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Effect of Sublethal Doses of Bifenthrin and Chlorpyrifos Administered Alone and in Combinations on Esterases of Stored Grain Pest, *Trogoderma granarium*

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ABSTRACT

Toxic effect of sub-lethal concentration (LC_{20}) of bifenthrin, chlorpyrifos and their combinations was studied on the specific activities of esterases in the 4th and 6th instar larvae of susceptible laboratory strain (Lab-S) and deltamethrin resistant population (GUW) of stored grain pest, *Trogoderma granarium*. After exposure to LC_{20} of bifenthrin, chlorpyrifos and their combinations of 3:1 and 1:3, the activities of total esterases, cholinesterase and carboxylesterase increased significantly in both the 4th and 6th instar larvae of Lab-S and GUW populations when compared with their respective controls. Acetylcholinesterase (AchE) activity was significantly decreased (28.94 and 42.42%) in the 4th instar larvae of Lab-S population after treatments with bifenthrin and bifenthrin: chlorpyrifos combination 3:1, respectively. After exposure to chlorpyrifos and bifenthrin: chlorpyrifos combination 1:3 the AchE activity was increased 45.24 and 64.28%, respectively. This increase was much more prominent in the 6th instar larvae *viz*., 93.95, 45.57, 182.52 and 132.61% following treatments with bifenthrin, chlorpyrifos, 3:1 and 1:3 combinations of two insecticides, respectively. In the resistant population this increase was 35.71, 48.10 and 75.61% in the 4th instar, respectively and 9.47, 28.17 and 60.29% in the 6th instar after chlorpyrifos, 3:1 combination and 1:3 combination treatments, respectively. This activity was decreased 63.99% in the 6th instar but increased in the 4th instar 13.36% after bifenthrin exposure. Arylesterase activity was declined after exposure to bifenthrin, chlorpyrifos and 3:1 combination in both the 4th instar (89.47, 64.33 and 36.65%, respectively) and 6th instar (84.49, 54.01, 4.28%, respectively) of Lab-S as well as in the 4th instar (77.73, 55.81 and 35.96%, respectively) of resistant population. This activity was upregulated (84.92, 141.86 and 192.88%, respectively) in the 6th instar of resistant population, following exposures to bifenthrin, chlorpyrifos and 3:1 combination. Whereas, this activity was increased in the 4th instar (53.22%) and 6th instar (124.60%) of Lab-S population as well as in the 4th instar (35.19%) and 6th instar (234.75%) of resistant population after treatment with 1:3 combination, respectively. The findings of current investigation suggests that insects upregulate the synthesis of esterases in general cholinesterase and carboxylesterase in response to exposure to insecticide to combat with the stressful conditions of exposure to toxic chemicals. The activity of the arylesterase, on the contrary is inhibited to variable extent after exposure to the insecticides.

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Authors' Contribution
AF performed experimental work, statistically analyzed the data and wrote this article. FRS and ARS designed and supervised the work and also reviewed the entire manuscript.

Key words
***Trogoderma granarium*, Bifenthrin, Chlorpyrifos, Synergistic combinations, Acetylcholinesterase, Arylesterase**

INTRODUCTION

Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) has tremendous economical significance. It has also been categorized as extremely destructive pest of stored products including cereals throughout the globe and Pakistan because of its voracious larval stages (Lowe *et al*., 2000; Ahmedani *et al*., 2007, 2009, 2011; Naeem, 2016; Khalique *et al*., 2018). In Pakistan it is usually controlled with deltamethrin spray as a pre storage treatment and fumigation of phosphine as a post storage treatment (Irshad and Iqbal, 1994; Tarakanov *et al*., 1994; Saxena and Sinha, 1995; Kumar *et al*., 2010). In several developing countries including Pakistan, cases of resistance development against insecticides like carbamates, organophosphates, pyrethroids and phosphine has further highlighted this pest as a major threat to economy (Chahal *et al*., 1991; Alam *et al*., 1999; Khalique *et al*., 2018). Use of commonly formulated insecticides mixtures like combinations of either pyrethroids, organophosphates or carbamates have been highly recommended to overcome wide variety of insect pests (Palumboa *et al*., 2001; Ahmad, 2009; IRAC, 2010). It has been documented in various studies that different components of mixture

