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ORIGINAL ARTICLE

Role of metabolic enzymes in resistance to chlorpyrifos-methyl in the cowpea aphid, *Aphis craccivora* (Koch)

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Abstract

The cowpea aphid, *Aphis craccivora* management relies mainly on chemical control. As a result extensive and repeated treatment of insecticides has led to the development of aphid resistance to commonly used insecticides. To investigate chlorpyrifos-methyl resistance in *A. craccivora*, a field strain was selected for 24-generations to achieve a resistance factor of 82.3 fold compared with a susceptible strain. In the resistant strain, malathion and lambda-cyhalothrin exhibited obvious cross-resistance; while fenvalerate and dinotefuran showed moderate cross-resistance. In contrast, slight or no cross-resistance was obtained with the other tested insecticides. To investigate metabolic resistance mechanisms, integration of biochemical and synergism assays was conducted. Results showed the key role of esterase (EST) and mixed function oxidases (MFO); however, glutathione-s-transferase (GST) contributed less to resistance. Cross-resistance studies showed the need for rotation with non-cross resistant insecticides as a resistance management tactic.

Key words: *Aphis craccivora*, chlorpyrifos-methyl, metabolic enzymes, resistance

Introduction

The cowpea aphid, *Aphis craccivora* (Koch) (Homoptera: Aphididae) is an important legume insect pest in Egypt (El-Ghareeb *et al.* 2002). Aphid infestation causes major yield losses as a result of their deleterious effects through either honeydew excretion or viruses' transmission (Laamari *et al.* 2008). Pest management relies mainly on insecticide applications. Organophosphate is a main class of insecticides which is used extensively due to its favorable characteristics (Costa 2006). Intensive and repeated use of insecticides in agriculture has generated a strong selection leading to resistance in over 600 arthropod species including aphid (Anonymous 2009; Mokbel and Mohamed 2009). Insecticide resistance hinders seriously agricultural pest control (Andrew *et al.* 2006). Risks associated with the development of new insecticides have led to the need to preserve sustainable efficacy of used active ingredients.

Strategies must be developed to preserve the efficiency of these insecticides (Wang *et al.* 2002). Means of resistance management can be designed by investigating cross-resistance and resistance mechanisms (Criniti *et al.* 2008). Investigating characteristics of resistance is necessary to develop strategies to manage resistance. Therefore the current study investigated the development of chlorpyrifos-methyl resistance, cross-resistance to other insecticides and explores the role of detoxifying enzymes in resistance.

Materials and Methods

Chemicals and insecticides

Piperonyl butoxide (PBO), tribufos S,S,S-tributyl phosphorothioate (DEF), glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma-Aldrich. α-naphthyl acetate was obtained from MPBio. Diethyl maleate (DEM) was obtained from Alfa-Aesar. All chemicals were technical grade (99%). Insecticides used in the current work are shown in Table 1.

Insects

Two strains of the cowpea aphid were utilized. The laboratory strain was acquired from the Plant Protection Research Institute and reared free from insecticide exposure under constant laboratory conditions [22±2°C, 70±5% relative humidity (RH) and 12 : 12 light-dark photoperiod]. Aphids were reared on broad bean seedlings (*Vicia fabae*) grown in plastic pots (15 cm diameter) until needed. This strain was considered as the susceptible (S) strain. The other strain was the chlorpyrifos-methyl resistant (R) strain. This strain was initially collected from faba bean fields in Sharkia Governorate, Egypt, and had been previously exposed to various recommended insecticides belonging to different classes. Selection for chlorpyrifos-methyl resistance was accomplished by utilizing the dipping technique according to Guo *et al.* (1996). Faba bean seedlings were infected with apterous adults of aphids for 24 h before treatment. Plants bearing aphids were dipped in the desired concentration for 10 s. They were allowed to air dry for around 1 h, and then set in the rearing room. The surviving aphids were placed on new plants and kept until apterous adults of the next generation were used for bioassay.

