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BIOCHEMICAL EFFECTS OF SUBLETHAL DOSES OF PHOSPHINE ON THE SIXTH- INSTAR LARVAE OF SUSCEPTIBLE AND RESISTANT POPULATIONS OF *Trogoderma granarium* EVERTS (COLEOPTERA: DERMESTIDAE)

Asma Naeem[1,*] and Sayeda S. Ahmed[2]

[1]Biochemistry and Toxicology Research Lab., Department of Zoology, University of the Punjab Lahore Pakistan;
[2]Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University, Giza, Egypt.
[*]Corresponding author's e-mail: talib.asma@gmail.com

Khapra beetle, *Trogoderma granarium* Evertsis a serious stored grain pest throughout the world. Misuse of pesticides and fumigants to control this pest is the major cause of resistance development. This study was planned to investigate susceptible and resistant populations of *T. granarium* by determining the LC_{50} doses and biochemical differences in the susceptible and resistant populations. For this purpose, two different populations i.e. Lahore (Lhr) and Khanewal (Khw) collected from various regions of Punjab province of Pakistan. Phosphine was generated in laboratory by FAO method. The LC_{50} values of Phosphine were determined against 6th instar larvae of these two populations of *T. granarium* in air tight glass desiccators. Following collection, they were transferred to lab and used to develop pure lab culture at 30±1°C and 65±5 R.H. of these populations used in this study. Mortality was calculated and subjected to probit analysis described. The LC_{50} of Phosphinefor 6th instar larvae was 8.4ppm in Lhr population while in Khw LC_{50} was 5.6ppm. On the basis of LC_{50} results it was concluded that Lhr was the resistant population. Toxicity of three doses (LC_{10}, LC_{20}, LC_{30}) of Phosphine used by analyzing the activities of the enzymes acid phosphatase (AcP), alkaline phosphatase (AkP), amylase, alanine aminotransferases (ALAT), aspartate aminotransferase (ASAT), lactate dehydrogenases (LDH), Isocitrate dehydrogenases (ICDH), cholinesterase (ChE), protease, aminopeptidase and trehalase. in T. *granarium*.The ASAT, aminopeptidase, trehalase and protease were increased in resistant population. Enzymes which increase in susceptible population are AcP, AkP, ALAT, LDH and ICDH. This study will help in understanding the biochemical basis of Phosphine resistance in *T. granarium* andprovide information for improving pests control programs.

Keywords: *Trogoderma granarium*, Enzymes, Phosphine resistance, Toxicity, Pest control.

INTRODUCTION

Stored grain pests are causing a huge damage to stored productsthroughout the world including Pakistan (Sherma, 1989; Hasan *et al.,* 1996; Ahmed *et al.,* 2006; Phillips and Throne, 2010; Nadur *et al.,* 2015). The major types of pests associated with stored grain products are Lesser grain borer *Rhyzopertha dominica*(Fabricius), Granary weevil *Sitophilus granarius*(Linnaeus), Saw-toothed grain beetle *Oryzaephilus surinamensis*(Linnaeus), Flat grains beetle *Cryptolestes* spp., Confused flour beetle *Tribolium confusum*Jacquelin du Val, Red flour beetle *Tribolium castaneum* (Herbst), Indian meal moth *Plodia interpunctella*(Hübner), Mediterranean flour moth*Ephestiakuehniella* Zeller, Cadelle *Tenebroidesmauritanicus* (Linnaeus), Ware house beetle *Trogoderma variable*Ballion and Khapra beetle *Trogoderma granarium*Everts.

In Indo-Pakistan, Khapra beetle *T. granarium* is one of the major pests of stored grains especially on wheat, it feeds directly on grains (Ram and Singh,1996; Pasek, 1998; Ali *et al.,* 2012). Various recommended pesticides e.g, organochlorines,organoph osphates, carbamates and pyrethroids have been used to reduce its population (White and Lessch, 1995; Lessard *et al.,* 1998; Hargreaves *et al.,* 2000). The continuous and indiscriminate use of above chemical pesticides led to failures in controlling this pest in different developing countries as well as in Pakistan (Chahal et al., 1991; Alam *et al.,* 1999). Khapra beetle is one of 100 worst invasive species because its larvae can go in diapause for long period of time, hiding themselves in cracks and crevices where insecticides cannot reach easily (Lowe *et al.,* 2000; Mujeeb *et al.,* 2011).