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can disturb physiology of insect in many ways because of diverse mechanism of toxicity (Bynum *et al*., 1997; Pereira *et al*., 1997; Martin *et al*., 2003; Ali *et al*., 2003, 2007; Ahmad *et al*., 2009). In many species of insects and ticks one of the most important component of metabolic resistance system is esterase-based resistance mainly employed for the detoxification of insecticides containing ester moiety such as organophosphates and pyrethroids. However, the involvement of particular mechanism for the evolvement of resistance has not been discovered yet (Hemingway *et al*., 1993; Rosario-Cruz *et al*., 1997; Downs *et al*., 2000; Jamroz *et al*., 2000; Zhu *et al*., 2004). Arylestearse, acetylcholinesterase, cholinesterase and carboxylesterase are known as most important types of esterases through which detoxification of insecticide occurs (Ellman *et al*., 1961; Fournier and Mutero, 1994). Both acetylcholinesterase and cholinesterase have vital function in nervous system and neuromuscular junction such as conduction of nerve impulse (Ollis *et al*., 1992; Walsh *et al*., 2001). The hydrolysis of carboxylic esters into alcohol and free acid anion is caused by carboxylesterase (Cygler *et al*., 1993; Satoh and Hosokawa, 2006; Hosokawa *et al*., 2007). Sub-lethal concentration such as LC_{20} cause low mortality but significant physiological/ biochemical responses have been recorded in many studies for the understanding of biochemical basis of insecticide modes of action in insects (Saleem *et al*., 2000; Ali *et al*., 2011, 2014).

So, this study has been designed to evaluate the toxic effect of LC_{20} of bifenthrin, chlorpyrifos and their most effective synergistic combinations on the total esterase, carboxylesterase, cholinesterase, acetylcholinesterase and arylesterase for understanding the mechanism that 4th and 6th instar larvae of insecticide susceptible and deltamethrin resistant populations of *T. granarium* use to cope with the insecticidal stress. There is no report pertaining to the effect of bifenthrin, chlorpyrifos and their synergistic combinations on the activities of esterases with reference to stored grain insect pests including *T. granarium*.

MATERIALS AND METHODS

Rearing and maintenance of insect larvae

The methods of FAO (1986) and Riaz *et al*. (2014) were followed to maintain the populations of stored grain pest *T. granarium* in age wise homogeneous stock. The insecticide susceptible laboratory strain (Lab-S) population was provided by Dr. Syed Shahid Ali to the Department of Zoology, University of the Punjab, Lahore and had been reared without any contact of insecticide or fumigant for more than 17 years.

While the other population was brought from the wheat storing warehouse of Pakistan Agricultural Storage and Service Corporation Ltd. located in Gujranwala. In this warehouse deltamethrin had been utilized for more than 37 years to overcome the several species of stored grain insect pests including *T. granarium*.

To conduct toxicological and biochemical analysis, 4th and 6th instar larvae of Lab-S and Gujranwala (GUW) populations were used from the pure stock of each population. Both larval instars of GUW populations had moderate level of resistance against deltamethrin according to their resistance ratios (RRs) which was estimated based on the criteria of Mazzarri and Georghiou (1995). For deltamethrin (1.5% EC) the RRs against 4th and 6th instar larvae of GUW population were 5.36 and 5.82 folds as against to LC_{50} of 4th and 6th instar larvae of Lab-S population, respectively.

Insecticides used

Bifenthrin, 10% EC [2-methylbiphenyl-3-ylmethyl (Z)-(1RS,3RS)-3-(2-chloro-3,3,3-trifluoroprop-l-enyl)-2,2-dimethylcyclopropanecarboxylate] was purchased from the Four Brothers Agri Services Lahore, Pakistan under the trade name of Benq. Chlorpyrifos, 40% EC [O, O-diethyl-O-(3,5,6-trichlor-2-pyridyl) phosphorothioate] was purchased from Sky Agro Chemicals Lahore, Pakistan.

Determination of LC_{20}

The original liquid stock solutions of deltamethrin (1.5% EC), bifenthrin (10% EC) and chlorpyrifos (40% EC) were used to prepare their respective working stock solutions of 2000ppm using absolute acetone as diluent. These working stocks were further used to prepare combinations of 3:1 (bifenthrin: chlorpyrifos, v/v) and 1:3 (bifenthrin: chlorpyrifos, v/v). The concentrations of bifenthrin and chlorpyrifos and their combinations used against 4th and 6th instar larvae of Lab-S and GUW populations were as follow: 10, 40, 70, 100, 130, 160, 190, 220, 250, 280, 310, 340, 370, 400, 430, 460, 490, 520, 550, 580 and 610 ppm.