Bioassay

Leaf-dip bioassay according to Moores *et al.* (1996) was used. Faba bean leaves were dipped in insecticide aqueous solution for about 10 s, and left to dry on a paper towel. Then, the leaves were placed upside down on an agar bed in Petri dishes (60 mm diameter). Ten apterous adults were placed on the treated leaf for each replicate. Leaves dipped in water served as control. Five replicates (i.e. 50 insects) were used per each insecticide concentration, and 5–7 concentrations were used per each insecticide. Petri dishes containing aphids were kept in the rearing chamber until mortality was recorded after 48 h. The resistance ratio (RR) was calculated by dividing LC_{50} of R-strain/LC_{50} of S-strain. Cross-resistance was examined against the other tested insecticides by the aforementioned leaf-dipping bioassay. LC_{50} values from both S and R strains were converted to the RR as mentioned above.

Synergism study

To investigate the role of detoxification enzymes in causing resistance the following synergists were used: DEF (esterase inhibitor), DEM as glutathione-s-transferase (GSTs) inhibitor and PBO (oxidase inhibitor). A constant concentration of each synergist (maximum

Table 1. List of insecticides with their trade names, active ingredients, IRAC classification and their producers

Active ingredient (common name)	Trade name	Manufacturer	Chemical group	IRAC MoA
Chlorpyrifos-methyl	Reldan 50% EC	Agrin Serve	organophosphates	Group 1B
Imidacloprid	Best 50% WP	Syngenta	neonicotinoid	Group 4A
Acetamiprid	Mospilan 20% SP	Nippon Soda	neonicotinoid	Group 4A
Dinotefuran	Ocean 20% SG	Mitsui Chem. Inc.	neonicotinoid	Group 4A
Thiamethoxam	Actara 25% WP	Syngenta	neonicotinoid	Group 4A
Malathion	Malason 57% EC	Ficom Organics	organophosphates	Group 1B
Pirimicarb	Aphox 50% DG	Syngenta	carbamates	Group 1A
Carbosulfan	Marshal 25% WP	FMC	carbamates	Group 1A
Lambda-cyhalothrin	Lambda 5% EC	Barghat	pyrethroids	Group 3A
Es-fenvalerate	Sumi-gold 10% EC	Sumitomo	pyrethroids	Group 3A
Pymetrozine	Chess 25% WP	Syngenta	pyridine azomethine derivatives	Group 9B
Diafenthiuron	Polo 50% SC	Syngenta	diafenthiuron	Group 12A

IRAC MoA Classification Version 8.0, December 2015

concentration of synergist that showed no observed mortality in the susceptible strain) was obtained by adding synergists to the prepared insecticide concentrations to get an insecticide + synergist solution. Faba bean leaves were dipped in the insecticide + synergist solution for 10 s. Leaves dipped in the synergist alone served as control. The synergistic ratio (SR) was calculated as follow:

$$SR = LC_{50} \text{ of insecticide alone}/LC_{50} \text{ of insecticide + synergist.}$$

Biochemical assays

Total esterase activity was measured according to Van Asperen (1962) with α-naphthyl acetate (α-NA) as a substrate with the modification of Cao *et al.* (2008). Fifty wingless adults from each strain were homogenized in 1 ml of ice-cold phosphate buffer (0.04 M, pH 7.0). The homogenate was centrifuged at 12,000 g for 15 min, 4°C. The resulting supernatants were diluted 20-fold with the homogenization buffer and used for subsequent esterase activity assay, then stored at 20°C. The α-naphthyl acetate concentration was 0.3 mM, diluted from their respective 0.03 M stock. The assay mixture contained 50 µl enzyme preparation, 450 µl 0.04 M phosphate buffer and 1.80 ml 0.3 mM substrate solution. The reaction was stopped by adding 0.9 ml of stop solution (two parts of 1% Fast Blue BB and five parts of 5% sodium dodecyl sulfate). Incubation at 30°C for 15 min at room temperature allowed color development. The absorbance was measured at 600 nm for the hydrolysis of α-NA. Mean levels of total esterase activity were calculated based on protein content and α-naphthol standard curves.

Glutathione-s-transferase (GST) activity was determined according to Habig *et al.* (1974). 1-chloro-2,4-dinitrobenzene (CDNB) was used as the substrate in ultraviolet (UV) semi-micro cuvettes (4 ml) by sequential addition of 0.1 M phosphate buffer pH 6.5 (1.78 ml), enzyme preparation (0.1 ml), 50 mM of reduced GST solution in buffer (0.1 ml) and 50 mM CDNB solution in acetonitrile (0.02 ml), giving 2 ml final volume of the incubation mixture. Enzyme activity was determined by monitoring continuous changes in absorbance at 430 nm for 3 min at 25°C with a spectrophotometer.