Fumigants are better agent than pesticides for such type of insects control due to the following reasons: i) they are toxic for all pests ii) fumigant can reach to that places where powder or other chemical insecticide cannot reach, iii) fumigants do not leave harmful residues so they can

applicable near food, iv) it is rapid method of controlling pests, v) fumigants are less expensive (Banks, 2012),vi) the mortality rate is huge in insect that treated with fumigant because it reaches the respiratory system of insect through spiracles (Aggarval, 1988). Methyl bromide is associated with depletion of the ozone layer (WMO, 1995; Sekhon *et al.,* 2010; Nath *et al.,* 2011; Tumambing and Dikin, 2013). Phosphine gas has been used worldwide as a famous fumigant for more than half a century for the protection of stored grains from the infestation of insects (Chaudhry, 2000; Lorini *et al.,* 2007).

However, widespread resistance to Phosphine has emerged in several species of stored-product insects in many countries in which Phosphine control failure has been detected (Chaudhry, 2000; Collins *et al.,* 2005; Lorini and Collins, 2006; Pimentel *et al.,* 2007, 2010; Collins, 2009). Several strong Phosphine-resistant stored-product insects have been found in Morocco (Benhalima *et al.,* 2004), Brazil (Ansell *et al.,* 1990; Mills and Athie, 2001; Athie and Mills, 2005), Vietnam (Bui, 1999), China (Yan *et al.,* 2004; Cao, 2006), Australia (Valmas and Ebert, 2006) and Pakistan (Ahmedani *et al.,* 2007; Mujeeb, *et al.,* 2011; Stevens *et al.,* 2011; Kaur *et al.,* 2012; Kaur *et al.,* 2013; Ridley *et al.,* 2012; Chen *et al.,* 2015) Phosphine Resistance has also been reported in theses notorious stored grain pests species, including *R.dominica* (F.), *Sitophilus oryzae* (Linnaeus), *Sitophiluszeamais* (Motschulsky), *T. castaneum* (Herbst) *T. granarium* Evert, *O. surinamensi* (L.), *Cryptolestes ferrugineus* (Stephens), *Liposcelis bostrychophila* (Badonnel), *L. entomophila* (Enderlein)and *L. decolor* (Pearman) (Leong and Ho,1994; Nayak *et al.,* 2002, 2003;Cao *et al.,* 2003; Athie and Mills, 2005; Nayak and Collins, 2008; Jagadeesan *et al.,* 2012; Schlipalius *et al.,* 2008;Collins, 2009; Pimentel *et al.,*2010; Ridley *et al.,* 2012; Kaur *et al.,* 2015;).

In Pakistan, only few reports are available on the impact of fumigant especially phosphine on stored grain insect pests (Hussain *et al.,* 2005; Mujeeb, *et al.,* 2011; Nighat, *et al.,* 2011). No work on khapra beetle *T. granarium* has ever been done on the basic biochemical and metabolic changes in susceptible and resistant populations causing extensive damage to stored grain in godowns in Pakistan. This study has been planned to evaluate the effect of fumigation by phosphine on the intermediary metabolism of *T. granarium* to determine the biochemical differences in susceptible and resistant populations.

MATERIALS AND METHODS

Fresh culture was collected from wheat godowns of Lahore(Lhr)and Khanewal(Khw) citylocated in Punjab Province of Pakistan. Farmers however told that Lahore farm houses had never been properlyfumigated with phosphine due to the lack of trained staff, gas tightness of the fumigated space and inadequate management has led to repeated fumigations to protect that stored areas from attackof insect pests.

Another sample collected from Khanewal go downs where they use seldom phosphine. The wheat samples containing T.granarium (Everts) were collected in sterilized plastic bags and brought to laboratory for study.

***Maintenance of culture*:** The master cultures of *T.granarium* (two populations) were maintained in temperature and humidity controlled room at 35±1°C and 65±5% RH (Riaz*etal*.,2014; Shakoori*etal*.2016). Apurehomogeneous stock of each population was developed in the culture room of Biochemistry and toxicology laboratory of Zoology department, University of Punjab. Pakistan.

