Advances in Entomology, 2016, 4, 167-182
Published Online July 2016 in SciRes. http://www.scirp.org/journal/ae
http://dx.doi.org/10.4236/ae.2016.43018

Scientific Research Publishing

Biochemical Characterization of Detoxifying Enzymes in Dimethoate-Resistant Strains of Melon Aphid, *Aphis gossypii* (Hemiptera: Aphididae)

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Received 12 April 2016; accepted 19 July 2016; published 22 July 2016

Abstract

The melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a highly polyphagous sap sucking pest on wide varieties of crops including cotton and vegetables. It is a notorious vector of many plant viruses that are persistently and non-persistently transmitted. In nature, aphids are regulated by their natural enemies. However, chemical control remains a major management tool even though resistance to insecticides has been documented worldwide. A better understanding of mechanisms by which insecticide resistance occurs and its early detection is desirable to develop effective management strategies. The present investigation was conducted to study the development of resistance to an organophosphate (OP) compound-dimethoate, identify biochemical mechanism(s) involved in resistance and study cross-resistance to imidacloprid in laboratory selected *A. gossypii* strains in comparison to susceptible strains. Bioassay studies revealed that the LC_{50} values increased dramatically with dimethoate selection in resistant strains and the resistance ratio (RR) was 270-, 243- and 210-fold greater than that of the susceptible strains by 30th

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How to cite this paper: Lokeshwari, D., Kumar, N.K.K., Manjunatha, H. and Shivashankar, S. (2016) Biochemical Characterization of Detoxifying Enzymes in Dimethoate-Resistant Strains of Melon Aphid, *Aphis gossypii* (Hemiptera: Aphididae). *Advances in Entomology*, **4**, 167-182. http://dx.doi.org/10.4236/ae.2016.43018

generation. Further, biochemical assays revealed enhanced activities of carboxylesterases (CarE), glutathione S-transferases (GSTs) and cytochrome P450-mediated *p*-Nitroanisole *O*-demethylase (PNOD) in resistant strains supporting their role in dimethoate detoxification. This study thus revealed that enhanced activity of detoxifying enzymes *viz*., CarE, GSTs and PNODs is one of the mechanisms underlying dimethoate resistance in *A. gossypii* collected from South India. Interestingly, the possibility of negatively correlated cross-resistance to imidacloprid was identified in three OP-resistant strains exhibiting 2.97-, 2.56- and 3.76-fold sensitivity to imidacloprid (a novel neonicotinoid). This indicated that the latter was less affected by the resistance mechanism(s) present.

Keywords

***Aphis gossypii*, Carboxylesterases, Dimethoate, Glutathione S-Transferases, LC_{50}, Melon Aphid, *p*-Nitroanisole *O*-Demethylase, Resistance**

1. Introduction

The melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is the most important cosmopolitan, highly polyphagous aphid species that infests 200 economically important crops worldwide [1]. Damage occurs as a result of direct feeding on the plant phloem sap, honeydew deposition and physiological disorders. It is the most versatile insect vector capable of transmitting >75 plant viruses [2]. It can easily reach outbreak population levels when environmental conditions are suitable and is regulated by their natural enemies in nature. However, chemical control using insecticides remains the main method of melon aphid control [3] and means of virus management in the absence of resistant varieties. Due to widespread intensive application of organophosphates (OPs), melon aphids have developed high levels of resistance ensuing in eventual control failures and reduced crop yield [4] [5]. It is considered to develop elevated levels of resistance to OPs in a relatively short period of time due to its high reproductive potential by means of parthenogenesis and likelihood to develop resistance [6] [7] [8]. Due to which it is one among top 12 resistant arthropods with reported resistance to 49 unique compounds [9]. In addition, the ability of this aphid species to travel over long distances could widely distribute resistant populations dispersing pathogenic viruses across host plants ensuing in huge crop loss [10]. Thus, resistance problem in *A. gossypii* has become a greater concern around world year by year and it is the most difficult case to manage. In the last three decades, remarkable strides have been made in understanding pesticide resistance in arthropods especially aphids. However, there are no reports available on the underlying mechanisms conferring OP resistance in Indian populations of *A. gossypii*. Among the OPs, dimethoate remains the management choice in aphid and aphid transmitted virus control for farmers and seed industries for the past 40 years [11]. Although effective for several years, a decrease in OP efficacy has been reported in populations collected from South India especially Guntur where this insecticide was applied intensively for many years [12]. It is anticipated that melon aphids have developed resistance to dimethoate that has been applied intensively for four decades and if so, biochemical basis behind such resistance needs to be determined and documented.

Earlier studies indicate that melon aphids develop resistance to OPs rapidly and the mechanisms underlying resistance are diverse [13] [14]. In *A. gossypii*, resistance to OPs is due to enhanced metabolic destruction by detoxifying enzymes *viz*., carboxylesterases (CarE), glutathione S-transferases (GSTs), cytochrome P450-mediated *p*-Nitroanisole *O*-demethylase (PNOD) [4] [14] [15] and altered acetylcholinesterase (AChE) carrying point mutations [5] [16]. Generally, CarE combines with OPs (sequester) and hydrolyses insecticide esters forming non-toxic products and thereby prevents access of OPs to the target site, AChE [17]. Elevated levels of CarE (E4 and FE4) caused by gene duplication or amplification resulted in increased detoxification of OPs, carbamates and pyrethroids in *Myzus persicae* Sulzer [18] [19]. Similarly, in *A. gossypii*, enhanced levels of CarE caused either by gene amplification or upregulation of transcription coupled with increased activity are implicated in OP resistance [4] [15] [20] [21]. In addition, structural substitutions (non-silent point mutations) on CarE gene are also associated with OP resistance [4] [22]. In contrast to CarE, GSTs catalyze the conjugation of a diverse array of electrophilic compounds with glutathione resulting in metabolism, detoxification and excretion of large number of pesticides and plant toxins [23]. The products formed are less toxic, more water soluble

and could be excreted more readily than non-GSH conjugated substrates from the cell. They also act as nonenzymatic binding proteins (ligandins) that participate in the intracellular transport [24] [25]. In insects, studies suggest that GSTs play an important role in resistance against several classes of insecticides including OPs [26]. The multifunctional monooxygenases catalysed by cytochrome P450 (MFOs) comprise the most versatile metabolic system for the detoxification of insecticides in insects [17]. Due to their broad substrate spectra, they potentially affect several classes of insecticides and thereby confer resistance [27]. Reports of enhanced activity of MFOs conferring insecticide resistance have been documented in insects such as *Culex pipiens pallens* Caguillett [28], *Plutella xylostella* (Linnaeus) [29], *Bemisia tabaci* Gennadius [30] and *M. persicae* [31]. MFOs are involved in the activation and detoxification of OPs (e.g. diazinon) [32]. In *A. gossypii*, an increased activity of MFOs, in particular, cytochrome P450-mediated O-demethylase (PNOD) is implicated in omethoate (OP) resistance [14]. Similarly, an increased PNOD activity was responsible for different levels of fenvalerate resistance in *Helicoverpa armigera* (Hubner) [33].