For determination of LC_{20} each concentration was administered according to the residual film method (Busvine, 1971; Saleem and Shakoori, 1989, 1990; Shakoori *et al*., 2004). Approximately 1ml of each concentration was applied on petri dish (9cm diameter) which was air dried to allow the formation of thin film of insecticide. Control petri dishes were prepared in the same way using 1ml absolute acetone. The entire bioassay was conducted in triplicate. Ten 4th and 6th instar larvae of Lab-S and GUW populations of *T. granarium* were introduced separately into each petri dish for 48 hrs at 30±2°C and 60±5% RH. Mortality of larvae in each concentration of the insecticide was recorded. Criteria of mortality was

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determined according to Lloyd (1969). The mortality data obtained was subjected to Probit analysis (Finney, 1971) using IBM SPSS software for the determination of LC_{20} values along with fiducial limits at 95% and values of slope (Tables I and II).

Experimental design

Almost 90 larvae for each biochemical test from the Lab-S population of 4th and 6th instar larvae were exposed separately in three replicates (30 larvae per replicate) to their respective sub-lethal concentration (LC_{20}) of bifenthrin (225.47 and 131.34ppm), chlorpyrifos (103.98 and 46.31ppm), 3:1 combination (28.64 and 8.88ppm) and 1:3 combination (13.88 and 0.16ppm), respectively for 48 hrs at 30±2°C and 60±5% RH. Similarly, the LC_{20} of bifenthrin (409.62 and 288.39ppm), chlorpyrifos (324.44 and 153.9ppm), 3:1 combination (133.31 and 87.61ppm) and 1:3 combination (80.03 and 51.81ppm) were used to expose 90 larvae (30 larvae per replicate) from the GUW population of 4th and 6th instars, respectively. In control groups, larvae from the both populations of 4th and 6th were exposed to absolute acetone in three replicates as described above. Subsequent to the exposure alive twenty larvae from each replicate were weighted separately prior to the preparation of their respective homogenates in suitable medium based on the criteria of protocol for estimation of different esterases.

Estimation of total esterase and carboxylesterase activities

After each treatment, twenty 4th and 6th instar larvae of Lab-S and GUW populations were homogenized by crushing in 2 ml of 0.1 M phosphate buffer (pH 7) at 4°C and used for the determination of total esterase activity. Likewise, larval homogenate for the determination of carboxylesterase activity was prepared at 4°C by homogenization of twenty 4th and 6th larvae of both populations in 2ml of 0.02 M phosphate buffer (pH 7) containing 10 µM eserine sulfate. Larval homogenates were centrifuged separately at 12000×g for 15 min at 4°C and clear supernatants were used for the estimation of total esterase and carboxylesterase activities according to Cao *et al*. (2008). The total activities of total esterase and carboxylesterase activities were calculated from standard curve of α-naphthol and expressed into mmole/min/mg of body wt.

Estimation of acetylcholinesterase activity

Estimation of acetylcholinesterase activity was performed according to the procedure of Devonshire (1975) by crushing twenty 4th and 6th instars larvae from each treatment in 2 ml of 0.1 M phosphate buffer (pH 8) at 4°C. The homogenate was used for estimation of acetylcholinesterase activity. The Beer-Lambert law was used to convert the absorbance of each sample into enzyme total activity units (µmole/min/mg of body wt.) using 13.6 mM^{-1} cm^{-1} extinction coefficient of TNB^{2-} (2-nitro-5-thiobenzoate ion) according to Worek *et al*. (1999).

Estimation of arylesterase and cholinesterase activities

Twenty larvae (4th and 6th instars) of both populations after each treatment were homogenized in 2 ml of 0.89% saline at 4°C. Supernatants were obtained by centrifugation at 3000×g for 30 min at 4°C. Clear supernatants were used for the estimation of arylesterase activity based on method of Haagen and Brock (1992) using extinction coefficient of phenol (1.31 mM^{-1} cm^{-1}) according to Beer-Lambert law. Total activity of arylesterase was expressed in µmole/min/mg of body wt. The above prepared supernatants were also used for the estimation of cholinesterase activity according to Rappaport *et al*. (1959). Then total cholinesterase activity was calculated from the standard curve prepared from cholinesterase purified from pooled serum and it was expressed in µg/30 min/mg of body wt. (Rappaport Units/mg of body wt.).

Statistical analysis

Data corresponded to effect of LC_{20} of bifenthrin, chlorpyrifos and their most effective combinations for the different esterases of 4th and 6th instar larvae of Lab-S and GUW populations was compared through Tukey's test after one-way analysis of variance (ANOVA) at 95% confidence limit for the determination of the significant difference between treatments at $p<0.05$ using IBM SPSS software. This data was represented as mean ± standard error of mean (S.E.M).