Statistical analysis

Mortality was corrected using Abbott's formula (Abbott 1925) and data were analyzed by probit analysis (Finney 1971) using the software package EPA probit analysis version 1.5. Mean enzyme activities recorded from the R strain were compared with those from the S strain colony with the Student's t-test using SPSS version 19. Significance was accepted at $\alpha = 0.05$ in the Student's t-test.

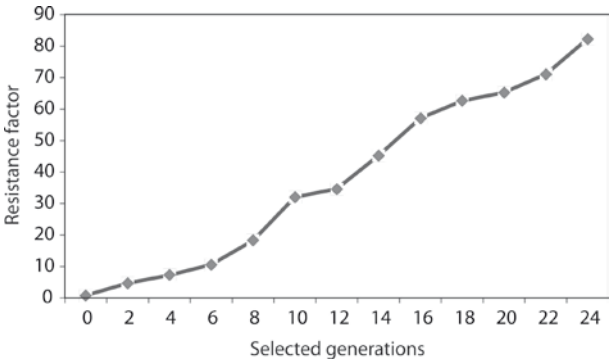

Fig. 1. Resistance development rate to chlorpyrifos-methyl in *Aphis craccivora* which selected every generation for a total of 24 generations

Results

Selection for resistance

Data in Figure 1 revealed that the initial LC_{50} value was 0.18 ppm (for the 1st generation); resistance level was increased proportionally by continuous selection. Resistance ratio increased from 0.78 fold in the 1st generation to 82.3 fold after the 24th generation. Ten-fold resistance required six successive generations. Resistance increased gradually until the 12th generation and further elevated dramatically up to the 24th generation.

Cross-resistance study

Results summarized in Table 2 exhibited cross-resistance against the organophosphate malathion (15.62 fold) and the synthetic pyrethroid, lambda-cyhalothrin (21.4 fold). Es-fenvalerate showed moderate cross-resistance with a resistance ratio of 8.8 fold. On the other hand, carbamate insecticides (pirimicarb and carbosulfan) showed no or slight cross-resistance, 1.06 fold and 3.51 fold, respectively. The neonicotinoid, acetamiprid, thiamethoxam, dinotefuran and imidacloprid showed negative or low RR with values of 1.3, 0.89, 5.53 and 3.03 fold, respectively. Similar trends were noticed with pymetrozine and diafenthiuron which exhibited resistance ratios of 1.43 and 3.06 fold, respectively.

Enzymes and synergism assay

Figure 2 presents the mean activities of esterase in the susceptible and resistant strains. By using the model

Table 2. Cross-resistance of chlorpyrifos-methyl (R strain) of *Aphis craccivora* to the tested insecticides

Insecticide	Susceptible strain LC_{50} (95% CL)	Resistant strain LC_{50} (95% CL)	Resistant ratio (RR)
Imidacloprid	0.77 (0.49–1.10)	2.34 (1.46–3.73)	3.03
Acetamiprid	0.13 (0.087–0.187)	0.17 (0.09–0.28)	1.30
Dinotefuran	0.95 (0.59–1.34)	5.26 (3.04–14.77)	5.53
Thiamethoxam	0.44 (0.29–0.60)	0.39 (0.10–0.85)	0.89
Malathion	9.47 (6.21–12.88)	147.9 (101.5–231)	15.62
Pirimicarb	1.02 (0.55–1.37)	1.09 (0.71–1.54)	1.06
Carbosulfan	4.56 (3.25–6.54)	16.03 (7.8–26.06)	3.51
Lambda-cyhalothrin	0.007 (0.00–0.019)	0.15 (0.07–0.32)	21.40
Es-fenvalerate	0.15 (0.11–0.19)	1.32 (0.73–2.33)	8.80
Pymetrozine	6.12 (4.18–9.14)	8.79 (4.42–14.46)	1.43
Diafenthiuron	23.46 (11.37–46.80)	71.8 (46.44–92.44)	3.06

