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Pesticide Biochemistry and Physiology 74 (2002) 17–26
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Biochemistry & Physiology
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Pyrethroid resistance mechanisms in the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) from West Africa

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Received 10 May 2002; accepted 18 September 2002

Abstract

In West Africa, the cotton bollworm *Helicoverpa armigera* has recently developed resistance to deltamethrin and cypermethrin. Resistance mechanisms of the strain BK99R9 collected in Bouaké, Ivory Coast in 1999 and selected with deltamethrin were investigated by comparison with a susceptible strain BK77 collected in the same area in 1977. Several approaches were performed: evaluation of the cross-resistance spectrum to various pyrethroids and DDT, effect of a synergist, and by determination of the biochemical characteristics of three enzyme systems (esterases, glutathione-*S*-transferases, and mixed function oxidases). Deltamethrin resistance in BK99R9 was correlated to an increase of mixed function oxidase. Enhanced monooxygenase levels were then confirmed in several *H. armigera* field strains collected in cotton areas of West Africa from 1999 to 2001.

Keywords: Resistance mechanism; *Helicoverpa armigera*; Oxidase; Pyrethroids; Cotton; West Africa

1. Introduction

Helicoverpa armigera (Hübner) is an economically important pest of cotton and vegetable crops. Control is usually achieved with insecticides especially pyrethroids. In Asia and Australia, *H. armigera* has developed resistance to virtually all the insecticides that have been applied against it in any quantity [1]. In West Africa, deltamethrin and cypermethrin susceptibility in *H. armigera* was surveyed annually from 1984. Pyrethroid resistance was detected in 1996 [2,3]. At the same time, pyrethroid resistance was also detected in South Africa [4]. A resistance management strategy based on the restriction of pyrethroid use was rapidly implemented in all cotton farmers of West African countries [5].

In most countries, pyrethroid resistance mechanisms of *Helicoverpa armigera* are multiple.

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0048-3575/02/$ - see front matter
PII: S0048-3575(02)00117-7

Evidence for the involvement of a nerve insensitivity mechanism (*kdr*), the target site resistance mechanism to pyrethroids, has been shown in *H. armigera* from Australia, India, China, and Thailand [6–10]. In every case, the presence of cross-resistance between DDT and pyrethroids was observed. In *H. armigera* the *kdr* mechanism was generally associated with other mechanisms, such as enzymatic detoxification of pyrethroids. The main systems revealed by biochemical studies are oxidation by microsomal P450 monooxygenases and hydrolysis by esterases [1]. In central India, enzyme assay data indicated that high cytochrome P450 levels generally coincided with low esterase activity and vice versa [11]. Glutathione-*S*-transferases are also involved in resistance to pyrethroids in Indian strains. In Australia an important study suggested that the pyrethroid resistant *H. armigera* have enhanced etsterase activity and that the esterases were acting as insecticide-sequestering agents [12]. An other mechanism which reduced penetration (*Pen*) appeared to be important for esfenvalerate resistance in an Australian resistant strain of *H. armigera* [13]. In comparison, the African species could be an exception as resistance seems to be limited to pyrethroids (submitted for publication). It has been shown that deltamethrin resistance in West African *H. armigera* was largely suppressible by the piperonyl butoxide (PBO)[1] [14].

Most of the classical methods to evaluate oxidase activity with chromogenic substrates in insects require the purification of microsomal fractions [15–19]. But these methods cannot be used to measure differences in oxidases from a single insect. Another alternative is to measure the level of heme-containing enzymes, which includes the cytochrome oxidase enzymes [20]. The amount of oxidases is correlated with the peroxidase activity of the heme groups. Such a technique would provide a useful means for measuring large-scale differences in oxidase levels characteristic of resistance and oxidase induction.

In the present investigation, the physiological mechanisms responsible for the deltamethrin resistance of *H. armigera* from West Africa were identified by analysing: (1) the synergistic effect of PBO, (2) the resistance spectrum to various pyrethroids and DDT, and (3) the activity of various enzymes known to be involved in pyrethroid resistance. Then the resistance mechanism was confirmed in field strains since resistance mechanisms in *H. armigera* can change markedly over a single cropping season or between assays on laboratory-reared insects as shown in India [11].