Crushed wheat was used as a supporting medium. Wheat was initially fumigated with phosphine to kill the insects if any present. Following fumigation, wheat was spread in fresh air for 4-5 h. The wheat was placed in oven overnight at 60°C, and then it was shifted into sterilized jars for culture rearing. The300ml glass jam jars were filled $1/4^{th}$ with wheat and 50 adult beetles of *T.granarium* were added inside it. The jars were covered with muslin cloth to prevent escape of beetles and entry other small organisms. Adult beetles were left in the culture medium for 5-6 days to ensure egg laying. By using separating sieve and camel hair

Brush dead beetles were discarded and flour containing eggs were separated. The eggs developed into adult beet levialarval and pupal period. These adult beetles were again transferred ton ext jars for continuity of the culture and homogeneous stock was maintained

For further studies. From homogeneous stock of each population 6^{th} instar larvae (obtained after 42±1 days) were used to record LC_{50} and other toxicological data.

Toxicants Used :Generic name of this chemical isphosphine while hydrogen phosphide and phosphorus trihydride are the common names of phosphine gas.The EPA chemical code of this insecticide is 066500.It belongs to Inorganic Phosphine Family.Empirical Formula:PH3 (CAS #: 7803-51-2). Chemical characteristics of this toxicant are given below:

Appearance	Colorless gas
Density	1.379 g/l, gas (25 °C)
Melting point	−132.8 °C (−207.0 °F; 140.3 K)
Boiling point	−87.7 °C (−125.9 °F; 185.5 K)
Solubility in water	31.2 mg/100 ml (17 °C)
Viscosity	1.1 x 10^{-5} Pa s

For farm use, pellets of aluminium phosphide, calcium phosphide, or zinc phosphide release Phosphine upon contact with atmospheric water. These pellets also contain agents to reduce the potential for ignition or explosion of the released phosphine. Phosphine is the only widely used, cost-effective, rapidly acting fumigant that does not leave residues on the stored product.

***Procedure adopted*:** For determination of LC_{50} against *T.granarium*, phosphine was generated from aluminium phosphide in the laboratory. Commercially available aluminium phosphide (AlP) pellets containing (approximately0.2g) are recommended as the most suitable source of phosphine(PH_3).

***Generation of phosphinegas*:** Phosphine was generated in the laboratory according to the technique given in FAO plant protection bulletin (1975). All procedure for phosphine generation was carried out in a fumehood.

Administration of phosphine: All glass vacuumdesiccators were used for phosphine administration to insects.The volume of desiccators was measured to evaluate the dose volume of phosphine. The lid of the desiccators was covered with rubber sheet. Athin layer of grease was applied on the edge of the lid to akeitairtight. Saturated solution of sodium nitritein Petridish was placed to maintain the RH65±5%. Aceramic plate with holes was placed over the bottom narrow compartment of desiccators to place the insect vials with holed lid.

The first thing to do for LC_{50} determination was the generation of Phosphine gas, which was done according to the technique given in FAO method (Bulletin,1975). Phosphine was generated from aluminiumphosphide tablets, collected over acidified water. Three glass vials(5gm), containing twenty healthy larvae of 6th instar of *T. granarium* in each, were placed in the desiccators. Gas was injected into desiccators with Hamiltonmicrosyringe through a rubber septum fitted on the desiccator lid. The PYREX desiccators were kept in the lab at 30±1 °C and 65±5 % R.H. for 20 h after which observations on mortality were made.

***Mortality assessment*:** After 20 hours, desiccators were opened and insect vials were taken out. The 6thinstarlarvaeweretransferredtoseparatecrushedwheatmediu mandmaintainedat30±1°Cand60±5%RH. Mortality of 6thinstarlarvaewasassessedafter48hoursfromtheendoftheexpo sureperiod.Lloyd(1969) criterion wasfollowed "insect judged to be dead when the pressure from abrush failed toreproducearesponse".The%mortalitywascorrectedbyAbbot 'sformula(Abbot, 1925).

Data was analyzed by the method outlined by Busvine (1971) and described by Finny (1971). Each treatment was repeated four times. Then the mortality data was subjected to logit analysis using POLO-PC (LeOra Software, 1987) to estimate different lethal concentrations up to LC_{90} and confidence limitand regression lines (in ppm Phosphine) for 6th instar larvae of *T. granarium*. Mortality at different concentrations, used to estimate the concentration-mortality curves.