CL – confidence limits
RR = LC_{50} of selected strain/LC_{50} of susceptible strain

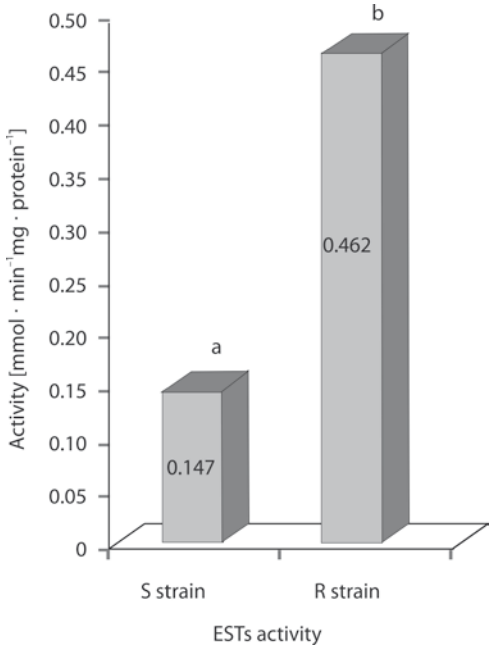

Fig. 2. Activity of esterases (ESTs) in susceptible (S strain) and chlorpyrifos-methyl resistant (R strain) of *Aphis craccivora*. Graph bars containing similar letters on the top are not significantly different (p = 0.05)

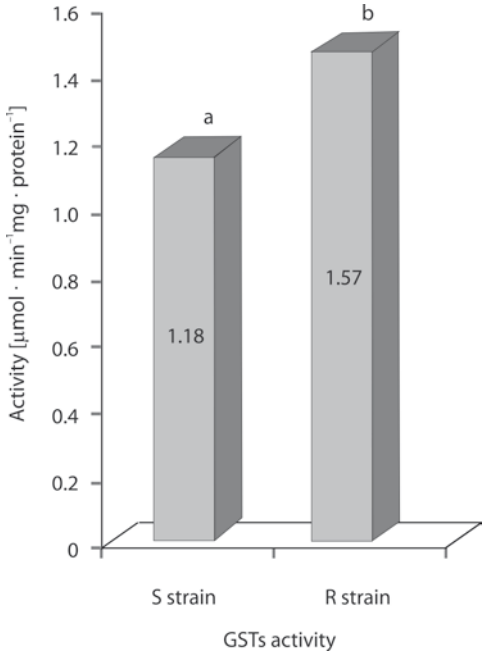

Fig. 3. Activity of glutathione-S-transferases (GSTs) in susceptible (S strain) and chlorpyrifos-methyl resistant (R strain) of *Aphis craccivora*. Graph bars containing similar letters on the top are not significantly different (p = 0.05)

substrate α-NA, esterase activity elevated (3.14 times) in R-strain compared with S-strain. Furthermore, Figure 3 showed that R-strain exhibited a slight change in glutathione-s-transferase activity with a ratio of 1.33 compared with S strain. Results in Table 3 indicated that PBO showed a synergistic ratio value of 5.69 while DEF exhibited a synergistic ratio of 11.6, in contrast DEM showed only a synergistic ratio value of 1.58.

Discussion

In our study, laboratory selection with chlorpyrifos-methyl increased resistance level to approximately 10-fold after six generations and 82-fold after 24 generations. Our results are in harmony with Mokbel (2015) who found that the cowpea aphid had the potential to develop

Table 3. Synergistic effect of piperonyl butoxide (PBO), tribufos S,S,S-tributyl phosphorothioate (DEF) and diethyl maleate (DEM) to (S strain) and chlorpyrifos-methyl (R strain) *Aphis craccivora*

Strain	Treatment	Slope±SE	LC_{50} [mg · l^{-1}] 95% CL	Synergistic ratio (SR)	Resistance ratio (RR)
S strain	Alone	2.03±0.40	0.23 (0.14–0.33)	–	–
	+DEF	1.33±0.24	0.16 (0.09–0.26)	1.39	–
	+PBO	1.46±0.25	0.17 (0.10–0.26)	1.35	–
	+DEM	1.53±0.31	0.20 (0.11–0.25)	1.13	–
R strain	Alone	2.28±0.59	18.9 (12.75–24.97)	–	82.30
	+DEF	3.27±0.61	1.63 (1.33–2.07)	11.60	7.09
	+PBO	1.76±0.28	3.32 (2.39–4.70)	5.69	14.46
	+DEM	2.05±0.49	12.02 (10.34–16.21)	1.58	52.26

