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Biochemical Characterization of the Cotton Bollworm *Helicoverpa armigera* Resistance to Pyrethroids in Burkina Faso

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Abstract: The bollworm *Helicoverpa armigera* is cotton plant main pest in most parts of the world. The mechanisms of the resistance of the bollworm to the pyrethroid deltamethrin were studied by comparing field strains to the reference and susceptible strain (BK77). Resistance to deltamethrin was studied using bio-assays. Results showed that the field collected strains had susceptibility 11 to 43 fold lower than that of the susceptible standard strain BK77. Activities of two types of enzymes i.e., oxidases and Glutathione-S-Transferases (GST) were significantly higher in field strains, whereas esterase activities were lower compared to that of standard strain. The increase of oxidases and GST activities and the decrease of esterase activity are at least in part, responsible for the development of resistance of *H. armigera* to pyrethroids.

Key words: Strains, deltamethrin, susceptibility, enzymatic activity, oxidases

INTRODUCTION

Cotton (*Gossipium barbadense*) is classified in the plant family of Malvaceae. In many countries of West Africa, cotton is the main export crop which is grown for its fibre and the oil extracted from the seeds. In Burkina Faso, cotton represents over 50% of the country export earnings (Yartey, 2008). Cotton plant is subject to serious damages by many pests, particularly insects which feed upon the leaves and the fruits. For most cultivated varieties, yield losses may represent 90% of potential yields if there is no control of pests and diseases (Michel *et al.*, 2000).

The bollworm *Helicoverpa armigera*, (Hübner) (Lepidoptera: Noctuidae) is the most important insect pest of cotton in the world (Pearson and Darling, 1958; Vaissayre and Cauquil, 2000). The larvae of the insect are mainly responsible of damages. At the larval stage, *H. armigera* is polyphagous and occurs all over the year. During the rainy season it infests mainly cotton, sorghum, maize and wild plants such as *Cleome viscosa*. Vegetables, particularly tomato and okra remain the main host plants during the dry season (Nibouche *et al.*, 2003). The lifecycle of the insect includes three to four generations a year if suitable host plants are available

only during the rainy season. If vegetable host crops are grown during the dry season, like in many West African countries, six to nine generations may occur (Nibouche *et al.*, 2003).

Since, the years 1980, cotton in West African sub-region was protected from caterpillar damage by the applications of binary insecticides containing both pyrethroids and organophosphates. From years 1995 and 1996, in few areas such applications of insecticides were not fully successful in controlling insect pests particularly larvae of *H. armigera* (Martin *et al.*, 2000). This led to the increase in the number of insecticides applications by cotton growers in order to get acceptable levels of pest control. The consequences were higher production costs and adverse effects on human health and environment. Worldwide *H. armigera* resistance to different classes of insecticides has been reported for Australia in 1983 (McCaffery, 1998), Thailand in 1985 (Ahmad and McCaffery, 1988), China and India in 1987 (McCaffery, 1998), Pakistan in 1991 (Ahmad *et al.*, 1995) and Côte d'Ivoire in 1995 (Vassal *et al.*, 1997). In Burkina Faso, several studies were carried out by the Cotton Research Programme in order to establish the levels of susceptibility of *H. armigera* to few insecticides including pyrethroids (cypermethrin and deltamethrin),

organophosphates (profenofos), cyclodiene (endosulfan) and other chemicals such as indoxacarb, acetamipride and diafenthiuron. Unfortunately, all these studies did not include standard susceptible strains. Therefore, results obtained from these studies could not show clearly the extent of *H. armigera* resistance to insecticides used in the country and what is the underlying mechanism of the resistance.

This study aimed to establish the levels of resistance of field collected *H. armigera* strains to deltamethrin and to understand the mechanisms involved in the resistance to this insecticide.

MATERIALS AND METHODS

Area of studies. All the tests were conducted in Burkina Faso. Bio-essays studies were conducted in the laboratory of INERA at Bobo-Dioulasso from the year 2003 to 2004; biochemical tests were conducted in the laboratory of CIRDES (Centre International de Recherche/Développement sur l'Élevage en Zones Sub-humides) at Bobo-Dioulasso in 2004 for insects collected in 2003 and 2005 for insects collected in 2004.