RESULTS

Both combinations were synergistic according to their relative toxic units (RTUs) calculated at LC_{50} level based on the criteria of Otitoloju (2001). The 3:1 combination showed 4.37 and 5.01 RTU against 4th and 6th instar larvae of Lab-S population whereas these values were 2.07 and 2.01 against 4th and 6th instar larvae of GUW population, respectively. Against 4th and 6th instar larvae of Lab-S population 1:3 combination showed RTU of 4.22 and 4.08 while these values were 4.00 and 2.84 against 4th and 6th instar larvae of GUW population, respectively.

Table III shows the effect of LC_{20} of bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination on different types of esterases of 4th and 6th instar larvae of Lab-S and GUW populations of *T. granarium* after 48 hours of exposure. In Figures 1-5, percentage changes in specific activities of esterases of 4th and 6th instar larvae

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Table I. LC_{20} values of bifenthrin and chlorpyrifos administered alone and in combination against 4th instar larvae of susceptible (Lab-S) and resistant (GUW) populations of *T. granarium*.

Treatments used	Ratio	Population	LC_{20} (ppm)	95% Fiducial limits	Slope ± SE	χ^2	P^*
Bifenthrin	1:0	Lab-S	225.47	196.12–307.88	0.609 ± 0.04	3.87	0.29
		GUW	409.62	390.84–504.18	0.75 ± 0.10	3.84	0.28
Chlorpyrifos	1:0	Lab-S	103.98	99.98–198.76	1.56 ± 0.07	4.87	0.84
		GUW	324.44	298.94–401.98	1.34 ± 0.01	2.06	0.99
Bifenthrin: Chlorpyrifos combinations	3:1	Lab-S	28.64	25.87–30.88	1.87 ± 0.03	3.84	0.28
		GUW	133.31	109.88–200.99	0.86 ±0.02	6.25	0.10
	1:3	Lab-S	13.88	11.09–15.19	1.15 ± 0.01	7.42	0.12
		GUW	80.03	78.13–85.92	1.43 ± 0.02	1.61	0.80

*Degree of freedom for all experiments, 19

Table II. LC_{20} values of bifenthrin and chlorpyrifos administered alone and in combination against 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations of *T. granarium*.

Treatments used	Ratio	Population	LC_{20} (ppm)	95% Fiducial limits	Slope ± SE	χ^2	P^*
Bifenthrin	1:0	Lab-S	131.34	100.31–201.14	2.21 ± 0.01	1.84	0.87
		GUW	288.39	201.11–301.08	3.05±0.04	0.28	1.00
Chlorpyrifos	1:0	Lab-S	46.31	41.14–50.69	1.00 ± 0.05	6.44	0.26
		GUW	153.90	143.77–198.09	0.92 ± 0.02	1.65	0.89
Bifenthrin: Chlorpyrifos combinations	3:1	Lab-S	8.88	5.16–11.17	2.02 ± 0.12	7.75	0.11
		GUW	87.61	82.10–97.00	1.63 ±0.02	3.58	0.73
	1:3	Lab-S	0.16	0.09–0.20	2.62 ± 0.04	12.74	0.01
		GUW	51.81	48.78–54.10	0.84 ± 0.01	4.34	0.36

*Degree of freedom for all experiments, 19

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of both population are shown with reference to their respective controls after each treatment.

Total esterase activity

The specific activity of total esterase was significantly increased 42.22, 75.66, 106.67 and 171.11% with respect to control in the 4th instar larvae of Lab-S population subsequent to treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination, respectively. Likewise, in the 6th instar larvae of Lab-S population significant increase in activity was 2250, 100, 364.29 and 171.43%, respectively.

After treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination specific activity of total esterase was significantly increased 71.67, 536.67, 848.33 and 251.67%, respectively with reference to control in the 4th instar larvae of GUW population. Similarly, in the 6th instar larvae of GUW population significant increase was 136.00, 56.00, 244.00 and 96.00%, respectively (Fig. 1 and Table III).