2. Materials and methods

2.1. Insects

A susceptible *H. armigera* strain (BK77) was originally collected in the Ivory Coast in 1977 and reared in CIRAD Entomological Laboratory in Montpellier, France [21]. To establish the deltamethrin resistant strain (BK99R9), about a hundred larvae were collected in October 1999 in the Cotton Research Station of Bouaké, Ivory Coast, where field control failures have been observed since 1995. This strain was then homogenised by nine selections for deltamethrin resistance as previously described for the Australian *H. armigera* [22]. Selection was done for five generations by retaining the survivors at a discriminating dose (0.6 μg/g, the LD_{99} of the susceptible strain) topically applied on third-instar larvae. Fifty to ninety percent of survival was obtained with the selecting dose. Larvae were reared on artificial diet at 25 °C, 75% humidity, and at 12 h/12 h photoperiod in the laboratory as previously described [14].

Field samples of different stages of *H. armigera* were collected from 1998 to 2001. Samples were obtained from strongly infested crops in identified farmers' fields from the West African cotton-growing areas. The strains were named according to the nearest large town (KDG: Konodougou, Mali; BK: Bouaké, Ivory Coast; KHO: Korogho, Ivory Coast; MK: Mankono, Ivory Coast; FKB: Farakoba, Burkina Faso; BOU: Boundiali, Ivory Coast) with the collect date (year/month) and the crop name: *c* for cotton (*Gossypium hirsutum*), *t* for tomato (*Lycopersicum esculentum*), and *g* for gumbo (*Hibiscus esculentus*). For example, the strain collected in Bouaké area in 1999, October, from cotton was named BK99/10*c*. A minimum of 50 larvae were collected in each field and reared in the laboratory on an artificial diet for one generation at 25 °C. The adults were placed in cages and fed on a 5% honey solution. Their eggs were collected on sterilised gauze and washed with 1%

[1] *Abbreviation used:* PBO, piperonyl butoxide; αNA, α-naphthyl acetate; PNPA, *p*-nitrophenyl acetate; GST, glutathione-*S*-transferase; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; TMBZ, 3,3′,5,5′-tetramethyl benzidine; BCA, bicinchoninic acid.

bleach. For each strain, 25 second-instar larvae were frozen at −75 °C for biochemical assays and 60 third-instar larvae were used for topical application with the discriminating dose of deltamethrin (0.6 μg/g). Insects were weighed before treatment and the dose was adjusted for larval weight.

2.2. Chemicals

The insecticides used were all technical grade materials. Deltamethrin (99%) and the synergist PBO (99%) were obtained from Aventis CropScience, France. Bifenthrin (93.5%) and cypermethrin (93.2%) were obtained from FMC, USA. Fenvalerate (95%) was provided by Sumitomo and etofenprox (99%) by Mitsui, Japan. Acetone was used for dilutions. BCA Protein Assay was from Pierce, The Netherlands. Cytochrome *C*, 3,3′,5,5′-tetramethyl benzidine (TMBZ), α-naphthyl acetate (αNA), Fast Garnet salt, *para*-nitrophenyl acetate (PNPA), glutathione (GSH), and 1-chloro-2,4-dinitrobenzene (CDNB) were from Sigma, France. Solutions of CDNB (64.3 mM) and TMBZ (8.3 mM) were prepared in methanol just before using.

2.3. Bioassays

Standard third-instar larvae topical bioassays were used to determine insecticide toxicity [14,22]. Five serially diluted concentrations were prepared. For each concentration, 10 third-instar larvae (35–45 mg) were treated with 1 μl solution applied by microapplicator to the thorax. Each test was replicated three times and included acetone treated controls. Mortality in the controls was less than 10%. PBO was applied at 10 μg/larva 1 h before application of deltamethrin. This dose did not result in any toxicity. After dosage, the test larvae were held individually at 25 °C and 75% humidity. Mortality was assessed 72 h after treatment. Larvae were considered dead if unable to move in a coordinated way when prodded with a needle. With the same method, a discriminating dose of deltamethrin (0.6 μg/g), corresponding to LD_{99} of the susceptible strain (BK77), was applied in 60 third-instar larvae of *H. armigera* field strains collected from 1998 to 2001.