Insect strains: Field-collected strains of *H. armigera* and a standard susceptible strain (BK77) were used. BK77 was previously collected at Bouake (Côte d'Ivoire) in 1977 and maintained in the laboratory without any contact with insecticides. Field strains were collected between 2003 (two strains) and 2004 (five strains) in different sites located in the main cotton growing areas of Burkina Faso. Insect strains were identified by site location and the year of collection (Table 1).

All insects were reared in the laboratory at $25^{\circ}\text{C}\pm 1$, with relative moisture of $70\%\pm 5$ and 12/12 h light/dark. Field-collected insects were kept first in a separate room until chrysalises were got. The chrysalises were then disinfected with water containing sodium hypochlorite (0.1% active chlorine) and transferred to the mass rearing room. Emerged butterflies were fed with sugared water. Eggs from these butterflies were collected and kept in the dark on artificial diet as described by Ahmed *et al.* (1998). First generation larvae were used in all subsequent tests. Larvae of 35 to 44 mg were used for testing susceptibility to insecticides whereas larvae of (10 to 15 mg) were kept at eighty Celsius degrees under zero (-80°C) to be used in subsequent biochemical tests.

Bio-assay: Larvae were tested by groups with 30 individuals. Larvae of the same group were exposed to a particular dose of insecticide. For the field-collected strains, the following six doses were tested: 0.6, 0.951, 1.507, 2.388, 3.785 and $6\text{ }\mu\text{g g}^{-1}$. For the standard

Table 1: Insect strains collecting areas

Name	Area	Year	Location
BK77	Bouaké	1977	Centre of CI*
BIT03	Bittou	2003	South east of BF**
DAT03	Datomo	2003	West of BF
BAL04	Balavé	2004	West of BF
BIT04	Bittou	2004	South east of BF
DAT04	Datomo	2004	West of BF
PO04	Pô	2004	South of BF
TIE04	Tiébé	2004	South of BF

*CI: Côte d'Ivoire, **BF: Burkina Faso

susceptible strain (BK77), the tested doses were 0.5, 0.0707, 0.0997, 0.141 and $0.2\text{ }\mu\text{g g}^{-1}$. Acetone was the only solvent in the commercial formulation of deltamethrin. Therefore, acetone was used as solvent for the preparations of insecticide doses and also for the control solution.

One microliter of insecticide or control solution was applied on the thorax of larvae using a microapplicator composed of a syringe equipped with a bent needle (Martin *et al.*, 2003). After treatments, each larva was placed separately in small plastic box containing artificial diet and mortality was scored after 48 h. Larvae were considered dead if they were unable to move or could not get to the upright position when they were placed on one side. Groups of larvae were compared on the basis the insecticide Lethal Dose (LD_{50}) which is the dose that kills half of the group.

The Resistance Index (RI) is then obtained from the ratio LD_{50} (field-collected strain)/ LD_{50} (standard susceptible strain). Experiments where mortality rates for the control were higher than 10%, were not taken into account.

Enzyme preparation: Enzyme samples were prepared from 60 sec-instar larvae (10-15 mg). Insect extracts were first prepared by grinding the larva individually in eppendorf tubes. To avoid the degradation of enzymes in insect extracts, the tubes were maintained on ice during the extraction procedure. Each larva was ground in (200 μL) of cold and sterile water. Crude extracts were clarified by centrifugation at $12,000 \times g$ and 4°C for 4 min. Supernatants (190 μL) were collected and kept in wells of chilled micro titration plates. Biochemical and enzyme assays were done on supernatants and all assays were replicated.

Biochemical tests: Martin *et al.* (2002) had shown that the main biochemical mechanism involved in strains of *Helicoverpa armigera* collected in Côte d'Ivoire was the detoxification of pyrethroids by the oxidase. Esterases and GST were also studied. As Burkina Faso is closed to Côte d'Ivoire, we have thought that the prospection could be oriented to these enzymes.

Total protein dosage: Protein content was determined in 10 μ L of insect extract using the Pierce bicinchoninic acid (BCA) Protein Assay as described by Martin *et al.* (2002). Bovine Serum Albumin (BSA) was used as standard and absorbance was read at 590 nm.