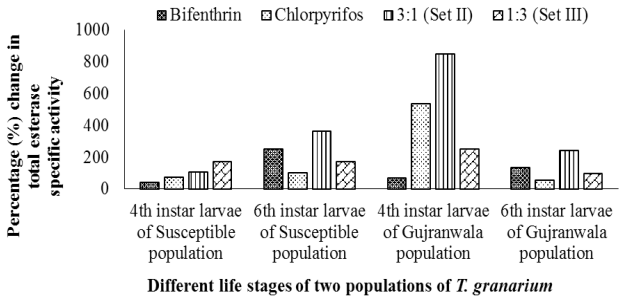

Fig. 1. Percentage changes in specific activity of total esterase of 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations with respect to control.

Carboxylesterase activity

The specific activity of carboxylesterase was significantly increased 65, 180, 275 and 375% with respect to control in the 4th instar larvae of Lab-S population subsequent to treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination, respectively. Likewise,

in the 6th instar larvae of Lab-S population significant increase in activity was 180, 360, 420 and 800%, respectively.

After treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination specific activity of carboxylesterase was significantly increased 104.35, 313.04, 486.96 and 743.48%, respectively with reference to control in the 4th instar larvae of GUW population. In the 6th instar larvae of GUW population significant increase was 161.54, 123.08 and 330.77% following treatments with chlorpyrifos, 3:1 combination and 1:3 combination, respectively but after bifenthrin exposure 38.46% upregulation was non-significant (Fig. 2 and Table III).

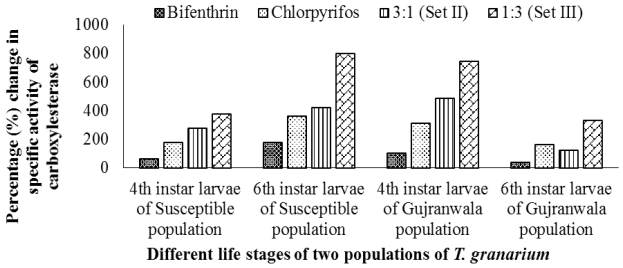

Fig. 2. Percentage changes in specific activity of carboxylesterase of 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations with respect to control.

Cholinesterase activity

The specific activity of cholinesterase was significantly increased 100.00, 225.75 and 171.67% with respect to control in the 4th instar larvae of Lab-S population subsequent to treatments with bifenthrin, 3:1 combination and 1:3 combination, respectively but after chlorpyrifos treatment upregulation of 27.47% in activity was insignificant. In the 6th instar larvae of Lab-S population significant increase in activity was 134.9, 88.64, 427.27 and 344.32% with reference to control subsequent to treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination, respectively.

After treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination specific activity of cholinesterase was significantly increased 67.44, 28.40, 119.14 and 90.90%, respectively with reference to control in the 4th instar larvae of GUW population. Similarly, in the 6th instar larvae of GUW population significant increase was 58.36, 50.99, 86.69 and 123.51%, respectively (Fig. 3 and Table III).

Acetylcholinesterase activity

In the 4th instar larvae of Lab-S population, the specific activity of acetylcholinesterase was significantly decreased 28.94 and 42.42% after treatments with bifenthrin and 3:1 combination respectively but after exposure to chlorpyrifos and 1:3 combination significant increase of 45.24 and 64.28% was recorded, respectively.

In the 6th instar larvae of Lab-S population the specific activity was significantly increased 93.95, 45.57, 182.52 and 132.61% subsequent to treatments with bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination, respectively. Similarly, significant increase was 13.36, 35.71, 48.10 and 75.61%, respectively in the 4th instar larvae of GUW population.

Whereas, in 6th instar larvae of GUW population the specific activity was increased significantly 9.47, 28.17 and 60.29% as a result of chlorpyrifos, 3:1 and 1:3 combinations exposures, respectively but bifenthrin exposure decreased the activity significantly 63.99% (Fig. 4 and Table III).

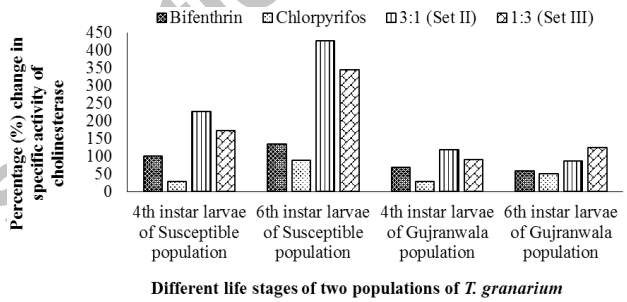

Fig. 3. Percentage changes in specific activity of cholinesterase of 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations with respect to control.