2.4. Enzyme preparation

For enzyme preparations, 40 second-instar larvae (5–10 mg) from the susceptible and the resistant strains and 25 for each field strain were used [23]. Individual insects were homogenised in 200 μl distilled water at 4 °C. The homogenate was spun at 14,000*g* for 2 min at 4 °C in a microfuge.

2.5. Oxidase assay

The assay mixture consisted of 80 μl of 62.5 mM potassium phosphate buffer, pH 7.2, added to a 20-μl aliquot of enzyme source. Two hundred μl solution was added, containing 13 mg TMBZ dissolved in 6.5 ml methanol with 19.5 ml of 0.25 M sodium acetate buffer ($NaC_2H_3O_2$), pH 5.0. Then 25 μl hydrogen peroxide (3%) was added. Absorbance at 630 nm was read against blanks after 30 min incubation at 25 °C. Total oxidase activity was expressed as nmol equivalent cyt-P450/mg protein. The standard curve of cytochrome *C* is accurately described by a linear equation.

2.6. Esterase assay

Hydrolysis of αNA was performed by incubating 10 μl sample with 90 μl of 1% Triton X-100, 10 mM phosphate buffer, pH 6.5, 136 mM NaCl, and 2.6 mM KCl, for 10 min at 25 °C. One hundred μl solution containing 0.5 ml of 15 mM αNA plus 2.5 ml of 1% Triton X-100 of 10 mM phosphate buffer, pH 6.5, 136 mM NaCl plus 7 ml H_2O was added and the mixture was incubated for 30 min at 25 °C. The reaction was stopped by addition of 100 μl distilled water containing 0.08 mg Fast Garnet salt. Absorbance at 550 nm was read against blanks. Hydrolysis of PNPA was performed using a 10-μl sample with two replicates and incubated with 200 μl of 0.05 M phosphate buffer, pH 7.4, 10 mM PNPA. The microplate was maintained for 5 min at 25 °C. Absorbance at 420 nm was read against blanks.

2.7. Glutathione-S-transferase assay

Ten μl samples were mixed with 200 μl of 0.1 M sodium phosphate buffer, pH 6.5, containing 10 mM GSH and 6 mM CDNB. Kinetic assays were immediately performed on a microplate reader taking absorbance readings (340 nm) automatically for 5 min.

2.8. Protein assay

The Pierce BCA Protein Assay, a detergent-compatible formulation based on bicinchoninic

acid (BCA), was used for the colorimetric detection and quantification of total protein. Ten-μl aliquot was incubated for 30 min at 25 °C with 200 μl solution containing 20 ml BCA reagent A and 400 μl BCA reagent B. Absorbance at 590 nm was read against blanks.

2.9. Analysis

LD_{50} (lethal dose 50%) was determined by using the Finney method [24]. Transformations and regression lines were automatically calculated by DL50 1.1 software of CIRAD. Readings and the transformations were made automatically by microplate reader using KC4 Kinetical Windows software from Bio-Tek Instruments. As resistant strains were not homogeneous, a Mann–Whitney test was used to check the equality of the means of two populations based on Minitab software.

3. Results

3.1. Bioassays

Bioassay results of deltamethrin with or without PBO on *H. armigera* susceptible strain (BK77) and deltamethrin-selected strain (BK99R9) are shown in Table 1. The strain BK99R9 was 189-fold more resistant to deltamethrin than BK77. PBO pre-treatment had no effect on deltamethrin toxicity in the susceptible strain, but it almost fully suppressed resistance in the deltamethrin resistant individuals, confirming results obtained in 1997 in field strains collected in Côte d'Ivoire [14] and in Benin (Djihinto, personal communication). The deltamethrin resistance factor decreased from 189-fold without PBO to 4-fold with PBO. The BK99R9 strain was 5-, 15-, and 163-fold more resistant to the three other pyrethroids etofenprox, bifenthrin, and fenvalerate, respectively, than to the susceptible strain BK77. Alterations in the pyrethroid structure, notably etofenprox which is not an ester compound, did not completely overcome the resistance (Fig. 1).