The resistance patterns and biochemical basis underlying OP resistance, especially dimethoate, in Indian populations of *A. gossypii* is lacking and thus remains an issue for in-depth clarification. A better understanding of mechanisms by which insecticide resistance occurs and its early detection is desirable to develop effective management tactics [17]. To date, no major work has been done on surveillance of resistance of *A. gossypii* to dimethoate in India. It is proving impossible to combat resistance by embarking on a chemical arms race and only by monitoring and characterizing resistance mechanisms, can the existing chemical tools be used in a sustainable manner. In this regard, the present study was conducted to identify biochemical mechanism(s) involved in dimethoate resistance, evaluate the activity of detoxifying enzymes *viz*., CarE, GSTs and MFOs as well as to study cross-resistance to imidacloprid in resistant vs. susceptible strains of *A. gossypii* collected from South India.

2. Materials and Methods

2.1. Insects

A total of 20 different *A. gossypii* strains were collected from unsprayed domestic backyards during 2009-2010 in and around Karnataka. Baseline susceptibility LC_{50} values of these 20 strains varied between 5.81 - 8.23 $mg \cdot l^{-1}$ for dimethoate. Two strains LKS-1 and LKS-2 collected during August, 2009 from untreated cucumber and watermelon plants cultivated (separated by approx. 2000 m from each other) at the Indian Institute of Horticultural Research (IIHR), Bengaluru, India (12˚58˚N, 77˚35˚E) with least LC_{50} values of 5.81 and 6.82 $mg \cdot l^{-1}$ were chosen as susceptible strains. Live aphids along with the plant material was brought to the laboratory and reared without exposure to insecticides (60 generations) on cucumber (*Cucumis sativus*) plants (hybrid Malini) in separate insect-proof cages at 30˚C ± 2˚C, 70% - 80% RH and a photoperiod of 16:8 (L:D) h and used as reference strains. Susceptibility of these two strains to dimethoate was monitored at an interval of 10 generations up to 60 generations. A total of 30 different strains of *A. gossypii* were randomly collected from fields prone to extensive dimethoate/OP sprays during 2009-2011 in and around South India (Andhra Pradesh, Karnataka and Tamil Nadu) as well from Maharastra and screened. LC_{50} values of these 30 strains ranged between 300.14 - 496.38 $mg \cdot l^{-1}$ for dimethoate. Of the 30 strains, three strains LKR-1 (IIHR, Karnataka), LKR-2 (Mangalagiri, Andhra Pradesh) and LKR-3 (Guntur, Andhra Pradesh) collected during April, 2011 from treated cucumber plants with highest LC_{50} values of 387.57, 496.38 and 339.83 $mg \cdot l^{-1}$ were chosen and reared on cucumber (*C. sativus*) plants (hybrid Malini) to develop field-derived dimethoate resistant clones under continuous selection in separate insect proof cages at 30˚C ± 2˚C, 70% - 80% RH and a photoperiod of 16:8 (L:D) h in the laboratory.

2.2. Insecticides and Chemicals

To understand resistance development and cross-resistance, the aphid populations were bioassayed against commercial formulations of the two insecticides extensively used for melon aphid control *viz*., dimethoate (Rogor® 30EC; Insecticides India Ltd., Bharuch, Gujarat) from organophosphate group and imidacloprid (Confidor® 17.8SL; Bayer Crop Science Pvt. Ltd. Bayer Crop Science, Monheim, Germany) from neonicotinoids following the leaf dip method. Concentrations were calculated on the basis of $mg \cdot l^{-1}$ of active ingredient. In order to establish mortality ranging from 20% to 85%, the ranges of concentrations for dimethoate and imidacloprid were examined in preliminary tests and selected. Enzyme substrates α-naphthyl acetate (α-NA), α-naphthyl propionate (α-NPr), β-naphthyl acetate (β-NA), 1-Chloro, 2-4-dinitrobenze (CDNB), Glutathione (GSH and Coomas-

sie Brilliant Blue G-250 were purchased from Quest International, Inc. (CA). *α*-naphthyl butyrate (*α*-NB), p-Nitroanisole, Eserine, Bovine serum albumin (BSA) and Fast Blue BB salt were from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used were of reagent grade.

2.3. Leaf-Dip Bioassay

Five laboratory strains of melon aphid, *A. gossypii* were investigated; two susceptible strains *viz*., LKS-1 and LKS-2; three resistant strains *viz*., LKR-1, LKR-2 and LKR-3. Dimethoate toxicity in the resistant and susceptible melon aphids were determined by methods described by the Insecticide Resistance Action Committee (IRAC) (Method No. 019, http://www.iraconline.org/content/uploads/Method_019_v3.2_May12_aphid.pdf). Serial dilutions of insecticides were prepared by dissolving appropriate quantity of respective insecticide in distilled water containing 0.05% (vol:vol) Triton X-100. The bioassay arena consisted of individual 90 by 15 mm disposable plastic petri dishes with a 5 mm layer of 1% agar solution in the bottom. A 50 mm diameter hole was cut into each lid and sealed with a piece of cotton cloth to allow excess moisture to escape. Discs (80 mm diameter each) were cut from non-treated cucumber leaves and dipped in insecticide solution for 10 s, placed abaxial surface uppermost on an agar bed in petri dishes and allowed to air-dry in the shade. The edges of each leaf disc were gently pressed into the agar to minimize desiccation. Apterous female adults of same size and colour from each strain (30 per plate) were gently placed on the treated leaf surface with a very fine camel hair brush. Leaf discs dipped in distilled water containing 0.05% (vol:vol) Triton X-100 were used as controls. Bioassay petri dishes were maintained at 30˚C ± 2˚C, 70% - 80% RH under ambient day light conditions. All bioassays were scored at 24 h after treatment. Insects were considered alive if they showed any sign of movement; each bioassay used triplicates of 10 concentrations along with a control. Bioassay for each insecticide was repeated at least five times and results were pooled for probit analysis. Tests in which mortality exceeded 5% in control dishes were not included in analyses.

2.4. Selection Regimes

Apterous, female adults (500 - 1000) were subjected to dimethoate selection from each strain and the surviving aphids after 24 h of treatment were reared for the next generation. The LKR-1, LKR-2 and LKR-3 resistant clones were continually selected for 30 generations with different concentrations of dimethoate LC_{50} (that kills 50% of the aphids) by the leaf-dipping method [34]. For the first generation, the selection pressure was applied at a concentration of 500 $mg \cdot l^{-1}$ (the LC_{50} value). In subsequent generations, selection concentrations were gradually increased as the response increased based on the results of bioassays ($\approx LC_{50}$) from the previous generation. The resistance level (resistance ratio [RR]) of the selected strain was calculated by dividing the LC_{50} of selected strain by the corresponding LC_{50} (Mean LC_{50} = 5.47 $mg \cdot l^{-1}$) of the susceptible strains. These selected strains exhibiting high RR values at 30th generation were used for biochemical studies.

