ray film for one week to trace radioactivity. After that, the silica gel plate was cut to small pieces (5mm width), scratched and introduced to a scintillation glass vial containing 5 ml aqueous counting scintillant, ACS II® then radioactivity was measured using a liquid scintillation spectrophotometer. Ultraviolet light was used to detect the authentic unlabeled compounds.

***In vitro* assay of carboxyamidase:**

Fourth instar larvae of black cutworm were homogenized in 0.05M, pH 7.5 Tris-HCl buffer, centrifuged at 1000g and the supernatant of the homogenate was used as enzyme source. The assay of carboxyamidase with acetyl p-nitroanilide as a substrate was performed at 30$^{o}$C (Woods *et al*., 1979).

## RESULTS

The synergistic effect of triphenyl phosphate (TPP), a carboxylesterase inhibitor, on chlorfluazuron was tested in chlorfluazuron resistant and susceptible strains of the black cutworm larvae and the results are shown in Table (1). TPP did not exert any remarkable synergistic action in the chlorfluazuron susceptible strain of the black cutworm. On the other hand, addition of TPP in the resistant insects has led to a clear synergistic effect on chlorfluazuron, evidenced by the sharp decline in the $LC_{50}$ value (from 445 to 48.11 ppm).

Table 1: Synergism of chlorfluazuron by triphenyl phosphate (TPP) in chlorfluazuron susceptible and resistant black cutworm.

| Insecticide | Susceptible strain | | Resistant strain | |
|---|---|---|---|---|
| | $LC_{50}$ ppm (95% c.i.)[b] | SR[a] | $LC_{50}$ ppm (95% c.i.)[b] | SR[a] |
| Chlorfluazuron (alone) | 4.00 (2.90-5.12) | ---- | 445 (290-402) | ---- |
| Chlorfluazuron + TPP | 3.40 (2.61-4.30) | 1.17 | 48.11 (33.5-51.1) | 9.24 |

[a] Synergistic ratio = $LC_{50}$ of chlorfluazuron alone/$LC_{50}$ of chlorfluazuron + TPP
[b] 95% confidence interval

I*n vitro* metabolism of $C^{14}$-chlorfluazuron, with and without the addition of TPP, was also investigated in the larvae of the susceptible and resistant strains of the black cutworm and the results are given in Table (2).

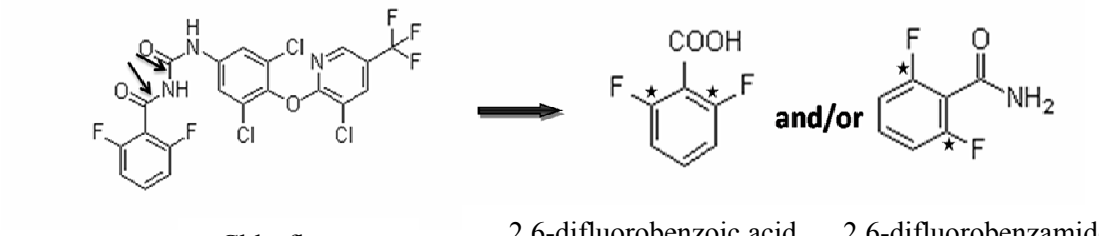
Table 2: In vivo metabolism of $C^{14}$-chlorfluazuron 96 hours after larval treatment with and without the addition of TPP.

| | | Percentage of $^{14}$C-radioactivity recovered (±SD) | | | |
|---|---|---|---|---|---|
| | | Susceptible strain | | Resistant strain | |
| Metabolites | RF | without TPP | + TPP | without TPP | + TPP |
| Polar metabolites | 0.00 | 3.3 ± 1.1 | 2.9 ± 0.8 | 4.9 ± 1.4 | 3.9 ± 09 |
| 2,6-difluorobenzoic acid | 0.60 | 2.4 ± 0.3 | 1.9 ± 0.5 | 8.7 ± 0.3 | 1.9 ± 1.0 |
| 2,6-difluorobenzamide | 0.68 | 1.6 ± 0.1 | 1.2 ± 0.1 | 7.9 ± 0.9 | 2.0 ± 0.5 |
| (Parent) Chlorfluazuron | 0.88 | 91.2 ± 1.3 | 92.3 ± 1.7 | 74.4 ± 1.6 | 90.5 ± 1.1 |
| Others | ---- | 1.5 ± 0.2 | 1.7 ± 0.3 | 4.1 ± 0.4 | 1.7 ± 0.2 |
| % Total recovery ± SD | | 91.2 ± 0.7 | 89.9 ± 1.1 | 92.1 ± 2.0 | 90.7 ± 2.2 |