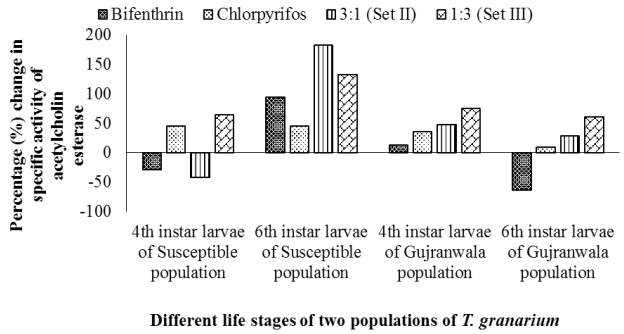

Fig. 4. Percentage changes in specific activity of acetylcholinesterase of 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations with respect to control.

Arylesterase activity

The specific activity of arylesterase was significantly decreased 89.47, 64.33 and 36.65% with respect to

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Table III. Effect of sublethal doses of bifenthrin and chlorpyrifos administered alone and in combination on the specific activities of different types of esterases in 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations of *T. granarium*.

Parameter*	Population	Life stage	Treatments				
			Control	Bifenthrin	Chlorpyrifos	3:1 combination	1:3 combination
Total esterase (IU/µg soluble protein)	Lab-S	4th instar	0.45±0.03^{A}	0.64±0.02^{B}	0.79±0.02^{C}	0.93±0.01^{D}	1.22±0.04^{E}
		6th instar	0.14±0.02^{A}	0.49±0.01^{B}	0.28±0.01^{C}	0.65±0.01^{D}	0.38±0.01^{E}
	GUW	4th instar	0.60±0.01^{A}	1.03±0.08^{B}	3.82±0.09^{C}	5.69±0.16^{D}	2.11±0.03^{E}
		6th instar	0.25±0.03^{A}	0.59±0.01^{B}	0.39±0.03^{C}	0.86±0.03^{D}	0.49±0.01^{E}
Carboxylesterase (IU/µg soluble protein)	Lab-S	4th instar	0.20±0.01^{A}	0.33±0.05^{B}	0.56±0.02^{C}	0.75±0.01^{D}	0.95±0.04^{E}
		6th instar	0.05±0.03^{D}	0.14±0.02^{C}	0.23±0.05^{B}	0.26±0.02^{B}	0.45±0.02^{A}
	GUW	4th instar	0.46±0.01^{A}	0.94±0.01^{B}	1.90±0.07^{C}	2.70±0.03^{D}	3.88±0.02^{E}
		6th instar	0.13±0.01^{D}	0.18±0.05^{D}	0.34±0.03^{B}	0.29±0.02^{C}	0.56±0.02^{A}
Cholinesterase (RU/µg soluble protein)	Lab-S	4th instar	2.33±0.08^{D}	4.66±0.11^{C}	2.97±0.03^{D}	7.59±0.26^{A}	6.33±0.13^{B}
		6th instar	0.88±0.09^{A}	2.06±0.04^{B}	1.66±0.10^{C}	4.64±0.26^{D}	3.91±0.04^{E}
	GUW	4th instar	6.48±0.19^{A}	10.85±0.07^{B}	8.32±0.23^{C}	14.20±0.13^{D}	12.37±0.16^{E}
		6th instar	3.53±0.05^{D}	5.59±0.04^{C}	5.33±0.03^{C}	6.59±0.13^{B}	7.89±0.10^{A}
Acetylcholin esterase (mIU/µg soluble protein)	Lab-S	4th instar	13.13±0.39^{A}	9.33±0.25^{B}	19.07±0.33^{C}	7.56±0.11^{D}	21.57±0.26^{E}
		6th instar	4.63±0.57^{A}	8.98±0.36^{B}	6.74±0.28^{C}	13.08±0.22^{D}	10.77±0.41^{E}
	GUW	4th Instar	17.67±0.73^{A}	20.03±0.52^{B}	23.98±0.96^{C}	26.17±0.79^{D}	31.03±0.55^{E}
		6th instar	15.94±0.38^{A}	5.74±0.91^{B}	17.45±0.42^{C}	20.43±0.87^{D}	25.55±0.91^{E}
Arylesterase (IU/µg soluble protein)	Lab-S	4th instar	5.13±0.40^{A}	0.54±0.03^{B}	1.83±0.11^{C}	3.25±0.18^{D}	7.86±0.34^{E}
		6th instar	1.87±0.11^{B}	0.29±0.05^{D}	0.86±0.04^{C}	1.79±0.13^{B}	4.20±0.19^{A}
	GUW	4th instar	16.88±0.84^{A}	3.76±0.16^{B}	7.46±0.30^{C}	10.81±0.50^{D}	22.82±0.63^{E}
		6th instar	5.90±0.22^{A}	10.91±0.44^{B}	14.27±0.34^{C}	17.28±0.42^{D}	19.75±0.71^{E}

*n, 3 (No. of replicates used in each experiment); Means followed by different letter in same row are significantly different with 95% confidence limit (Tukey's Test, $p < 0.05$).