2.5. Biochemical Assays

2.5.1. Carboxylesterase (CarE) Activity

Carboxylesterase (EC 3.1.1.1) activity was measured using various substrates *viz*., *α*-naphthyl acetate (*α*-NA), *β*-naphthyl acetate (*β*-NA), *α*-naphthyl butyrate (*α*-NB) and *α*-naphthyl propionate (*α*-NPr) [4] [21] with suitable modifications. One hundred wingless adult females similar in size and color from each strain were collected and homogenized in 500 µL of ice-cold phosphate buffer (0.04 M, pH 7.0). The homogenate was centrifuged at 10,000 g for 20 min at 4˚C and the resulting supernatant was collected and used as an enzyme source. The assay mixture contained 100 µL of enzyme preparation, 450 µL of 0.04 M phosphate buffer, and 1.80 mL of 0.3 mM substrate solution. The reaction was stopped by addition of 0.9 mL of mixture containing two parts of 1% Fast Blue BB salt (Diazotized 4-amino-2,5-diethoxybenzanilide zinc chloride salt) and five parts of 5% sodium dodecyl sulfate and the reaction mixture was incubated at 30˚C for 20 min under natural light conditions. The color was allowed to develop at room temperature. The absorbance of the reaction product was measured after 15 min at 600 nm for *α*-NA, *α*-NB and *α*-NPr using Beckman DU-64 UV-Vis spectrophotometer, USA against the blank. Similarly, the absorbance was measured at 550 nm for *β*-NA. Carboxylesterase activity was calculated based on *α*-naphthol standard curve and on protein content. The specific activity of carboxylesterase was ex-

pressed as µmol naphthol formed $min^{-1} \cdot mg^{-1}$ protein.

2.5.2. Glutathione-S-Transferase (GST) Activity

GST (EC 2.5.1.18) activity was measured using 1-Chloro, 2-4-dinitrobenze (CDNB) as substrate [35]. A total of 100 wingless adult females of similar size and color from each strain were homogenized in 500 µL of ice-cold phosphate buffer (66 mM, pH 7.0). The homogenate was centrifuged at 10,000g for 20 min at 4˚C and the clear supernatant was collected and used for the determination of GST activity. 200 µL of enzyme extract was mixed with 100 µL of 30 mM CDNB (in 95% ethanol) and 2.4 mL of 66 mM phosphate-buffered saline (pH 7.0). The reaction was initiated by adding 300 µL of 50 mM Glutathione (GSH). Activity was measured by recording the change in absorbance at 340 nm and 25˚C using the kinetic mode for 5 min at 1 min interval against the blank. The specific activity of GST was determined using the extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$ for CDNB-GSH conjugate (2, 4-dinitrophenyl glutathione) formed and was expressed as µmol of 2, 4-dinitrophenyl glutathione formed $min^{-1} \cdot mg^{-1}$ protein.

2.5.3. Cytochrome P450-Mediated O-Demethylation (PNOD) Activity

Cytochrome P450-mediated p-Nitroanisole O-demethylase activity was measured using p-nitroanisole [36] with slight modifications. A total of 100 wingless adult females of similar size and color from each strain were homogenized in 500 µL of 50 mM Tris-HCl buffer on ice (pH 7.7, containing 1.15% KCl and 1.0 mM EDTA). The homogenate was centrifuged at 14,000 g for 20 min at 0˚C. The supernatant was collected and used for determination of PNOD activity. The assay mixture containing 1.6 mL tris-HCL buffer, 1 mL 50 mM p-Nitroanisole (in ethanol) and 100 µL enzyme extract was incubated at 34˚C for 3 min. 200 µL of 10.0 mM NADPH (in 0.1 M NaPHO4 buffer, pH 7.8) was added and the reaction mixture was incubated for 30 min at 34˚C. The activity of PNOD forming O-demethylated product p-Nitrophenol was immediately measured at 405 nm at 34˚C at intervals of 15 sec for 15 min against the blank. The specific activity was determined using the extinction coefficient of 3.32 $mM^{-1} \cdot cm^{-1}$ for p-Nitrophenol [37] [38] and presented as nmol p-nitrophenol released $min^{-1} \cdot mg^{-1}$ protein.

2.5.4. Protein Assay

The protein content in enzyme extracts was estimated using Coomassie Brilliant Blue G-250 [39]. The absorbance was measured at 595 nm and the protein content was calculated from the standard curve prepared from Bovine Serum Albumin (BSA) as standard protein.

2.6. Statistical Analysis

Mortality data obtained in the bioassays were subjected to probit analysis for determination of lethal concentration (LC) value at 50 level, 95% fiducial limits (FL), slope and Chi sq. values. Correction for the control mortality was made by using Abbott's formula [40]. Probit analysis [41] of the concentration dependent mortality data were carried out using the software program POLO-PC software [42]. LC_{50} values were calculated in $mg \cdot l^{-1}$. Mean of LC_{50} values of susceptible strains was calculated and used as reference strain in this study to calculate resistance ratios (RRs). The LC_{50} of the resistant strain was divided by the LC_{50} of the susceptible strain to obtain RRs. Resistance ratio is used as an indicator of an increasing resistance due to selection by dimethoate. The slope values were taken as an indication of the degree of homogeneity of the strain to insecticide. A high slope value suggests less heterogeneity in population sensitivity to a particular insecticide, while a shallow slope represents high heterogeneity in sensitivity among individuals of a population [43]. Lethal concentration ratios (LCR) *i.e.* LC_{50} (Resistant strain)/LC_{50} (Susceptible strain) and their 95% confidence intervals (CI) were calculated using LCR significance test [44]. Significant differences among LCR values were determined based on the 95% CI of LCR [44]. The biochemical enzyme assays comprising of two replicates for each strain were repeated for five times and the data were pooled. Results of two replicates were averaged from protein preparations made on five times (n = 10). Mean value of enzyme activities in susceptible strains was utilized to calculate the relative ratio in resistant strains. Enzymatic activity levels reported from the linear response portions of enzyme reactions in each resistant strain were compared with those obtained for the susceptible strain. Comparisons of the means were made by Fisher's least significant difference (LSD) test ($P < 0.01$) using SAS program V9.3. available at IIHR, Bengaluru [45]. All data in this study are expressed as mean ± standard error (SEM).

3. Results

3.1. Dimethoate Toxicity Assays

The LC_{50} values of dimethoate for two susceptible populations are presented in **Table 1**. At the beginning, the LC_{50} values for two susceptible strains, LKS-1 and LKS-2 were 5.81 and 6.82 $mg \cdot l^{-1}$ respectively. At the 60th generation, the LC_{50} values were 4.93 and 6.01 $mg \cdot l^{-1}$ for LKS-1 and LKS-2 respectively. A small but consistent decrease in measured LC_{50} was observed for susceptible strains. This observation suggested that the strains chosen were not fully susceptible when initially chosen (**Table 1**). However, *A. gossypii* resistant strains LKR-1, LKR-2 and LKR-3 developed high level of resistance to dimethoate rapidly with LC_{50} values of 1476.89-, 1326.27- and 1146.49 $mg \cdot l^{-1}$ after 30 consecutive generations through continuous selection (**Table 2**). As the selection progressed, the resistance ratio (RR) in relation to susceptible strain as well increased from 79-, 101- and 69-folds (generation 1) to 270-, 243- and 210-folds (generation 30) in LKR-1, LKR-2 and LKR-3 respectively. The LCR at LC_{50} varied significantly based on their corresponding 95% confidence intervals suggesting the possibility of different mechanisms or at least different detoxifying enzyme activities predominating in the different resistant strains (**Table 3**).