Mortality obtained with DDT for the deltamethrin-selected strain BK99R9 did not differ from mortality observed with the susceptible strain except that the slope of the mortality–dose curve was weak, suggesting that the pyrethroid resistant strain was more heterogeneous (Fig. 2). Mortality was similar to a cotton field strain recently collected, BK01.

3.2. Biochemical analysis

The technique of heme peroxidation to analyse variation in oxidase levels allowed rapid determination of resistance frequency. The simplicity of the method made it feasible in laboratories surveying pyrethroid resistance of *H. armigera*. The level of oxidase enzymes in single insects is correlated with hemoprotein level and with the peroxidase activity of the heme group [20]; however, this method does not provide information on specific oxidases. We used the method to estimate

Table 1
LD_{50}, resistance factor (RF), and synergistic factor (SF) for deltamethrin with and without PBO, three other pyrethroids (fenvalerate, bifenthrin, and etofenprox) on susceptible and resistant strains of *H. armigera*

Active ingredient	Strain[a]	LD_{50} (μg/g)	95% confidence intervals	Slope ± SEM	RF[b]	SF[c]	χ^2
Deltamethrin	S	0.055	0.043–0.066	2.16 ± 0.29	—	—	4.9
	R	10.40	6.45–14.07	2.44 ± 0.37	189	—	10.7
Deltamethrin + PBO	S	0.044	0.017–0.064	3.35 ± 0.74	—	1.3	1.3
	R	0.192	0.074–0.341	1.56 ± 0.25	4	54	10.3
Fenvalerate	S	0.145	0.108–0.176	1.62 ± 0.24	—	—	6.2
	R	23.64	13.47–35.74	1.50 ± 0.22	163	—	5.7
Bifenthrin	S	0.129	0.09–0.14	4.62 ± 1.16	—	—	1.7
	R	1.931	1.299–2.574	2.32 ± 0.34	15	—	3.7
Etofenprox	S	2.937	1.898–3.850	2.91 ± 0.37	—	—	5.8
	R	15.79	9.83–21.72	2.20 ± 0.41	5	—	9.8

[a] S for the susceptible strain BK77 and R for the pyrethroid resistant strain BK99R9.
[b] Resistance factor (RF), calculated as the ratio of LD_{50} resistant/LD_{50} susceptible.
[c] Synergistic factor (SF), calculated as the ratio of LD_{50} deltamethrin/LD_{50} (deltamethrin + PBO).