Biochemical Analysis: About 90 larvae were homogenized in 0.89% saline with a help of motor driven glass homogenizer under cold conditions. The homogenate was centrifuged at 4200xg for 45 min.The supernatant thus obtained was used for the estimation of various enzyme activities like acid phosphates (AcP; orthophoshoric monoester phosphohydrolase, acid optimum, EC:3.1.3.2) activity according to Andersch and Szcypinski (1947); alkaline phosphates (AkP; orthophosphoric monoester phosphohydralase alkaline optimum EC: 3.1.3.1) activity as mentioned in Bessey *et al.* (1946); lactate dehydrogenase (LDH; L-lactate NAD: oxidoreductase; EC: 1.1.1.27) activity by a method based on Cabaud and Wroblewski (1958); isocitrate dehydrogenase (ICDH); Threo-Ds-isocitrate: NADP: oxidoreductase, EC: 1.1.1.42) activity by a procedure described by Bell and Baron (1960); Asparatate aminotransferase (ASAT: L-asparatate: 2-oxoglutrate aminotransferase, EC: 2.6.1.1 and alanine aminotransferase E: 2.6.1.2) and alanine aminotransferase (EC: 2.6.1.2) activities according to Reitmann and Frankel (1957); cholinesterase ChE: acetylcholine acetylhydrolase, EC: 3.1.1.7) cholinesterase ChE: acetylcholine activity according to Rappaport *et al.* (1959); amylase (1,4-D glucan, glucanhydrolase, EC: 3.2.1.1) activity according to the procedure described in Wootton and Freeman (1982); trehalase activity by the procedure described by Dahlqvist (1966); Protease activityby the method of Yasunobu and Mcconn, (1970).

RESULTS

The calculated LC_{50} Values of Khw 5.6 ppm and Lhr 8.4 ppm clearlyindicated that Lhr is resistant population whileKhw is susceptible population.

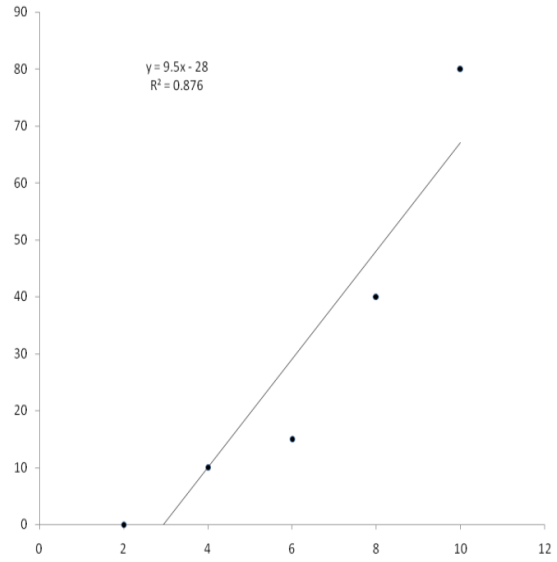

Figure1: Mortality curve of 6th instar larvae of *Trogoderma granarium (Lhr)*againstphosphine.

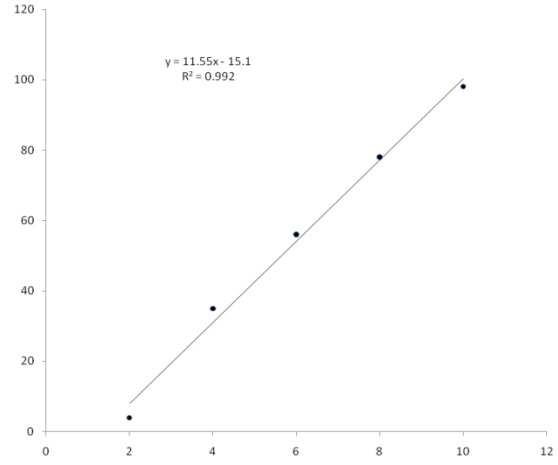

Figure 2: Mortality curve of 6th instar larvae of *Trogoderma* granarium *(Khw)* againstphosphine.

Table 1: Effects of Phosphine on some enzyme activities of 6th instar larvae of Lhr Population *T. granarium*.