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control in the 4th instar larvae of Lab-S population following treatments with bifenthrin, chlorpyrifos and 3:1 combination, respectively. But after exposure to 1:3 combination significant upregulation of 53.22% was recorded with reference to control.

In the 6th instar larvae of Lab-S population the specific activity was also significantly reduced 84.49 and 54.01% subsequent to treatments with bifenthrin and chlorpyrifos, respectively. But after exposure to 3:1 combination, decrease of 4.28% in arylesterase activity was not significant. However, reverse trend was observed after 1:3 combination treatment because significant upregulation of 124.6% was observed in activity.

Following bifenthrin, chlorpyrifos and 3:1 combination exposures the significant decrease in arylesterase specific activity was 77.73, 55.81 and 35.96%, respectively in the 4th instar larvae of GUW population. While opposite trend was observed subsequent to 1:3 combination as activity was increased significantly 35.19%.

Whereas, in the 6th instar larvae of GUW population the specific activity was increased significantly 84.92, 141.86, 192.88 and 234.75% as a result of bifenthrin, chlorpyrifos, 3:1 combination and 1:3 combination exposures, respectively (Fig. 5 and Table III).

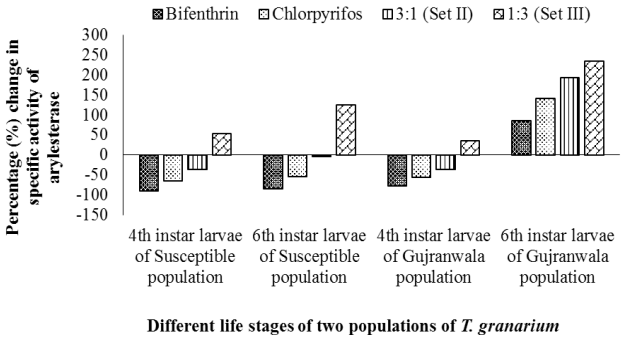

Fig. 5. Percentage changes in specific activity of arylesterase of 4th and 6th instar larvae of susceptible (Lab-S) and resistant (GUW) populations with respect to control.

DISCUSSION

In the current study toxic effect of bifenthrin, chlorpyrifos and their most effective synergistic combinations such as 3:1 and 1:3 was analyzed on different types of esterases of 4th and 6th instar larvae of Lab-S and GUW populations of stored grain pest *T. granarium* as data regarding interaction of pyrethroid and organophosphate in mixture form and their mode of toxicity on various physiological parameters is highly scarce.

Esterases are considered as an important class of detoxification enzymes in various insect pests like *Blattella germanica* (Anspaugh *et al*., 1994), *Culex quinquefasciatus* (Sahgal *et al*., 1994), *Tribolium castaneum* (Darvishzadeh and Sharifian, 2015) and *T. granarium* (Riaz *et al*., 2017). In current investigation the specific activities of total esterase, carboxylesterase and cholinesterase were increased after all treatments in both life stages of Lab-S and GUW populations as compared to control. But highest upregulation in activities was observed after treatments with 3:1 and 1:3 combinations of bifenthrin and chlorpyrifos. These results showed that larvae required increased activities of total esterase, carboxylesterase and cholinesterase to cope with the toxicity of synergistic combinations as compared to separate applications of bifenthrin and chlorpyrifos. Li *et al*. (2007) correlated the role of esterases with insecticide resistance in several insect species due to qualitative and/or quantitative alterations after exposure to insecticide. This kind of boosted intensities or altered activities of esterases either detoxify insecticide molecule by enzymatic breakdown or sequestration rather than quick hydrolysis (Haubruge *et al*., 2002; Oakeshott *et al*., 2005; Wheelock *et al*., 2005; Darvishzadeh and Sharifian, 2015). In acarine, dipterous and homopterous species detoxification of insecticides containing organophosphate group is mainly associated with increased activity of carboxylesterase (Devonshire, 1991). Levitin and Cohen (1998) also reported that upregulated cholinesterase activity in *Aonidiella aurantii* was due to organophosphate resistance. Sher *et al*. (2004) and Riaz *et al*. (2017) also reported upregulation in activities of total esterase, carboxylesterase and cholinesterase in *T. granarium* after exposure tophosphine and deltamethrin. They correlated such upregulated activities of esterases with the development of insecticide tolerance.