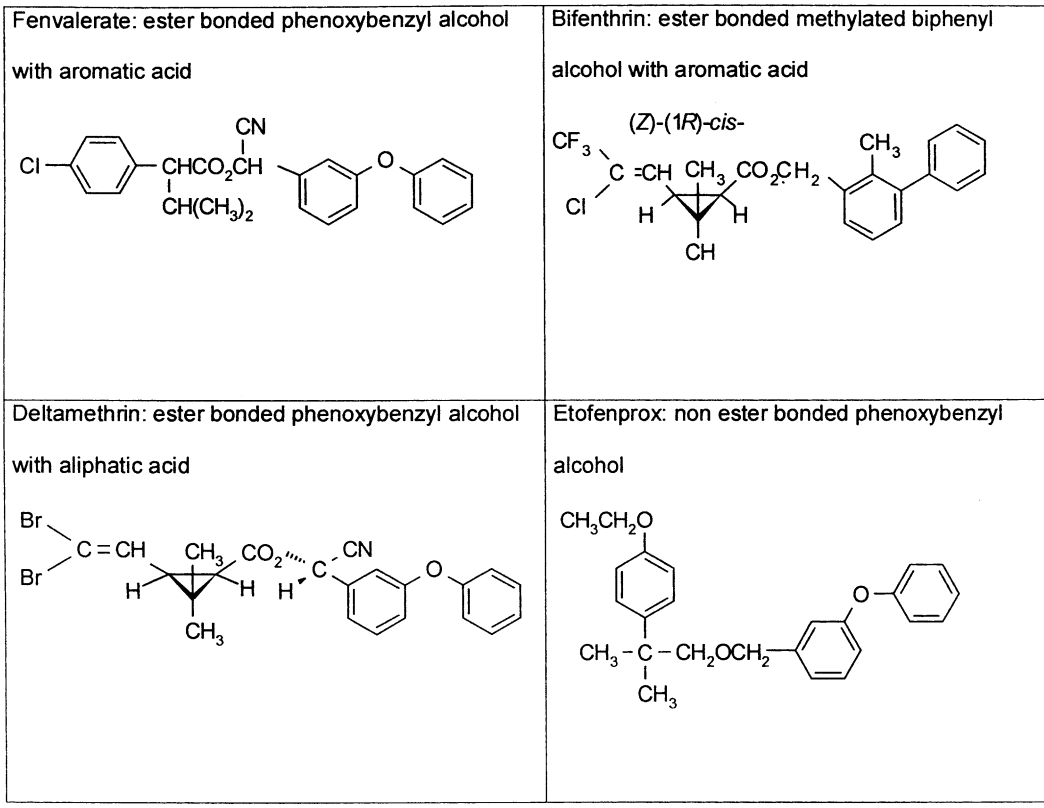

Fig. 1. Structures of pyrethroids tested in this study. Fenvalerate, deltamethrin, bifenthrin, and etofenprox represent the major type of structure.

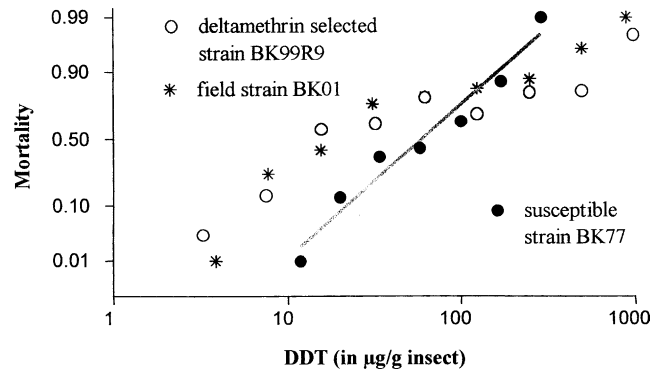

Fig. 2. Toxicity of DDT for the *H. armigera* field strain (✳), the deltamethrin selected strain (○), and the susceptible strain (●). Mortality obtained with DDT in the pyrethroid resistant strains was not linear, indicating heterogeneity of tolerance in these strains.

the hemoprotein level in each larva. A significant elevation of oxidases was observed in the resistant strain BK99R9 (Table 2). The deltamethrin-selected strain had 3.8-fold higher quantities of cyt-P450 than the susceptible one. An increased quantity of oxidase was observed to parallel the deltamethrin resistance factor of the successive generations selected with deltamethrin. This

Table 2
Mean esterase and glutathione-*S*-transferase activities and median oxidase amount for *H. armigera* pyrethroid resistant strain (BK99R9) and susceptible strain (BK77)

Strain	*N*[a]	Esterase[b] (αNA) in μmol/min/mg protein	Esterase[c] (PNPA) in μmol/min/mgF protein	Oxidase in nmol equiv. cyt-P450 U/mg protein	GST[d] in μmol/min/ mg protein
Susceptible	40	0.161	0.183	3.224	0.202
Resistant	40	0.074*	0.131*	12.352*	0.554*

[a] *N* indicates the number of larvae tested.
[b] Esterase activities obtained with α-naphthyl acetate like substrate.
[c] Esterase activities obtained with *para*-nitrophenyl acetate like substrate.
[d] GST: glutathione-*S*-transferase activities.
* Indicates a significant difference with the susceptible strain with $P < 0.05$ by Mann–Whitney test.

suggests that detoxification by oxidative enzymes may be a major resistance mechanism.

The level of cyt-P450 in individual insects from the susceptible and the pyrethroid resistant populations is illustrated in the frequency distribution of oxidase activity (Fig. 3). It revealed homogeneous, low levels in the susceptible population BK77 and a higher, more heterogeneous oxidase level in the resistant population BK99R9. In BK77, the quantity of cyt-P450 equivalent was always below 10 nmol/mg protein. The deltamethrin-selected strain contained only a small portion (25%) of individuals with less than 10 nmol cyt-P450 U/mg protein.