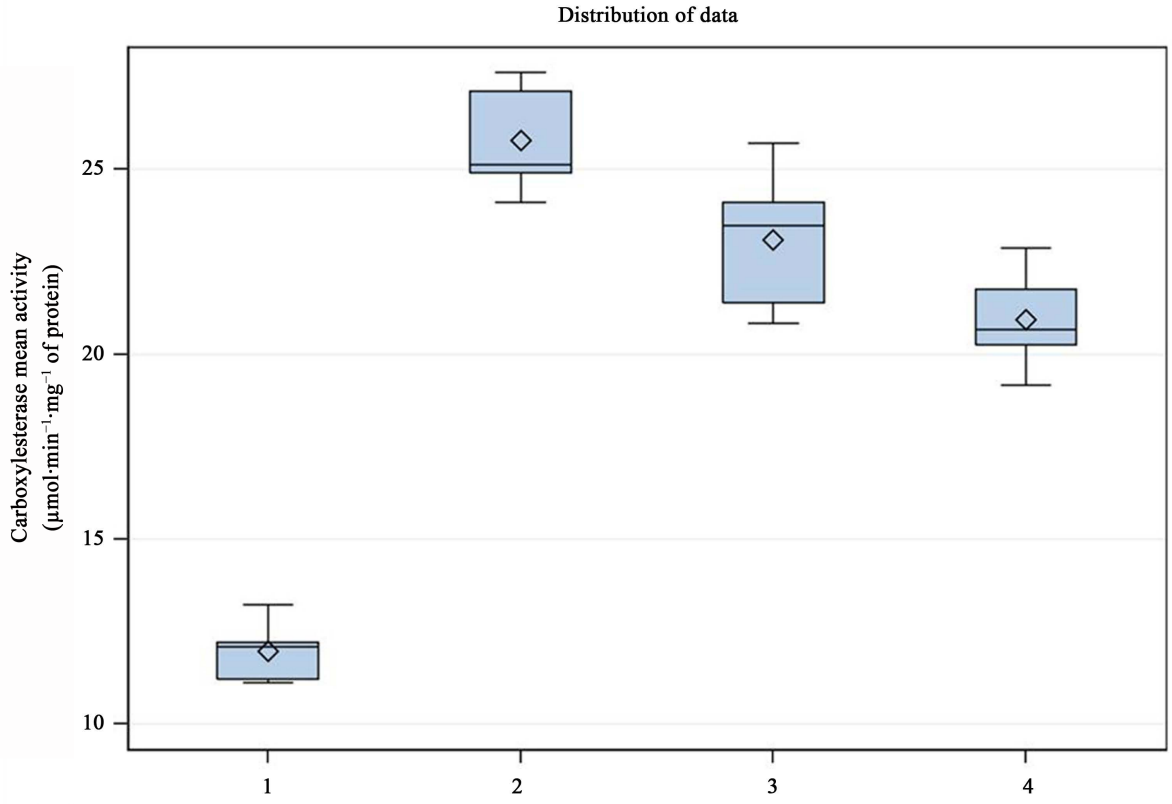
3.2. Biochemical Assays

The carboxylesterase activity measured using ester substrates: α -NA, α -NB, α -NPr, and β -NA is presented in **Table 4**. The esterase activity of the resistant strains was significantly higher than the susceptible strain (α-NA: LSD, $F = 90.1$, df = 3, $P < 0.01$; β-NA: LSD, $F = 562.1$, df = 3, $P < 0.01$; α-NB: LSD, $F = 382.2$, df = 3, $P < 0.01$ and α-NPr: LSD, $F = 574.3$, df = 3, $P < 0.01$) (**Figures 1(a)-(d)**). However, the CarE in these strains exhibited the lowest activity for hydrolyzing α-NPr among the four substrates analysed. The esterase activities of the resistant strains were 2.15-, 1.93- and 1.75-folds higher than the susceptible strain for α-NA, similarly, 2.13-, 1.78- and 1.55-folds for β-NA; 2.41-, 2.28- and 1.99-folds for α-NB; 1.68-, 2.15- and 1.90-folds for α-NPr respectively. The CarE activities towards β-NA and α-NPr varied significantly among three resistant strains ($P < 0.01$) in contrast to CarE activities towards α-NA. However, CarE activity of LKR-3 strain towards α-NB varied significantly from LKR-1 and LKR-2. These results clearly suggested that CarE activities differed predominantly among three different resistant strains with respect to ester substrates (LSD, $F = 24.1$, df = 9, $P < 0.01$). The GST activities shown in **Table 5** indicate that the resistant strains had significantly higher GST activities than the susceptible strain (LSD, $F = 440.0$, df = 3, $P < 0.01$) (**Figure 2**) and significant differences in GST activities were observed among three resistant strains ($P < 0.01$). GST activities in resistant strains were 2.30-, 1.94- and 1.52-fold higher for CDNB against susceptible strain respectively. The PNOD activity presented in **Table 5** showed that PNOD activities in resistant strain were significantly higher (LSD, $F = 115.1$, df = 3, $P < 0.01$) (**Figure 3**) recording 1.32-, 1.51- and 1.57-fold increase against susceptible strain respectively. The PNOD activity varied significantly in resistant strain, LKR-1 when compared with the other two strains.

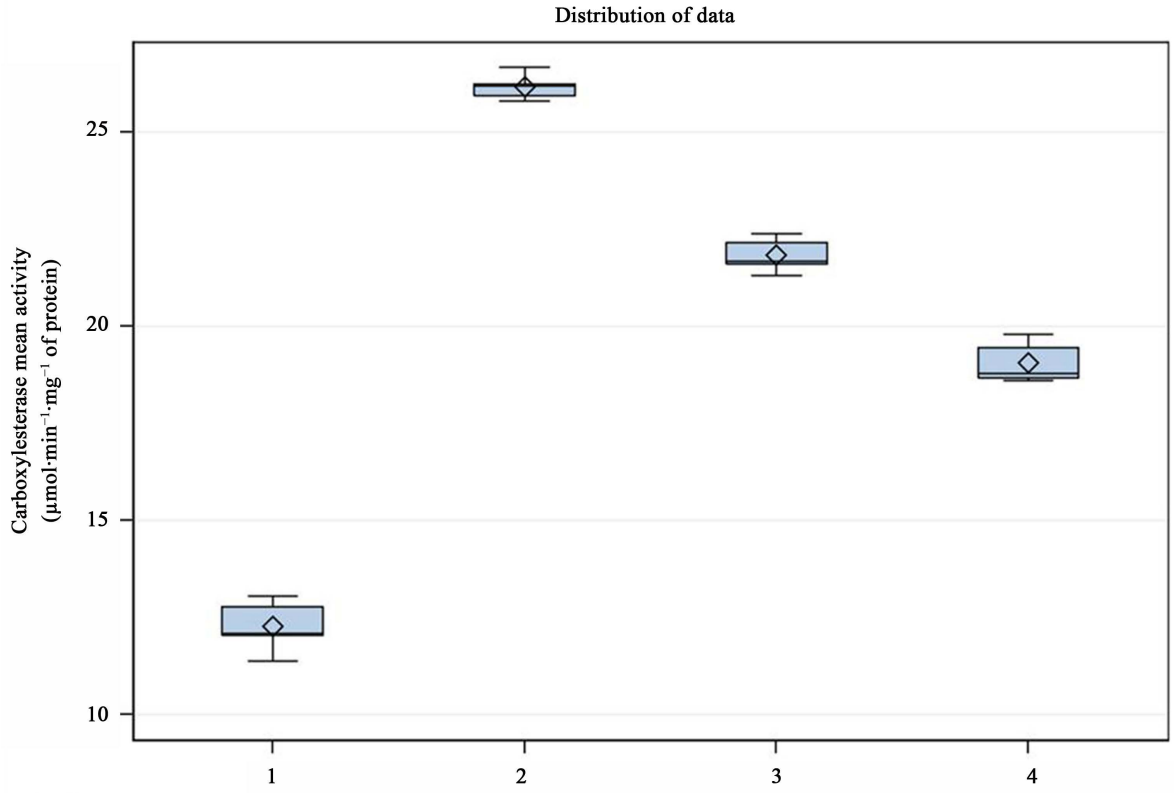
Table 1. Susceptibility of *Aphis gossypii* reared in the laboratory to dimethoate.