Parameters	Control	Phosphine treatment		
	n=4	10 ppm n=4	20 ppm n=4	30 ppm n=4
AcP (IU/mg)	* 0.684 ±0.001 a	0.602 ±0.002 ab	0.585 ±0.002 bc	0.545 ±0.002 c
AkP (IU/mg)	0.404 ±0.0177 b	0.366 ±0.0186 c	0.277 ±0.0187 d	0.455 ±0.018 a
Amylase (SU/mg)	0.119 ±0.001 a	0.113 ±0.002 ab	0.117 ±0.002 b	0.105 ±0.002 c
ALAT (IU/mg)	1.240 ±0.0354 a	1.180 ±0.0183 b	1.210 ±0.0422 bc	1.190 ±0.0332 c
ASAT (IU/mg)	0.950 ±0.0177 c	1.180 ±0.0186 b	1.350 ±0.0187 a	1.480 ±0.018 a
LDH (IU/mg)	8.521 ±0.0296 a	8.204 ±0.0112 b	8.421 ±0.009 a	8.022 ±0.0314 b
ICDH (SU/mg)	58.23 ±0.1 a	46.62 ±0.2 b	33.34 ±0.1 c	23.23 ±0.1 d
ChE (SU/mg)	5.530 ±0.0066 a	5.500 ±0.0032 a	5.480 ±0.0042 b	5.470 ±0.0046 b
Protease (IU/mg)	22.520 ±0.2573 a	21.420 ±0.3718 b	21.95 ±0.3721 b	23.120 ±0.5909 a
Aminopeptidase (IU/mg)	0.615 ±0.0187 b	0.712 ±0.0234 a	0.428 ±0.0252 c	0.643 ±0.0222 b
Trehalase (IU/mg)	0.450 ±0.0066 c	0.530 ±0.0032 b	0.560 ±0.0042 a	0.590 ±0.0046 a

* Mean ± SEM

The values in a row having different superscript (ab) are significantly different at 0.05 significant level according to DRMD.

Definitions of enzyme units: IU, international unit, the amount of enzyme, which under defined assay conditions, will catalyze the conversion of 1µ mol of substrate per minute; mSU, the amount of enzyme digesting 5000 mg of starch in the experimental conditions used here.

Table 2: Effects of Phosphine on some enzyme activities of 6th instar larvae of Khw population *T. granarium*.

Parameters	Control	Phosphine treatment		
	n=4	10 ppm n=4	20 ppm n=4	30 ppm n=4
AcP (IU/mg)	* 0.679 ±0.0069 b	0.698 ±0.0063 b	0.715 ±0.0108 ab	0.736 ±0.0075 a
AkP (IU/mg)	0.401 ±0.0219 c	0.522 ±0.0127 b	0.545 ±0.0184 a	0.372 ±0.0162 d
Amylase (SU/mg)	0.112 ±0.003 b	0.102 ±0.003 c	0.128 ±0.003 a	0.105 ±0.003 c
ALAT (IU/mg)	1.170 ±0.0296 d	1.220 ±0.0112 b	1.290 ±0.009 a	1.180 ±0.0314 c
ASAT (IU/mg)	0.928 ±0.0219 a	0.453 ±0.0127 c	0.430 ±0.0184 c	0.765 ±0.0162 b
LDH (IU/mg)	8.240 ±0.0296 d	8.550 ±0.0112 c	8.830 ±0.009 b	8.940 ±0.0314 a
ICDH (SU/mg)	58.64 ±0.1 d	76.32 ±0.1 c	82.36 ±0.1 b	98.36 ±0.1 a
ChE (SU/mg)	5.412 ±0.008 a	5.380 ±0.0046 b	5.340 ±0.0072 b	5.290 ±0.0093 b
Protease (IU/mg)	22.720 ±0.4294 bc	24.350 ±0.3483 a	21.250 ±0.4028 c	23.960 ±0.3695 b
Aminopeptidase (IU/mg)	0.636 ±0.0163 b	0.746 ±0.0152 a	0.450 ±0.027 d	0.573 ±0.0138 c
Trehalase (IU/mg)	0.490 ±0.008	0.460 ±0.0046	0.430 ±0.0072	0.450 ±0.0093

* Mean ± SEM

The values in a row having different superscript (ab) are significantly different at 0.05 significant level according to DRMD.

Enzymes analysis of sixth instar larvae of *T. granarium* that treated with Phosphine for 20 h. showed that AcP and AkP respond in a same manner at 10 and 20ppm doses. Both activities decreased in resistant populations and also increased in susceptible populations with respect to their control as shown in Tables 1 and 2.In Khw population AkP activity, between 10 to 20ppm doses, increased. Prominent decrease was observed in Lhr population by 24.44%. Between 10 to 30ppm doses significant decrease was seen in Khw population which was 28.75% (Table. 3).In Khw population, AcP activity increased at 30ppm as compared to 20ppm. In Lhr population, AcP activity decreased at 30ppm as compared to 20ppm. Comparison between AkP activity 20 to 30ppm showed significant increase in resistant population while decrease in Khw populations (Table. 3).