The glutathione-*S*-tranferase (GST) activity of the deltamethrin-selected strain BK99R9 was significantly (2.7-fold) higher than in the susceptible strain (Table 2). The esterase activities were measured with two substrates, αNA or PNPA, because some esterases may be specific. Mean values in the resistant strain of *H. armigera* were significantly lower than those of the susceptible strain.

Helicoverpa armigera field strains collected on various host plants from 1998 to 2001 were tested with a discriminating dose of deltamethrin (0.6 μg/g larva). The portion of resistant individuals varied from 15 to 77% compared with 89% in the deltamethrin-selected strain BK99R9 (Table 3). Biochemical assays have also been used in the same strains (Fig. 4). For five of them, the oxidase levels were significantly higher than for the susceptible strain. The mean of their oxidase contents varied between 2.3 and 8.9 nmol P450 U/mg protein compared with 2.1 nmol P450 U/mg protein for the susceptible strain. We found a positive

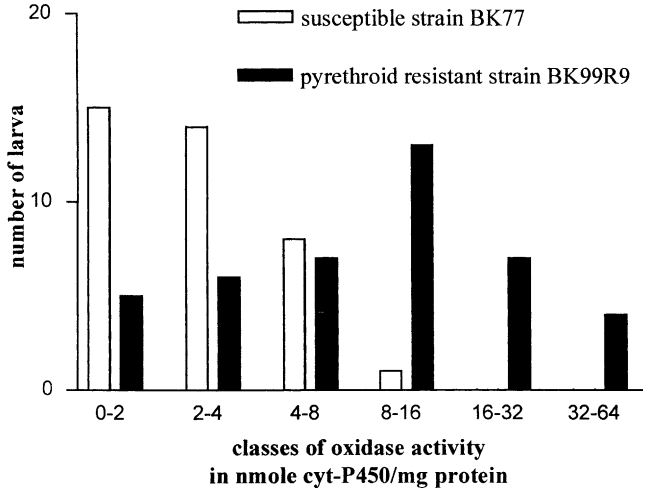

Fig. 3. Frequency distribution of oxidase level (nmol/mg) in individuals from the susceptible (BK77) and the pyrethroid resistant (BK99R9) *H. armigera* strains. For each strain, $n = 40$.

Table 3

Strains[a]	% surviving larva[b] ± SEM	Esterase[c] (αNA)	Esterase[c] (PNPA)	Oxidase[d]	GST[c]
BK77	1% ± 0.01	0.161	0.183	3.224	0.202
KDG98/10c	69% ± 0.06	0.140	0.162	5.124*	0.278*
BK99/03t	58% ± 0.06	0.123	0.155	6.536*	0.184
BK99/04g	—	0.103	0.174	4.861*	0.170
KHO99/06g	52% ± 0.07	0.111	0.017	8.950*	0.173
BF99/9c	—	0.055	0.091	2.356*	0.142
MB99/9c	—	0.058	0.113	1.656	0.133
BK99/10c	77% ± 0.05	0.064	0.117	3.770	0.105
KHO99/10c	55% ± 0.06	0.135	0.029	3.796	0.131
NIO99/10c	—	0.111	0.015	1.191	0.104
BK00/04t	—	0.146	0.159	3.975	0.094
BK00/10c	49% ± 0.07	0.070	0.055	2.775	0.103
MK00/10c	31% ± 0.06	0.108	0.096	5.875*	0.200
FKB01/08c	19% ± 0.05	0.017	0.065	2.150	0.107
BOU01/10c	15% ± 0.05	0.053	0.126	2.150	0.221
BK01/10c	54% ± 0.06	0.033	0.136	3.075	0.128

[a] Strains collected (year/month) from cotton (c), tomato (t) or gumbo (g): BK77: susceptible strain; KDG: Konodougou, Mali; BK: Bouaké, CI; KHO: Korogho, CI; BF: Bouaflé, CI; MB: M'Bengué, CI; NIO: Niofoin, CI; MK: Mankono, CI; FKB: Farakoba, Burkina Faso; BOU: Boundiali, CI; BK99R9: deltamethrin selected strain.

[b] Discriminating dose of deltamethrin (0.6 μg/g larva).

[c] Esterase and glutathione-*S*-transferase activities are expressed in μmol/min/mg protein.