Generation	Lab susceptible strain LKS-1*				Lab susceptible strain LKS-2*			
	Slope ± SE	LC_{50} ($mg \cdot l^{-1}$) (95% FL)	Chi sq	df	Slope ± SE	LC_{50} ($mg \cdot l^{-1}$) (95% FL)	Chi sq	df
1	0.89 ± 0.11	5.81 (3.46 - 8.89)	3.75	8	1.03 ± 0.11	6.82 (4.43 - 9.92)	2.45	8
10	0.92 ± 0.11	5.59 (3.37 - 8.47)	2.65	8	1.05 ± 0.12	6.75 (4.40 - 9.77)	2.86	8
20	0.93 ± 0.11	5.25 (3.15 - 7.96)	1.78	8	1.03 ± 0.11	6.49 (4.19 - 9.47)	2.69	8
30	0.91 ± 0.11	5.02 (2.96 - 7.68)	2.00	8	1.01 ± 0.11	6.25 (3.99 - 9.17)	3.11	8
40	0.91 ± 0.11	4.87 (2.85 - 7.47)	2.07	8	1.01 ± 0.11	6.11 (3.89 - 8.97)	3.70	8
50	0.92 ± 0.11	4.90 (2.89 - 7.51)	2.80	8	1.00 ± 0.11	6.22 (3.96 - 9.14)	2.19	8
60	0.96 ± 0.11	4.93 (2.98 - 7.44)**	2.94	8	0.99 ± 0.11	6.01 (3.79 - 8.88)**	2.51	8

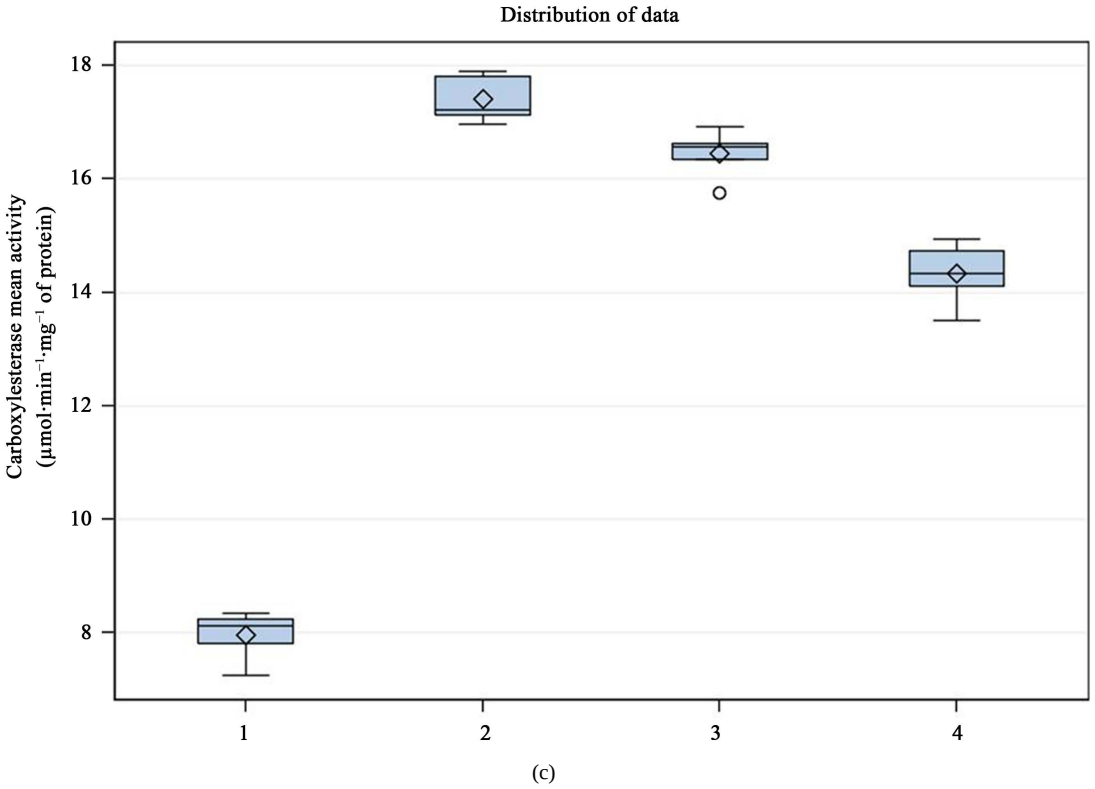
*Results of three replicates were averaged and made for five times (n = 15). FL: fiducial limits, df: degrees of freedom. **LC_{50} did not differ between susceptible strains based on overlapping FL.


Distribution of data
Carboxylesterase mean activity
(µmol·min⁻¹·mg⁻¹ of protein)
25
20
15
10
1
2
3
4

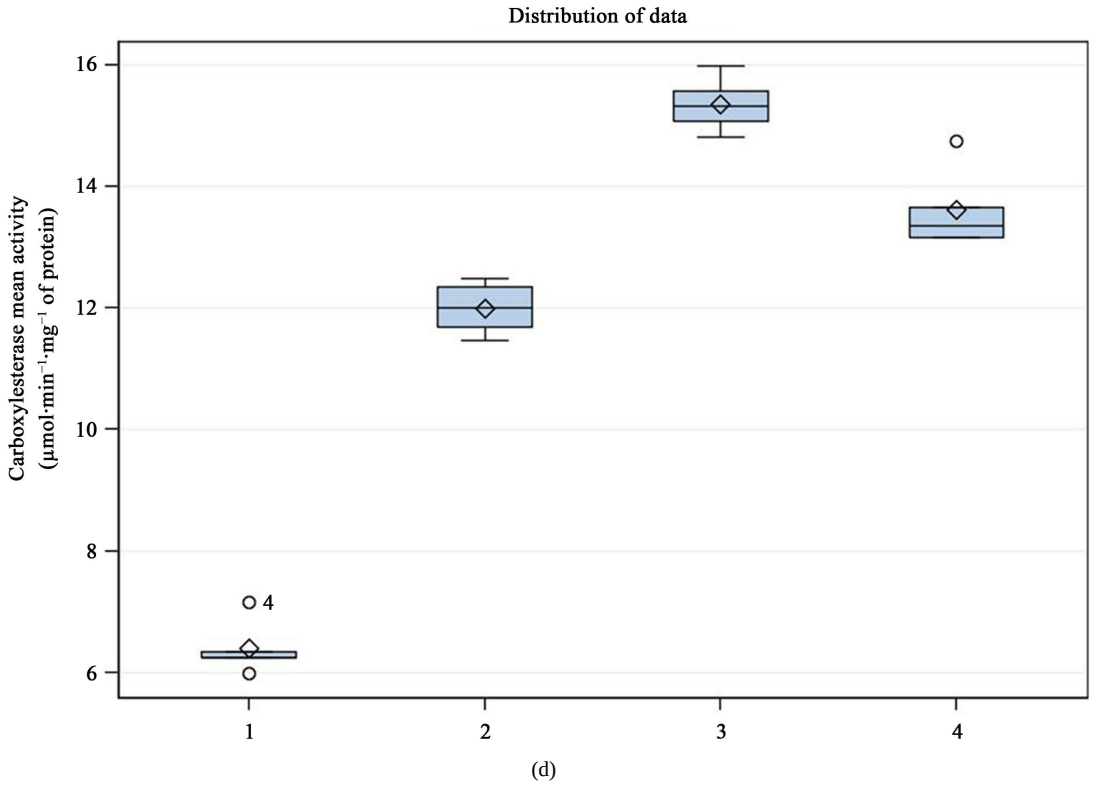
(a)


Distribution of data
Carboxylesterase mean activity
(µmol·min⁻¹·mg⁻¹ of protein)
25
20
15
10
1
2
3
4

(b)



(c)



(d)

Figure 1. Carboxylesterase activities to model substrates (a): *α*-NA, (b) *β*-NA, (c): *α*-NB, (d): *α*-NPr in susceptible and resistant *Aphis gossypii* strains is presented as distribution plots (Legends: 1: Mean enzyme activities of LKS-1 and LKS-2, 2: LKR-1; 3: LKR-2; 4: LKR-3). Bars above and below the means represent the standard errors of the mean.