In 4th and 6th instar larvae of Lab-S population and 4th instar larvae of GUW population the arylesterase activity was decreased with respect to control after treatments with 3:1 combination, chlorpyrifos and bifenthrin. But reverse trend was observed after 1:3 combination treatment as activity was increased. In many studies it has been observed that organophosphate in mixture with pyrethroids, impede the enzymes (monooxygenases and esterases) involved in metabolic detoxification in various species of insect pests (Bryne and Devonshire, 1991; Martin *et al*., 2003; Montella *et al*., 2012). Saleem and Shakoori (1986) also described the reduction in several enzyme activities and correlated them mainly with enzyme inhibition or activation of rapid catabolic process that is involved in detoxification of insecticidal harmful effects. Same type of rise and fall in esterases level and activities has also been reported by Tang *et al*. (1993) during their investigation on resistance profile of *Cavariella salicicola* to pyrethroid and organophosphate (OP) insecticides.

However, in 6th instar larvae of GUW population arylesterase activity was increased after each treatment as compared to control. Mujeeb and Shakoori (2012a) reported same kind of trend in population of *Tribolium castaneum* collected from the warehouses of Karachi (Pak strain) as arylesterase activity in 4th instar larvae was reduced but 6th instar larvae had upregulated activity following fury treatment (synthetic pyrethroid). Mujeeb and Shakoori (2012b) reported reduction in arylesterase activity in all the developmental stages of different populations of *Tribolium castaneum* subsequent to exposure topirimiphos-methyl (organophosphate). It has been suggested that variability in activities of esterases is dependent on food, quality of food, sex, strain, hormone, developmental stage, environmental conditions and many other aspects. It has been also observed from previous studies that high level of polymorphism in genes of esterases facilitate adaptive flexibilities in several insect species (Devorshak and Roe, 1999; Villatte and Bachmann, 2002; Li *et al*., 2005; Baffi *et al*., 2007). In 4th and 6th instar larvae of Lab-S and GUW populations, upregulated acetylcholinesterase activity was observed after exposures of chlorpyrifos and 1:3 combination with respect to control. But bifenthrin and 3:1 combination treatments upregulated acetylcholinesterase activity only in 6th instar larvae of Lab-S population and 4th instar larvae of GUW population with respect to control. Results showed that chlorpyrifos and 1:3 combination (bifenthrin: chlorpyrifos) were inducers for acetylcholinesterase activity in both life stages of two populations. Stumpf *et al*. (2001) associated the resistance in the two-spotted spider mite *Tetranychus urticae* with insensitive acetylcholinesterase against organophosphorus acaricides *viz*., demeton-S-methyl, ethyl paraoxon, chlorpyrifos oxon and the carbamate carbofuran. Correlation between organophosphate resistance and upregulated esterase activity due to amplification of detoxifying genes has been recognized in several species of insects (Cuany *et al*., 1993; Jayawardena *et al*., 1994; Callaghan *et al*., 1998). In this study bifenthrin and 3:1 combination (bifenthrin: chlorpyrifos) were repressing

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acetylcholinesterase activity in 4th instar larvae of Lab-S population. While 6th instar larvae of GUW population had repressed acetylcholinesterase after only bifenthrin treatment with respect to control. Mujeeb and Shakoori (2012a) documented in *T. castaneum* that the toxicity of fury decreased the acetylcholinesterase activity significantly in all developmental stages of the three strains excluding 15 days old beetles of Pak and CTC-12 strains.

CONCLUSION

From the biochemical analysis it has been conclusive that both susceptible and deltamethrin resistant 4th and 6th instar larvae of *T. granarium* defended stress full conditions due to toxicities of bifenthrin, chlorpyrifos and their most effective 3:1 and 1:3 combinations at sub-lethal level (LC_{20}) by the induction of total esterase, carboxylesterase and cholinesterase. The outcomes of the current research can be proved significant from the point of understanding of mode of action of bifenthrin, chlorpyrifos and their most effective combinations in susceptible and resistant populations of 4th and 6th instar larvae of *T. granarium* that may provide information for the resistance management in resistant insect pests.

Statement of conflict of interest

The authors have declared no conflicts of interest.

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