The detected amounts of total metabolites recovered from the chlorfluazuron resistant insects were three times higher than those recovered from susceptible ones (25.6% and 8.8%, respectively).. The recovered amounts of major metabolites resulting from the cleavage of chlorfluazuron molecule at the urea bond (C-NH-C) by the action of carboxyamidase i.e. 2,6-difluorobezoic acid and 2,6-difluorobenzamide were more than four times higher in the resistant insects than in the susceptible ones (16.6% and 4.0%, respectively). Consequently, the amount of unchanged parent compound (chlorfluazuron) in the susceptible strain was remarkably higher than the recovered amount from the resistant strain (91.2% and 74.4%, respectively).

Addition of TPP did not have any remarkable effect on the amounts of major metabolites of chlorfluazuron in the susceptible insects (without TPP = 4.0 and +TPP = 3.1). On the other hand, addition of TPP in the chlorfluazuron resistant insects has led to a remarkable decrease in the amount of major metabolites (without TPP = 16.6% and + TPP = 3.9%, respectively).

Cleavage of chlorfluazuron molecule took place at the urea bond C-NH-C by the action of carboxyamidase enzyme has resulted in two major metabolites which are shown in Fig. (1).




Chlorfluazuron 2,6-difluorobenzoic acid 2,6-difluorobenzamide

Position of suspected cleavage of chlorfluazuron
Detected major metabolites labeled at the 2,6 position of the difluorophenyl ring

Fig.1 Cleavage of chlorfluazuron molecule at the urea bond by carboxyamidase enzyme.

Carboxyamidase activity from the susceptible and resistant strains of the black cutworm was assayed *in vitro* using acetyl p-nitroanilide as a substrate. The results or this assay are shown in Table (3).

Table 3: *In vitro*, carboxyamidase activity in chlorfluazuron susceptible and resistant black cutworm.

| Strain | Carboxyamidase activity ($10^{-4}$ μ mole/min/mg protein) |
|---|---|
| Susceptible | 1.103 ± 0.121 |
| Resistant | 4.129 ± 1.227 |

Carboxyamidase activity was about four times higher in the chlorfluazuron resistant strain than the susceptible strain of the black cutworm.

## DISCUSSION

Triphenyl phosphate, TPP is known to be a typical carboxylesterase inhibitor (Ishaaya, 1992 and Haubruge *et al.*, 2002). In the present study, addition of TPP has synergized chlorfluazuron (through inhibition of carboxylesterases) in the resistant insects but not in the susceptible ones. This suggests that carboxylesterases are involved in the mechanism of resistance to chlorfluazuron in the black cutworm. The same pattern of synergist was also reported in chlorfluazuron resistant and susceptible strain of the diamondback moth (Fahmy and Miyata, 1998 and Wu QingJun, 1998). Usmani *et al*. (2001) have detected *S*,*S*,*S*-tri-*n*-butyl phosphorotrithioate (DEF)-sensitive enzymes that hydrolyzed *trans*-cypermethrin in adults and larvae of the black cutworm. DEF is also known to be an esterase inhibitor.

In order to confirm the role of carboxylesterases as a suspected mechanism of chlorfluazuron resistance in this insect pest, *In vivo* metabolism of $^{14}$C-chlorfluazuron was investigated. 2,6-difluorobenzoic acid and 2,6-difluorobenzamide were the major metabolites recovered and detected 96 hours after treating the larvae with $^{14}$C chlorfluazuron (Table 2). The amounts of these metabolites were four times higher in the resistant insects than

susceptible ones. Detected metabolites labeled at the 2,6 positions suggest that cleavage of chlorfluazuron molecule took place at the C-NH-C urea bonds and this strongly supports the hypothesis of involvement of a TPP-sensitive enzyme system (presumably carboxyamidase) in the metabolic breakdown of chlorfluazuron in resistant insects.

Again, addition of TPP did not affect the pattern of chlorfluazuron metabolism in the susceptible strain, but it led to a significant decrease in the amount of major metabolites in the resistant insects due to the fact that TPP inhibits the carboxyamidase enzymes responsible for chlorfluazuron degradation.

The responsibility of carboxyamidase enzyme for chlorfluazuron degradation was further confirmed by the *in vitro* assay of carboxyamidase activity in chlorfluazuron resistant and susceptible insects using p-nitroanilide as a substrate. Results clearly show that carboxyamidase activity was about four times higher in the resistant insects than the susceptible ones which confirm the same hypothesis.