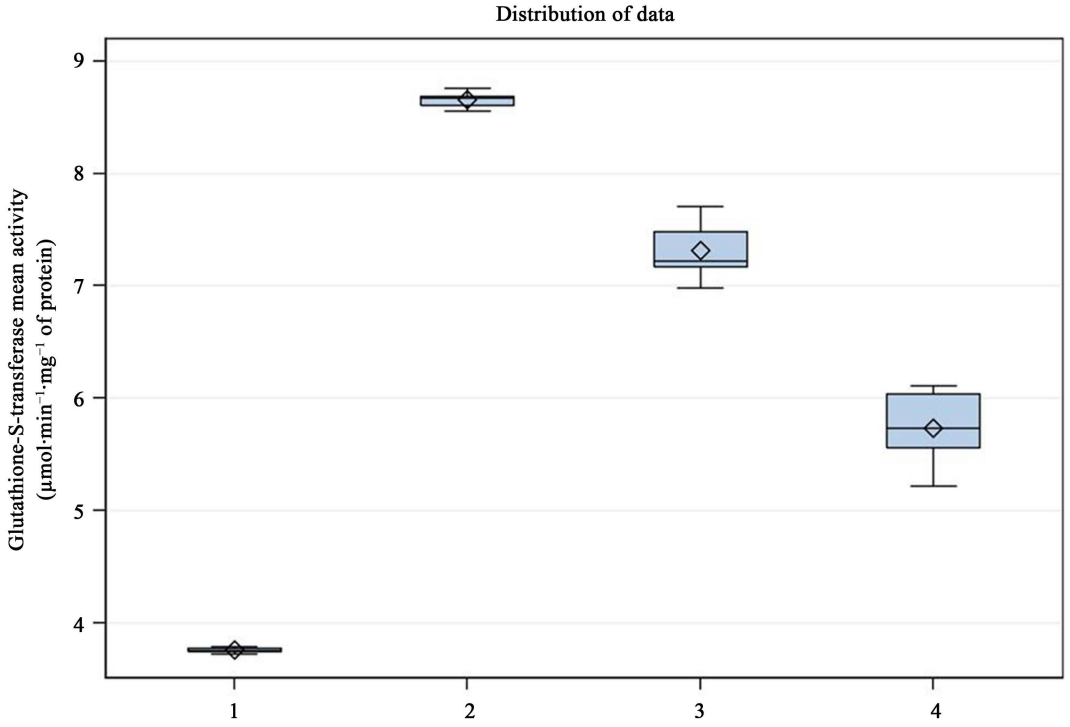


Figure 2. Distribution plot of Glutathione S-transferase activities in susceptible and resistant *Aphis gossypii* strains (Legends: 1: Mean enzyme activities of LKS-1 and LKS-2, 2: LKR-1; 3: LKR-2; 4: LKR-3). Bars above and below the means represent the standard errors of the mean.

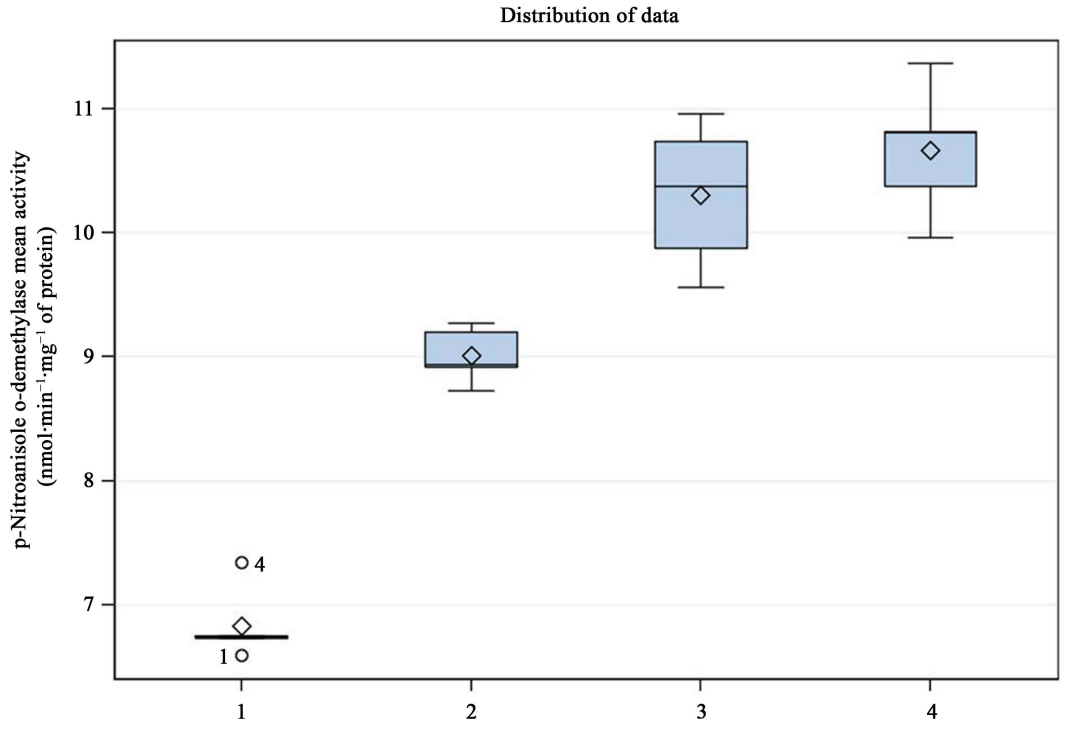


Figure 3. Distribution plot of *p*-Nitroanisole *o*-demethylase activities in susceptible and resistant *Aphis gossypii* strains (Legends: 1: Mean enzyme activities of LKS-1 and LKS-2, 2: LKR-1; 3: LKR-2; 4: LKR-3). Bars above and below the means represent the standard errors of the mean.

Table 2. Development of resistance in successive generations of *Aphis gossypii* strain (a) Lab resistant strain, LKR-1* (b) Lab resistant strain, LKR-2* and (c) Lab resistant strain, LKR-3* exposed to laboratory selection pressure with dimethoate.