Oxidase assay: Oxidase activity was assessed as described by Martin *et al.* (2002): mix first 20 μ L of enzyme extract with eighty microliters 80 μ L of 62.5 mM potassium phosphate buffer, pH 7.2. Then, the following solutions were added:

- Two hundred microliter of a mixture containing 10 mg of tetramethyl benzidine dissolved in methanol (6.5 mL) and 0.25 M sodium acetate buffer (19.5 mL) pH 5
- Eighty microliter of 62.5 mM potassium phosphate buffer, pH 7.2
- Twentyfive microliter of 30% hydrogen peroxide

The whole mixture was incubated at twenty Celsius degrees 20°C for thirty minutes 30 min and absorbance values were measured at 630 nm. Cytochrome C was used to build the standard curve and total oxidase activity was expressed as nmol equivalent cyt-P450 mg⁻¹ protein.

Esterase assay: The method used was described by Martin *et al.* (2002) using α -naphthyl acetate (α NA) or β -naphthyl acetate (β NA): and 10 μ L of enzyme extract were used for each larva.

The following procedure was performed:

- Ninety microliter of Phosphate Saline Borate (PBS) pH 6.5 with 1% triton were added to enzyme extract and incubated during 10 min at room temperature
- One hundred microliter of solution composed with 500 μ L of α -naphthyl acetate 0.3 M (or β -naphthyl acetate) + 2.5 mL PBS + 7 mL of distilled water were added and incubated during 30 min at room temperature
- One hundred microliter of solution of Fast Garnett Salt (FGBC) 8 mg dissolved in 10 mL of distilled water were added and incubated at the room temperature during 10 min
- Absorbance was read at 550 nm

Glutathione-S-transferase assay: Glutathione S-transferase (GST) activity was measured in samples of 10 μ L enzyme extract using DCNB (1-chloro-2,4-dinitrobenzene) as substrate following the procedure described by Habig *et al.* (1974). Absorbance readings were recorded at 340 nm using a microplate reader.

Data analysis: In bioassays, lethal dose 50% (LD₅₀) values were determined according the method developed by Finney (1971). The Windl software of CIRAD (France) was used to calculate transformations and regression curves.

For biochemical tests, the readings and the transformations of the data were made automatically on the microplate reader using PROCOMM 2.4.3 software from Datastorm Technologies, Inc. As non parametric test, data were statistically analyzed using Kruskal-Wallis and Mann-Whitney tests implemented in XLSTAT software version 6.1.

RESULTS

Bio-assays: Results of bioassays on *H. armigera* susceptibility to deltamethrin are shown in Table 1 and 2. In 2003, LD₅₀ of the standard susceptible *H. armigera* strain (BK77) was 0.087 μ g g⁻¹. The LD₅₀ for strains BIT03 and DAT03 were, respectively 1.241 and 3.765 μ g g⁻¹ (Table 2). Resistance Index (RI) was 14 and 43, respectively for BIT03 and DAT03. Confidence intervals indicated that the strain DAT03 had a significantly higher resistance level compared to that of the reference strain BK77.

The loss of susceptibility to deltamethrin observed in 2003 was confirmed with the strains collected in 2004. LD₅₀ values ranged between 0.97 and 2.54 (Table 3).

Confidence intervals indicated that all the field-collected strains had significantly higher resistance levels compared to the susceptible strain BK77. Compared to strain BK77, the strains TIE04, DAT04 and PO04 were respectively 11-fold, 17-fold and 21-fold, more resistant to the insecticide, while the two other strains BAL04 and BIT04 were 29-fold more resistant.

Table 2: LD₅₀ and Resistance Index (RI) to deltamethrin on the standard susceptible strain BK77 and field strains of *H. armigera* collected in 2003

<i>H. armigera</i> strain	LD ₅₀ (μ g g ⁻¹)	95% confidence intervals	Slope \pm SE*	RI
BK77	0.087	0.051-0.146	3.28 \pm 0.21	-
BIT03	1.241	0.037-2.365	2.53 \pm 0.23	14
DAT03	3.765	1.574-5.579	2.26 \pm 0.09	43

*SE: Standard error

Table 3: LD₅₀ and Resistance Index (RI) to deltamethrin on the standard susceptible strain BK77 and field strains of *H. armigera* collected in 2004