Control level of amylase activity can be observed from Tables 1, 2 and 3 which also showed that Amylase activity decreased in Lhr population 5.04, 9.24 and 2.52% at 10, 20 and 30ppm respectively. In case of Khw population, Amylase activity increased 14.29% at 20ppm and then decreased 6.25% at 30ppm. Amylase activity from 10 to 20ppm doses and 10 to

30ppm which was in the form of 3.54% increase, 7.08% decrease in case of Lhr population, 25.49 and 2.94% increase in Khw population noted. Amylase activity decreased 10.26 and 17.97% in Lhr and Khw populations at 30ppm as compared to 20ppm dose respectively.

Table 3: Percentage increase (+) or decrease (-) in Enzyme Activity in 6th instar larvae of *T. granarium*.

Enzyme	Population	Phosphine treatment (ppm)					
		10	20	30	10 vs 20	10 vs 30	20 vs 30
ACP	Lhr.	-12	-14	-20	-3	-9	-7
	Khw.	3	5	8	2	5	3
AKP	Lhr.	-9	5	-6	-13	-22	-10
	Khw.	30	36	-7	5	-29	-32
Amylase	Lhr.	-5	-2	-12	4	-7	-10
	Khw.	-9	14	-6	25	3	-18
ALAT	Lhr.	-20	-43	-60	-28	-50	-30
	Khw.	30	40	68	8	29	19
ASAT	Lhr.	24	42	56	14	25	10
	Khw.	-51	-54	-18	-5	69	78
LDH	Lhr.	-4	-1	-6	3	-2	-5
	Khw.	4	7	8	3	5	1
ICDH	Lhr.	-20	-43	-60	-28	-50	-30
	Khw.	30	40	68	8	29	19
ChE	Lhr.	-1	-1	-1	0	-1	0
	Khw.	-1	-1	-2	-1	-2	-1
Protease	Lhr.	-5	-3	3	2	8	5
	Khw.	7	-6	5	-13	-2	13
Aminopeptidase	Lhr.	16	-30	11	-12	39	58
	Khw.	17	-29	-10	-40	-23	27
Treahalase	Lhr.	18	24	31	6	11	5
	Khw.	-6	-12	-8	-7	-2	5

Tables 1 and 2 reflected the effect of 10, 20 and 30ppm doses of Phosphine after the exposure of 20 hours in ALAT and ASAT activity. Both showed the opposite response in resistant and susceptible populations when one decreased other increased at almost all doses as shown in Tables 2.1 – 2.6. Control values range of ALAT and ASAT examined were from1.17± 0.030 to 1.27±0.030 and 0.89± 0.017 to 0.96 ±0.02 IU/mg respectively (Table. 3).

In resistant population (Lhr population) ASAT activity rose at almost all doses at 30ppm. ASAT activity decreased in Khw by 5.04% at 20ppm as compared to 10ppm. An increase in ASAT activity was also observed in Khw which was 68.83% in population at 30ppm as compared to 10ppm. ASAT activity increased 9.63%, 77.79% in Lhr and Khw population respectively at 30ppm as compared to 20ppm.

Decrease in ALAT activity in LHR population was 30% at 30ppm as compared to 20ppm. ALAT activity increases in Khw population significantly at 30ppm as compared to 20ppm.

LDH and ICDH exhibited the same pattern of activity in resistant and susceptible populations of *Trogoderma granarium* (Table. 3). LDH activity decreased in resistant population at all doses 10, 20 and 30ppm of Phosphine Likewise ICDH activity gradually decreased in resistant population (Lhr population) at all doses 10, 20 and 30ppm of Phosphine. Decrease in ICDH was highly significant at 20ppm and 30ppm respectively. LDH activity increased in susceptible population (Khw population) at 20 and 30ppm 7% at 20ppm, 20% at 30ppm respectively. Significant increases were examined at 30ppm.

It was revealed that there was slightly decrease in ChE activity in Lhr population while decrease was prominent in Khw population as compared to resistant population and in other comparisons 10 vs. 20ppm, 10 vs. 30ppm and 20 vs. 30ppm same behavior depicted. It was seen an increase in trehalase activity in Lhr population by 17.78, 24.44 and 31.11% at 10, 20 and 30ppm respectively. On the other hand, it decreased 6.12, 12.24 and 8.16% in Khw population at 10ppm, 20ppm and 30ppm doses respectively. Tables 1 showed the change in trehalase activity at 20 and 30ppm when compared with 10ppm. Trehalase activity in Lhr population was observed 5.66 and 11.32% increase, in Khw population 6.52 and 2.17% decrease at 30ppm.