(a)

Generation	Slope ± SE	LC_{50} (mg·l^{-1}) (95% FL)	Chi sq	RR
1	1.97 ± 0.23	387.57 (310.91 - 467.31)	2.77	71
5	3.16 ± 0.36	715.56 (630.18 - 805.23)	1.90	131
10	3.85 ± 0.43	918.71 (831.92 - 1012.34)	4.82	168
15	4.68 ± 0.55	1066.31 (975.30 - 1152.70)	1.06	195
20	5.97 ± 0.63	1235.40 (1157.06 - 1316.93)	3.88	225
25	5.41 ± 0.62	1364.94 (1274.37 - 1469.06)	2.16	249
30	4.73 ± 0.60	1476.89 (1366.46 - 1620.52)	4.05	270

(b)

Generation	Slope ± SE	LC50 (mg·l^{-1}) (95% FL)	Chi sq	RR
1	2.49 ± 0.28	496.38 (419.51 - 577.43)	3.75	91
5	3.26 ± 0.37	693.64 (611.68 - 778.24)	2.98	127
10	3.98 ± 0.44	878.47 (795.82 - 964.87)	3.92	160
15	4.27 ± 0.48	972.34 (885.92 - 1059.08)	2.68	177
20	5.22 ± 0.57	1137.45 (1053.88 - 1220.68)	1.63	208
25	5.20 ± 0.59	1269.11 (1181.06 - 1364.47)	3.58	232
30	5.03 ± 0.59	1326.27 (1233.00 - 1432.48)	3.40	243

(c)

Generation	Slope ± SE	LC50 (mg·l^{-1}) (95% FL)	Chi sq	RR
1	2.74 ± 0.33	339.83 (294.30 - 388.05)	2.78	62
5	3.83 ± 0.43	465.99 (421.79 - 514.02)	4.40	85
10	3.53 ± 0.43	544.32 (490.65 - 610.88)	4.09	99
15	2.77 ± 0.33	662.33 (574.00 - 761.48)	2.87	121
20	3.25 ± 0.38	817.62 (726.42 - 919.64)	3.95	149
25	3.97 ± 0.47	972.87 (881.20 - 1065.21)	4.15	178
30	4.96 ± 0.56	1146.49 (1059.15 - 1233.96)	1.36	210

*Results of three replicates were averaged and made for five times (n = 15). RR: resistance ratio.

Table 3. Lethal concentration of dimethoate tested on susceptible and resistant *Aphis gossypii* strains.

Populations	LC_{50} (mg·l^{-1}) (95% FL)[a]	LCR[b] (95% CI)[c]
LKS-1	4.93 (2.98 - 7.44)	-
LKS-2	6.01 (3.79 - 8.88)	-
LKR-1	1476.89 (1366.46 - 1620.52)	270.21 (267.82 - 272.60)*
LKR-2	1326.27 (1233.00 - 1432.48)	242.66 (240.50 - 244.81)*
LKR-3	1146.49 (1059.15 - 1233.96)	209.76 (207.82 - 211.70)*

[a]95% fiducial limits calculated using POLO-PC (LeOra Software 1987). [b]LCR-lethal concentration ratio = LC_{50} (Resistant strain)/LC_{50} (Susceptible strain). [c]95% confidence intervals (CI) estimated using LCR significance test, Robertson *et al*. 2007. * Indicates that LCR was significant (α = 0.05), Robertson *et al*. 2007.

Table 4. Carboxylesterase activity to model substrates in susceptible and resistant *Aphis gossypii* strains.

Samples	Mean activity (μmol·min^{-1}·mg^{-1} of protein) ± SEM[#]			
	α-NA	*β*-NA	*α*-NB	*α*-NPr
LKS-1	10.60 ± 0.24	11.41 ± 0.15	7.48 ± 0.12	5.86 ± 0.14
LKS-2	13.34 ± 0.11	13.14 ± 0.10	6.93 ± 0.07	8.43 ± 0.11
LKR-1	25.78 ± 0.27*	26.18 ± 0.06*	17.40 ± 0.07*	11.99 ± 0.08*
LKR-2	23.11 ± 0.36*	21.83 ± 0.08**	16.44 ± 0.08*	15.35 ± 0.08**
LKR-3	20.95 ± 0.26*	19.06 ± 0.09***	14.33 ± 0.10**	13.61 ± 0.12***

[#]Results of two replicates were averaged from protein preparations made on five times (n = 10). Means ± SEM followed by * in same column indicates that enzyme activity values were significantly different with LSD test ($P < 0.01$, ANOVA).

Table 5. Glutathione-S-transferase and *p*-Nitroanisole *o*-demethylase activities in susceptible and resistant *Aphis gossypii* strains.

Samples	GSTs Mean activity ± SEM[#] (μmol·min^{-1}·mg^{-1} of protein)	PNODs Mean activity ± SEM[#] (nmol·min^{-1}·mg^{-1} of protein)
LKS-1	3.13 ± 0.02	6.14 ± 0.12
LKS-2	4.38 ± 0.02	7.48 ± 0.08
LKR-1	8.66 ± 0.01*	9.01 ± 0.04*
LKR-2	7.31 ± 0.05**	10.30 ± 0.10**
LKR-3	5.73 ± 0.07***	10.67 ± 0.09***

[#]Results of two replicates were averaged from protein preparations made on five times (n = 10). Means ± SEM followed by * in same column indicates that enzyme activity values were significantly different with LSD test ($P < 0.01$, ANOVA).

3.3. Imidacloprid Toxicity Assays

The dimethoate resistant *A. gossypii* strains were checked for cross-resistance to imidacloprid (a novel neonicotinoid). Interestingly, toxicity bioassay data revealed that the LC_{50} values of dimethoate resistant strains for imidacloprid were significantly lower when compared with susceptible strains. The LCR at LC_{50} varied significantly based on their corresponding 95% confidence intervals (**Table 6**) suggesting that, the data is consistent with the possibility of negative cross-resistance to imidacloprid.

4. Discussion

This study revealed that *A. gossypii* has an innate ability to develop resistance to dimethoate under continuous selection that might be attributed to evolution of general resistance [46]. The three resistant strains selected against dimethoate showed varied resistance levels and the rate of resistance development increased rapidly with selection. However, there were no marked changes in the LC_{50} values of two susceptible *A. gossypii* strains to dimethoate when it was reared away from insecticide exposure in the laboratory. These results suggested that the parthenogenetic mode of reproduction in *A. gossypii* allowed rapid changes in resistance development to dimethoate over time [15]. The resistance development trend is in accordance with those studied in *A. gossypii* selected for imidacloprid in the laboratory [47] [48]. The highest resistance was recorded in LKR-1 strain followed by LKR-2 and LKR-3 strain suggesting that *A. gossypii* population collected from Bengaluru, Karnataka developed resistance to high concentrations of dimethoate followed by those collected from Mangalagiri and Guntur, Andhra Pradesh regions. This study reports a drastic increase in RR in 30 generations similar to RR of >40-fold obtained in imidacloprid selected *A. gossypii* strain after 45 generations [48]; 29,035-fold and 700-fold fenvalerate resistance obtained on cotton and cucumber plants respectively after 16 consecutive generations of selection [49]. RR of >2000× was observed in Hawaiian *A. gossypii* populations to oxydemeton-methyl (OP) [50]. Correspondingly, ≥1000× RR was reported in *A. gossypii* resistant to deltamethrin from China [51]. Warangal (Telangana) population of *A. gossypii* was reported to acquire 2.19-, 2.12- and 1.32-fold resistance to

Table 6. Lethal concentrations of imidacloprid tested on dimethoate susceptible and resistant *Aphis gossypii* strains.