<i>H. armigera</i> strain	LD ₅₀ (μ g g ⁻¹)	95% confidence intervals	Slope \pm SE*	RI
BK77	0.087	0.051-0.146	3.28 \pm 0.21	-
BAL04	2.536	2.068-3.108	2.40 \pm 0.05	29
DAT04	1.507	1.223-1.848	2.37 \pm 0.05	17
PO04	1.870	1.495-2.340	2.05 \pm 0.05	21
TIE04	0.971	0.485-1.944	0.80 \pm 0.15	11
BIT04	2.508	1.744-3.605	1.31 \pm 0.08	29

*SE: Standard error

Table 4: Enzyme activities in extracts from *H. armigera* strains collected in 2003 and 2004

<i>H. armigera</i> strain	Oxidases (nmol cytochrome P450 mg ⁻¹ protein)	Esterase α	Esterase β	GST
		----- (μmol/min/mg/protein) -----		
BK77	2.746b	0.166a	0.286a	0.160a
BIT03	3.11b	0.139a	0.284a	0.116b
DAT03	5.038a	0.164a	0.232b	0.145a
BK77	2.337d	0.201b	0.228a	0.136c
BAL04	5.338a	0.292a	0.193b	0.188a
BIT04	3.530c	0.232ab	0.216ab	0.176ab
DAT04	5.647a	0.266a	0.206ab	0.164b
PO04	4.452ab	0.263a	0.179c	0.131c
TIE04	3.962b	0.266a	0.188bc	0.173ab

In each column, values followed by the same letter are not significantly different at 5%

Enzyme assays

Oxidase: In 2003, average concentrations in oxidases were 2.75 nmol equiv. cytochrome P450 U mg⁻¹ protein for the susceptible strain BK77, 3.11 nmol equiv. cytochrome P450 U mg⁻¹ protein for BIT03 and 5.04 nmol equiv. cytochrome P450 U mg⁻¹ mg protein for DAT03 (Table 4). With the Mann-Whitney test, oxidase concentration in BIT03 was equivalent to that of BK77 ($p > 0.05$). By contrast, strain DAT03 produced significantly higher oxidase levels than BK77 and BIT03 ($p < 0.001$).

Differences in oxidase concentrations between strains in 2004 were highly significant ($p < 0.0001$). Oxidase concentrations were 2.34, 5.34, 3.53, 5.65, 4.45 and 3.96 nmol equiv. cytochrome P450 U mg⁻¹ protein, respectively for susceptible strain (BK77), BAL04, BIT04, DAT04, PO04 and TIE04 (Table 4). Consequently, all the field-collected strains produced higher oxidase concentrations than the standard susceptible BK77. Among field strains, DAT04 had the lowest oxidase concentration.

Esterases: Results of esterase activities with α NA and β NA in 2003 are shown in Table 3. Field-collected strains BIT03 and DAT03 as well as standard susceptible strain (BK77) had similar esterase activities when β NA was used as substrate ($p = 0.12$). Esterase activities ranged between 0.139 and 0.166 μ mol/min/mg/protein. Esterase activities with β NA were higher than that with α NA for all strains. Moreover, significant differences were observed in the hydrolysis of β NA by *H. armigera* strains ($p = 0.003$). Strains BIT03 and BK77 had similar enzyme activities which were higher than that of the strain DAT03.

By contrast, esterase activities with α NA in 2004 significantly differed between field-collected strain and susceptible strain BK77 ($p = 0.02$). No significant difference was observed between strains BAL04, DAT04, PO04 and TIE04 for which enzyme activities ranged from 0.263 to 0.292 μ mol/min/mg/protein proteins. However, all these strains produced clearly higher enzyme activities than BK77. Significant differences were also observed in esterase activity with β NA ($p = 0.001$). Particularly, strains BAL04, PO04, TIE04 had lower activities compared to that

of BK77 (Table 4). Mean enzyme activity with strain BAL04 was 0.193 μ mol/min/mg/protein, which was quite similar to that of TIE04 (0.188 μ mol/min/mg/protein) but significantly higher ($p < 0.05$) than enzyme activity with PO04 (0.179 μ mol/min/mg/protein).