Table1 and 2 reflected the effect of 10, 20 and 30ppm doses of phosphine after 20 hours in Protease activity also. It was noticeable that in Lhr population Protease activity decreased at 10ppm and 20ppm by 4.88% and 2.53% but increased 2.66% at 30ppm dose. On the other hand it increased 7.17%, decreases 6.47% and increased 5.46% in Khw population at 10, 20 and 30ppm doses.Table 3 showed that Protease activity changes at 20 and 30ppm when compared with 10ppm by (2.47% and 7.94%) in Lhr population, 12.73 and 1.60% in Khw population. Here bracket "( )" represents 'increase' around percentages.

Normal values of Aminopeptidase activity in Khw and Lhr populations were 0.636 and 0.615 IU/g respectively as noted from tables. Aminopeptidase activity decreased in Lhr population increased 15.84%, decreased 30.43% and increased 4.63%, In Khw population increased 17.20, decreased 29.22 and 9.85% at 10, 20 and 30ppm doses respectively (Table. 3).

It was obvious from the Tables1 and 2 that Aminopeptidase activity changed between 10 to 20 and 10 to 30ppm. There was 39.94% and 9.68% decrease in Lhr population, 39.60% and 23.08% decrease in Khw population. Aminopeptidase activity enhanced in Lhr and Khw populations by 50.38 and 27.36% respectively at 30ppm as compared to 20ppm in 6th instar larvae.

DISCUSSION

Various enzymes have been correlated with the degradation of pesticides. Among them degradation of DDT has been correlated with DDT-dehydrochlorinase (Abedi *et al.*, 1963) whereas degradation of other organochlorines has been

correlated with this enzyme and oxidases (Sternberg and Kearns, 1950; Kimura and Brown, 1964; Oppenoorth and House, 1968). Degradation of organophosphates and carbamates has been correlated with general esterases, carboxyesterases, cholinesterases and mixed function oxidases (Rashan *et al.*,1979; Naqvi*et al.*, 1984; Naqvi and Rub, 1985; Jagadeesan, 2011)

In the present study esterases (acid phosphatae, alkaline phosphatase and cholinesterase) and oxidase (LDH, ICDH,GOT, GPT) and other enzymes have been determined in the Phosphine treated and un-treated 6th instar larvae of *Trogoderma granarium* to find any correlation or toxic effect of Phosphine with induction / inhibition of enzymes.

Tables 1 and 2 show the changes in the activities of various enzymes (AcP, AkP, amylase, ALAT, ASAT, LDH, ICDH, ChE, protease, aminopeptidase, trehalase) in 6th instar larvae of *T. granarium*in two different populations Lhr and Khw collected from different godowns of Punjab have been quantified after the exposure of Phosphine for 20 h.

The results of present study suggested that almost all the enzymes were found to be sensitive to Phosphine. This is the first study on *T. granarium* in Pakistan. From other laboratories effects of some insecticides have been reported on transaminases of *Culex fatigans* (Srivastava and Verma, 1980), on inhibition of phosphomonoesterases in desert locust with DDT (Naqvi *et al*., 1970), inactivation of LDH by organochlorines (Kocak *et al.,* 2015) and inhibition of trehalase activity in haemolymph of *Phormia reegina* (Friedman, 1961) etc. Shakoori and Saleem, (1989) have reported effects of different insecticides, mixture of insecticides on the enzyme, metabolites and macromolecules of 6th instar larvae of T. castaneum.

Phosphates are involved in dephosphorylation and their decreased activity may be attributed to inhibition of enzymes after insecticide treatment, this may be necessary for decrease amount of ATP due to low respiratory rate which results to overcome the sudden toxic stress (Pimental *et al*., 2010). In susceptible population (Khw) phosphatases were induced, which indicated higher concentration of enzymes due to decreased body weight of treated larvae and / or increased energy production through breakdown of phosphate bonds of energy production through breakdown of phosphate bonds of energy rich nucleotides and amino acids due to increased respiratory rate. AkP activity showed significant increase at 30ppm in both populations for increased energy requirement which results to overcome the sudden toxic stress (Shakoori and Saleem, 1998; Dow and Davis, 2001; Cabero *et al.*, 2004; Schlipalius *et al.*, 2012) and due to scleritizasion in 6th instar of larvae (Saleem and Shakoori.,1990). Psachoulia *et al.* 1989 reported that ALPase is involved in the sclerotization process of insect integument. In resistant population aliesterases decreased which is also confirmed by the current findings (Tables 1 and 2). Almost same results are described by Tufail *et al.* (1994) in adult beetles of *T. castaneum* after the treatment of cypermethrin insecticide at sub lethal dose. Contrast to this study, Vyjayanthi and Subramanyam, 2002 indicated that due to the organophosphorus insecticides, AcP and AkP was inhibited in *Oreochromis mossambicus* while exposure tenures was 3, 7, 15 and 30 days. Reduced activity of phosphatases also reported by Shakoori *et al.* (1994a,b,c).