CL – confidence limits
SR = LC_{50} of insecticide alone/LC_{50} of insecticide + synergist
RR = LC_{50} of R strain/LC_{50} of S strain

resistance to chlorpyrifos-methyl. Selection with chlorpyrifos-methyl for 24 generations resulted in 105 fold resistance compared with the susceptible strain.

Although chlorpyrifos-methyl and malathion belong to the same class and are expected to share a common resistance mechanism, the *A. craccivora* resistant strain (R strain) showed a lower resistance factor to malathion than that of chlorpyrifos-methyl. Similar findings were obtained with *Oryzaephilus surinamensis*. These differences were interpreted to result from the major differences in carboxylesterase isozymes between malathion and chlorpyrifos resistant strains (Lee and Lees 2001). Negative cross-resistance to thiamethoxam, pirimicarb, acetamiprid and pymetrozine may be due to the fact that these insecticides have different modes of actions. Although pirimicarb has a mode of action similar to organophosphates, it showed a unique mechanism of resistance (reduced sensitivity of acetylcholinesterase) (Kandil *et al.* 2017). Similar trends were found by Suzuki *et al.* (1993) who showed that there was no correlation between carboxylesterase activity and resistance to dimethyl carbamate in *A. gossypii*.

Integration between enzymes and synergism assays has a significant role in identifying metabolic resistance mechanisms. Metabolic resistance to organophosphates in aphids has been thought to be due to the elevated activity of a number of detoxification systems. It is generally expected that resistance to organophosphates is correlated with elevated esterase activity especially with the model substrate α-naphthyl acetate (Devonshire 1977). It is noteworthy that our results indicated an elevation of esterase activity in the R strain about 3-fold more than that in the S strain. Moreover, synergism assay showed that esterase and monooxygenases, as metabolic enzymes, may play a vital role in chlorpyrifos-methyl resistance. Adding either DEF or PBO suppressed resistance from 82.3-fold to 7.9 and 14.46-fold, respectively. So, R strain switched to tolerance level by using esterase inhibitor. This means that esterase plays the key role in the resistance mechanism of R strain, followed by monooxygenases. The greater activity of detoxifying enzymes, particularly carboxylesterase has a significant role in endowing resistance to thiamethoxamin in the cowpea aphid (Abdallah *et al.* 2016). These results agree with Fouad *et al.* (2016) who found that esterase activity in three field populations of cowpea aphid was higher than in the susceptible strain. The activity ratios ranged from 4.3 to 7.8-fold. Moreover, these results agree with Kandil *et al.* (2013) who found that DEF and PBO had significant synergism in the acetamiprid-resistant strain of cowpea aphid with a synergism ratio of 3.74 and 8.3-fold, respectively. Also, Lee and Lees (2001) found elevated levels of carboxylesterase activity in *O. surinamensis* resistant to malathion and chlorpyrifos-methyl. The role of GST's in conferring insecticide resistance is mainly due to the conjugation to insecticides or their primary metabolites. Biochemical determination of GSTs activity or synergist assay showed slight differences in activity of GSTs in either R strain or S strain of the cowpea aphid (Fig. 3).

In conclusion, the current data revealed the potential of *A. craccivora* to develop resistance to chlorpyrifos-methyl. Negative cross-resistance to thiamethoxam, acetamiprid and pymetrozine makes it possible to use it in rotation with chlorpyrifos-methyl to control cowpea aphid. Synergism and biochemical studies suggested that resistance is multifactorial. Esterase is the main metabolic enzyme contributing in chlorpyrifos-methyl resistance followed by oxidases but to a lesser degree. Moreover, GSTs plays a marginal role in resistance. Results indicate that careful selection and rotational use of non-cross-resistant insecticides should result in the satisfactory control of field populations of *A. craccivora*. Accordingly, an effective resistance management program is necessary to combat resistance development in *A. craccivora*.

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