[d] Oxidase level is expressed in nmol equivalent cyt-P450 U/mg protein.

* Indicates a significant difference with the susceptible strain BK77 with $P < 0.05$ by Mann–Whitney test.

correlation ($r^2 = 0.40$; slope is significantly non-zero) between the level of oxidase and resistance in the field strains (Table 3, Fig. 4). But we did not find any correlation between esterase and glutathione-*S*-transferase activities and deltamethrin resistance.

4. Discussion

The present investigation suggests that the resistance to pyrethroids in natural populations of *H. armigera* from West Africa is associated with an increase in oxidase metabolism as shown by the fact that resistance was abolished by PBO pre-treatment and that cyt-P450 levels were higher in resistant insects. Analysis of the deltamethrin-selected strain BK99R9 collected in Ivory Coast in 1999 showed this strain to have a higher level of cyt-P450, an increased GST activity, and a decreased esterase activity compared with the susceptible strain. Analysis of the resistant field populations collected from 1998 to 2001 allowed discrimination between these three mechanisms; since only the increase of cyt-P450 was correlated to resistance. The importance of oxidative attack: in resistance to pyrethroids has been already shown in *H. armigera* from India [11] and China [25] and in *Heliothis virescens* from the US cotton belt [26–28]. DDT did not show any cross-resistance to deltamethrin since toxicological studies did not discriminate between the susceptible and the resistant strains. This molecule has been used for more than 20 years in West Africa as the only one insecticide to control *H. armigera*. With the discovery of pyrethroids, DDT although still efficient was replaced in early 1980s and its utilization is now forbidden. Thus the absence of cross-resistance provides the information that pyrethroid resistance does not originate from previous treatments with DDT and most probably from other organochlorines. Therefore, the *kdr* mechanism was not involved.

The major involvement of cyt-P450 is indirectly confirmed by cross-resistance with all pyrethroids tested, as shown by the fact that all these chemicals have typical oxidation sites on the alcohol moiety or on the acid moiety [29]. The involvement of oxidases may explain the negative cross-resistance observed with triazophos in pyrethroid resistant strains compared with susceptible strains (manuscript submitted). Triazophos is an organophosphate activated by P450 to give an active oxon form. An increase of cyt-P450 responsible for this activation would increase the