Only little information is available in the literature on the metabolic breakdown of chlorfluazuron in insect bodies. In most studies on resistance mechanisms, esterases have been confirmed to be involved in resistance by the addition of synergists (esterase inhibitors) or through the *in vitro* assaying of esterase activity in resistant and susceptible insects (El-Saidy *et al*., 1998; Haubruge *et al*., 2002 and Xianchun *et al*., 2007). In this study the *in vivo* degradation pattern of $^{14}$C-chlorfluazuron elucidates the cleavage of chlorfluazuron molecule, metabolites resulted, amount of unchanged parent compound and the involvement of carboxyamidases.

However, it is still necessary to investigate the pattern of metabolite distribution in both larval body and the excreta separately, since the amount of unchanged chlorfluazuron might then show difference due to the speed by which chlorfluazuron reaches its target site inside insect body or excreted with excreta.

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## ARABIC SUMMERY

**دور الكربوكسي اميديز فى تكسير الكلورفلوازورون داخل الدودة القارضة السوداء**
***اجروتيس ابسيلون* (حرشفيات الأجنحة: الليليات)**

**عادل رمزي فهمي**
قسم علم الحشرات - كلية العلوم – جامعة عين شمس

تعتبر الديدان القارضة إحدى الآفات الهامة التى تسبب خسائر كبيرة للبادرات وهى تصيب كل من الذرة، البرسيم، القمح، الشعير و الفول. و نتيجة الأفراط فى استخدام المبيدات العضوية بدأت عمليات المكافحة تقل كفائتها و ذلك لظهور صفة المقاومة لمعظم المبيدات المستخدمة.

يظل الأمل قائما فى استخدام المبيدات غير التقليدية و منها مثبطات تكوين الكيتين التى تتميزبالأمان للأنسان و عدم وجود مقاومة خلطية مع المبيدات التقليدية. و لهذا لابد من استخدامها بحكمة و محاولة فهم طبيعة المقاومة لها فى وقت مبكر حتى يمكن تجنب او تأخيرظهورها.

الهدف من هذة الدراسة هو محاولة فهم الية المقاومة الناتجة عن تكسير المبيد داخل جسم اليرقة و معرفة دور الأنزيمات المسئولة عن ذلك. باستخدام عشيرتين من الدودة السوداء و هما من أصل واحد، احدهما مقاومة لكلورفلوازورون و الأخرى حساسة لة و حصلنا على النتائج التالية:

1- عند معالجة اليرقات من العشيرتين بالمبيد على حدة ثم بالمبيد مع اضافة ال TPP وهى مادة تثبط انزيمات الأستيريز المسؤلة عن تكسير بعض المبيدات الحشرية حدثت زيادة ملحوظة فى سمية المبيد مع اضافة ال TPP مع العشيرة المقاومة فقط و لم يحدث اى زيادة فى السمية مع العشيرة الحساسة و هذا يدل على ان المقاومة لهذا المبيد هى نتيجة وجود أو نشاط انزيمات الأستيريز فى العشيرة المقاومة و عدم وجودها فى العشيرة الحساسة.

2- تمت دراسة نمط تكسير مبيد الكلورفلوازورون داخل جسم الحشرة بعد معالجتها بالمبيد المشعع (كربون $^{14}$) عند موضع 2,6 دايفلوروبنزين فى العشيرتين، مع أو بدون اضافة ال TPP. كانت كمية نواتج تكسير الكلورفلوازورون ) (difluorobenzamide & 2,6 difluorobenzoic acid) فى العشيرة المقاومة اكبر أربع مرات من نظيرتها فى العشيرة الحساسة بدون ال TPP . اضافة ال TPP لم تؤثر على هذا النمط فى العشيرة الحساسة، فى حين أدت الى تقليل نواتج الأيض بصورة ملحوظة فى العشيرة المقاومة. و هذا يدل على مسؤلية انزيم الكاربوكسي اميديز فى تكسير الكلورفلوازورون فى موضع 2,6 دايفلوروبنزين.

3- دراسة النشاط الأنزيمى للكاربوكسى اميديز فى اليرقات من العشيرتين بعد طحنها اثبت ان هذا الأنزيم انشط اربع مرات فى يرقات العشيرة المقاومة للكلورفلوازورون عن العشيرة الحساسة.

من كل ما سبق يمكن استنتاج ان انزيمات الكاربوكسى اميديز هى المسؤلة عن تكسير مبيد الكلورفلوازورون داخل يرقات الدودة القارضة السوداء.