Glutathione-S-transferase: There was a highly significant difference in GST activity in 2003 ($p < 0.0001$). As shown in Table 3, enzyme extract from strain BIT03 yielded particularly lower activity (0.116 μ mol/min/mg/protein) compared to that of strains BK77 (0.16 μ mol/min/mg/protein) and DAT03 (0.145 μ mol/min/mg/protein). In 2004, differences in GST activities between strains were also highly significant ($p < 0.0001$). Except strain PO04, all the field-collected strains produced higher GST activities than the standard susceptible strain BK77 (Table 4).

DISCUSSION

In order to control the cotton pest *H. armigera*, insecticides have been widely used in different countries worldwide. This resulted in the development of resistances of this pest to several classes of insecticides (Ahmad and McCaffery, 1988; McCaffery, 1998; Tang *et al.*, 2000). Results obtained from this study showed clearly the loss of susceptibility of several *H. armigera* field-collected strains to the pyrethroid deltamethrin in Burkina Faso. LD₅₀ values with this insecticide were significantly higher for most strains (6 out of 7) compared to the standard susceptible strain BK77. Strains of *H. armigera* were collected in cotton fields when farmers had done four to six insecticide sprays with pyrethroids such as cypermethrin, deltamethrin, lambda-cyhalothrin. Selection pressure with pyrethroids applied on these strains in cotton fields and vegetable cultures during the season raining and the dry season, since the years 1970 had probably selected resistant individuals (Han *et al.*, 1999). Resistance of pest to insecticides was studied in few West African countries, the loss of susceptibility to deltamethrin and other

pyrethroids, previously reported in Côte d'Ivoire (Vassal *et al.*, 1997) and Benin (Martin *et al.*, 2000). The loss of susceptibility to deltamethrin in field-collected strains *H. armigera* was associated to higher oxidase concentrations. Whatever the year, insect strains which showed higher LD₅₀ values, also gave higher oxidase concentrations. This result confirmed the findings of Martin *et al.* (2002, 2003), who showed that increased oxidase levels were clearly associated with high resistance to deltamethrin. No clear-cut conclusions could be drawn from assays on esterases between the strains. No significant difference was found between *H. armigera* strains collected in 2003 by using α NA as substrate. While, using the same substrate in 2004 showed a significantly higher esterase activity for several strains in comparison with standard susceptible strain BK77. With β NA as substrate, most of the strains that showed a lower susceptibility to the insecticide also developed a lower esterase activity, whatever the year of collection. Martin *et al.* (2002) obtained also lower esterase activity in the resistant *H. armigera* strain BK99R9. Overall, discrepancies in esterase activities suggested that compared to oxidases, esterases probably play a minor role in the mechanism of the development of resistance to deltamethrin in West Africa (Martin *et al.*, 2002). *Helicoverpa armigera* showed higher GST activity in comparison with strain BK77. Several authors found positive correlation between the development of resistance by the insect to deltamethrin and other pyrethroids and GST activity (Tang *et al.*, 2000; Martin *et al.*, 2002; Ahmad, 2007). Consequently our results are in accordance with such findings.

In Côte d'Ivoire and in Burkina Faso, oxidases seemed to be responsible of pyrethroids detoxification whereas in Australia, esterases were known to be involved. In fact, in Australia, the treatments were done with single insecticides (Gunning *et al.*, 1999) and in West Africa they were always done with pyrethroid associated with organophosphate. This difference in the design even of the types of insecticides to be applied could have played a determining role in the enzymatic mechanism developed by the various strains to resist to pyrethroid.

CONCLUSION

Results obtained in this study evidenced the development of *H. armigera* resistance to the pyrethroid deltamethrin in Burkina Faso. Cypermethrin is another pyrethroid widely used in the country. As *H. armigera* resistance problems arose in areas where insecticides were used, this suggests that the insect has also developed resistance against cypermethrin. Oxidases,

esterases and GST are the three major enzymes involved in the mechanism of *H. armigera* resistance to insecticides by detoxification (Ahmad, 2007). However, the precise role of each of them is still to be investigated (Martin *et al.*, 2002). Possibly, the effectiveness of the resistance to insecticide is due to a combined effect of these enzymes.

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