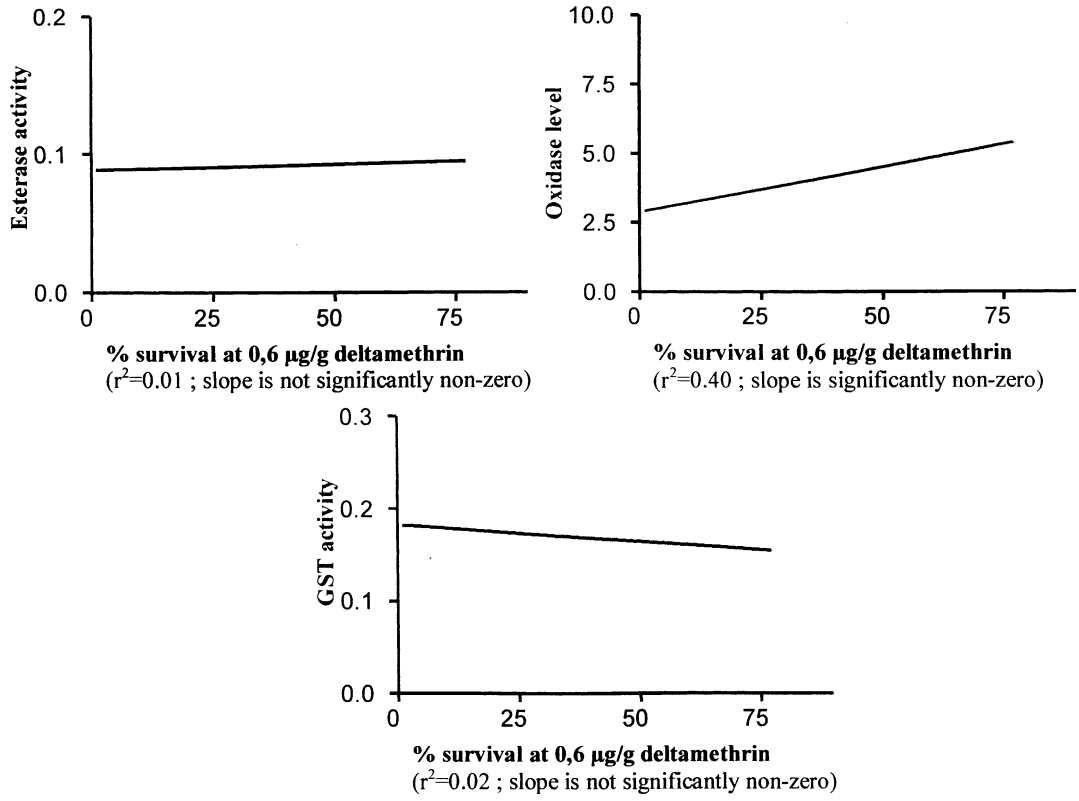

Fig. 4. Relation between the percentage of larvae of *H. armigera* surviving a discriminating dose of deltamethrin (0.6 µg/g) and enzymatic activities of esterase and glutathion-*S*-transferase and level of oxidase in *H. armigera* field populations compared with the susceptible strain (BK77) and the pyrethroid resistant strain (BK99R9).

concentration of the active form in pyrethroid resistant insects.

Other pyrethroid resistance mechanisms may exist. Several lines of evidence suggested that esterases can also be involved: (1) There was a lower esterase activity in the resistant strains showing that esterases are not identical in the susceptible and the resistant strains. That esterase would be different in the hydrolysis of classical chromogenous substrates [11,12,28]. (2) PBO is a moderately effective esterase inhibitor [30], suggesting that inhibition of esterases may contribute to the observed synergism. (3) We observed a low cross-resistance with etofenprox, a non-ester pyrethroid. The 5-fold resistance factor is very marginal compared to those obtained for the fenvalerate and deltamethrin and may be due to strain differences and not resistance. This would suggest that esterase may be involved in the resistance mechanism. Furthermore, we cannot eliminate the involvement of GST since their activity was significantly higher in BK99R9 strain. However, we did not find any correlation between esterase and GST activities and deltamethrin resistance, suggesting that if these enzymatic mechanisms exist, they are not widespread in field populations. In conclusion there was no doubt in the involvement of MFO in the resistance mechanism which does not exclude the involvement of esterases or glutathione-*S*-transferases. Further biochemical studies of the purified enzyme activities will bring information toward a better understanding of resistance mechanisms to pyrethroids in *H. armigera*.

Conservation of pyrethroid efficacy in bollworms for an extended period is a challenge for all West African countries. Knowing oxidase involved in the pyrethroid resistance of *H. armigera* allows the use of resistance-breaking molecules within existing conventional insecticide groups like the organophosphorus compounds. Thiophosphates are activated by mixed function oxidases in such a way that in their oxidised form a neurotoxic action occurs more rapidly in the insect

pest. Thus, an increase of oxidase activity in resistant *H. armigera* results in two different consequences, increased degradation of pyrethroids and increased activation of some organophosphates. Therefore, the following resistance management strategy in West Africa was adopted: endosulfan is used for the first two sprays, on the basis that no cross-resistance was detected and for the last four sprays; mixed products containing a pyrethroid in association with an organophosphate were used. This strategy proved successful during the last four years of its widespread use (1999–2001) on the regional scale as there was no longer any field infestation problem due to the bollworm *H. armigera* to such extent that it was difficult to find larvae for laboratory screening [31]. The use of OPs, especially proved to be activated by MFO, in insecticide mixtures would be a useful requirement for pyrethroid resistant management.

The ability to diagnose the precise nature of the mechanisms of resistance was a key component of the resistance management in *H. armigera*. But heliothines are especially flexible in the use of a variety of modifications in all their resistance mechanisms [1]. To keep the advantage in resistance management, it is necessary to rapidly improve understanding of the biochemical and molecular nature of the problem.

Acknowledgments

The authors acknowledge the technical assistance of T. Konate, I. Ouattara, and M.J. Mousso and thank Dr. D. Russell (NRI) and Dr. R.V. Gunning (NSW Agriculture) for their corrections and comments.

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