Populations	Mean (mg·l^{-1}) ± SEM[a]					
	Slope ± SE	LC_{50} (95% FL)[b]	Chi sq	df	LCR[c] (95% CI)[d]	Negative cross resistance ratio[e]
LKS-1	1.11 ± 0.11	3.78 ± 0.10 (2.68 - 5.45)	3.15	8	-	-
LKS-2	1.13 ± 0.12	4.49 ± 0.08 (3.19 - 6.46)	2.61	8	-	-
LKR-1	1.38 ± 0.13	1.39 ± 0.04 (1.02 - 1.88)	3.06	8	0.337 (0.314 - 0.359)*	2.97
LKR-2	1.44 ± 0.14	1.62 ± 0.02 (1.21 - 2.17)	3.71	8	0.391 (0.370 - 0.412)*	2.56
LKR-3	1.37 ± 0.14	1.10 ± 0.05 (0.80 - 1.48)	2.92	8	0.265 (0.256 - 0.274)*	3.77

[a]Results of three replicates were averaged and made for five times (n = 15). [b]95% fiducial limits calculated using POLO-PC (LeOra Software 1987). [c]LCR-lethal concentration ratio = LC_{50} (Resistant strain)/LC_{50} (Susceptible strain). [d]95% confidence intervals estimated using LCR significance test, Robertson *et al*. 2007. [e]Negative cross resistance ratio = 1/LCR *Indicates that LCR was significant (α = 0.05), Robertson *et al.* 2007.

dimethoate at LC_{50} relative to the populations from Kurnool (Andhra Pradesh), Adilabad (Telangana) and Prakasam (Andhra Pradesh) districts respectively [12]. Thus *A. gossypii* appears to be the notorious pest capable of developing resistance to various chemicals under selection pressure worldwide. The main contribution of the present report is that laboratory selection of field-collected strains exhibiting dimethoate resistance leads to a rapid increase in resistance level, supporting the pre-existence of these metabolic resistance factors in the field-collected strains of *A. gossypii*.

Metabolic detoxification of pesticides is one of the physiological resistance mechanisms in aphid species exhibiting resistance to OP and carbamate pesticides [4] [20] [21]. Detoxification is conferred by esterases (CarE and AChE), GSTs and MFOs. These enzymes are very important in many OP-resistant insect pests [15] [52] [53]. In this study, biochemical results revealed that an additive interaction of increased CarE, GSTs and PNOD activities in *A. gossypii* is correlated with resistance to dimethoate indicating metabolic resistance is the one of the means of dimethoate detoxification in the resistant strains. Earlier studies report that an increased esterase activity conferred resistance to malathion (OP) in *Tetranychus kanzawai* [54] and *A. gossypii* [4]. An omethoate (OP) resistant *A. gossypii* strain was reported to have significantly higher CarE activity and 11-fold elevated activity towards α-NA and β-NA, 12-fold for α-NB and 10-fold for α-NPr was observed [21]. Similarly, a malathion *A. gossypii* resistant strain had 3.7-, 3.0-, 2.9- and 2.0-fold elevated CarE activity for α-NA, β-NA, α-NB and α-NPr substrates respectively [4]. Elevated amounts of CarE and GSTs were related to insecticide resistance in *Tetranychus urticae* [55] [56]. So far GSTs were not implicated in conferring resistance to OPs in aphids especially *A. gossypii* and thus this study is the first to provide an evidence for active participation of GSTs in dimethoate detoxification. An omethoate resistant strain of *A. gossypii* from China had 3.7-fold elevated cytochrome P450-mediated O-demethylase activity [14]. Further, 4 - 60 fold elevated levels of *O*-demethylase activity was reported in fenvalerate resistant Chinese population of *H. armigera* [33]. These enzymes are responsible for insecticide resistance through increased detoxification or sequestration or both as cited in literature [14] [15].

In contrast to enhanced levels of CarE, GSTs and PNOD, there was no significant change observed in the activities of acetylcholinesterase (AChE) in resistant strains. However, AChE inhibitory assay and molecular analysis showed that 50% of the enzyme activity in resistant strains was inhibited at significantly higher concentration of dimethoate and an altered AChE encoded by *Ace2* gene carrying three point mutations respectively [57]. This indicated AChE insensitivity in resistant strains. Thus, the decrease in the efficacy of neurotoxic substance like dimethoate could be attributed to induced metabolic detoxification by enzymatic systems (CarE, GSTs and MFOs) coupled with AChE insensitivity due to point mutations in AChE. It is evident from the results that two different resistance mechanisms operate simultaneously and contribute to combat the toxic effects of dimethoate in resistant strains. So far there are no reports that indicate the involvement of GSTs with CarE and PNOD in degradation and sequestration of OPs. This study is the first to report that a complex of multiple detoxifying enzymes *viz*., CarE, GSTs and PNOD nullify the adverse effects of OPs and thus contribute to confer resistance to dimethoate in *A. gossypii*.

Results also confirmed that South Indian populations of *A. gossypii* have an inherent ability to develop high levels of resistance to dimethoate under selection through activation of its detoxification system. Intensive use and misuse of dimethoate may be responsible for the rapid evolution of high-level resistance in *A. gossypii* in

these regions. The data suggest that efficacy of dimethoate has reduced over a period of time and rendered it to be unsuitable for management of *A. gossypii* in South India and this requires further investigations at field levels. However, newer chemical molecules such as neonicotinoids *viz*., imidacloprid and thiamethoxam could be incorporated into a rotation strategy to preserve their efficacy against melon aphid. Use of botanicals such as neem oil and mineral oil could be used for management of melon aphids and aphid transmitted viral diseases with little or no harm to natural enemies such as cocconellids and syrphids besides parasitoids [58].

Lower LC_{50} values to imidacloprid by dimethoate selected strains are an intriguing phenomenon. This indicated that dimethoate resistant strains were 2.97-, 2.56- and 3.76-fold sensitive to imidacloprid suggesting the possibility of negative cross-resistance. Similar cases were reported in pirimicarb resistant *A. gossypii* population being 2.5-fold more sensitive to bendiocarb than the susceptible strain [59] and dimethoate resistant *A. gossypii* population of Cameroon being 7.8- and 19.2-fold hypersensitive to carbosulfan and pirimicarb respectively [60]. Comparable results were obtained in pirimicarb resistant population of *M. persicae* being sensitive to carbofuran [61]. This implies that imidacloprid is less severely compromised by resistance mechanism(s) present in *A. gossypii* selected populations. These results will help promote efficient control of this pest and have major implications for successful implementation of resistance management strategies for *A. gossypii*. Since, there are no reports available that can report the biochemical mechanisms underlying dimethoate resistance that governs explosion of melon aphid populations by repeated control failures in India. This study was taken up with the purpose to gain more knowledge about possible resistance mechanisms in *A. gossypii*. This study aids in providing data to support the development of effective resistance management strategies for sucking pests, an arena which has so far not been attempted on a national level. IPM is largely successful if one has technology to manage insecticide resistance and this is all the more true for aphids where the dimensions are many for the stability of the agro-ecosystem.

Acknowledgements

The authors thank Director, Indian Institute of Horticultural Research, Bengaluru for encouragement and provision of facilities to carry out the research. Thanks are also due to Indian Council of Agricultural Research (ICAR), New Delhi for financial support through the Out Reach Programme on Management of Sucking Pests of Horticultural Crops. This work is a part of the Ph.D. thesis of the senior author, D. Lokeshwari.

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