Induced activities of aminotransferases (ASAT and ALAT) may probably be due to induction process at molecular level to route amino acids into Kreb's cycle for production of energy or for gluconeogenesis in resistant populations of 6th instar larvae. These enzymes normally indicative of aminoacid catabolism and promote breakdown of aminoacids by transfer of amino groups of keto acids. In 6th instar larvae ALAT and ASAT showed opposite behavior in susceptible and resistant population (Table 1). If induced in susceptible then decreased in resistant population and vice versa (Table 1). Habig *et al.*, 1974 and Kriestoff *et al.*, 2010 describe the similar response of these two aminotransferases in *Clarias betrachus* and in *Oreochromis mossambicus* respectively in response to carbofuran and organophosphorus insecticides (Wang *et al.*, 2010).

The dehydrogenases belong to oxido-Rwductase major group of enzymes and involve in oxidation and reduction of metabolites. Increased LDHactivity indicated the switching over anaerobic glycolysis to aerobic respiration. Significant decrease in LDH activity was found in current studies at 30 ppm of Phosphine in resistant populations of 6th instar larvae of *T. granarium* in Lhr population (Table 1) This decrease indicate decrease in respiratory rate through inhibition of lactate to pyruvate interconversion. Similar results were described by DeLorenzo and Serrano, 2003 and Guidi *et al.*, 2010. LDH activity also decreased in T. castaneum due to diffusion of Phosphine observed by (Emery *et al.*, 2011). In susceptible population (Khw) LDH activity enhanced to support the respiration. LDH activity increased in 4th and sixth instar larvae of *T.* castaneum in response to cyhalothrin, Karate (The pyrethroids) and other insecticides (Saleem and Shakoori, 1986; Shakoori *et al.*, 1988; Shakoori *et al.*, 2000)

ICDH is another dehydrogenase which is normally utilize to evaluate the efficiency of Krebs cycle. Reduced leveled of ICDH in 6th instar larvae of resistant population (Lhr), however, suggested that citric acid cycle was probably deactivated or slowed down, which provided less energy to the insect at both the doses of 20 ppm and 30ppm of Phosphine, ICDH activity was enhanced in susceptible population of larvae (Khw). Which revealed that citric acid cycle more active in this stage (Opit *et al.*, 2012) and also inactive of high rapidly so respiratory rate. The finding of this study were supported by Shakoori *et al.*, 2000 working with *Tribolium castaneum*.

Cholinesterase is another important esterase which hydrolyze the acetylcholine ester which play important role in nervous co-ordination. Desturbation in its activity promotes in co-ordination in the systems. In the present study higher sub

lethal dose 20 ppm, 30 ppm of Phosphine depleted the activity of ChE in Khw population. whereas the activity of ChE remained unaffected in resistant population (Van Asperene,1962; De-Lima *et al.,* 2013; O'Brien, 1992; Mutero *et al.,* 1994; Ellman *et al.,* 1968; Putkome *et al.,* 2008). ChE is also invole in the detoxification of insecticides strongly recommended by Tim-Tim *et al.,* 2009; Valbonesi *et al.,* 2003; Gagnaire *et al.,* 2008 and Wang *et al.,* 2004.

Aminopeptidases are generally zinc metalloenzymes, which hydrolyze peptide bonds near the N- terminal end of peptides and polypeptides. Aminopeptidases are considered as one of the main pathways to neuropeptide inactivation and peptide activation (Mayas *et al.,* 2004) by the hydrolysis of their precursors (Barrett *et al.,* 1998) L-Aminopeptidases can hydrolyse bradykinins (Sanderink *et al.,* 1988). It can also participate in the brain rennin-angiogenesis system (Ahmad and Ward, 1990). Changes in aminopeptidases level do not reflect possible tissue damage. This complex interaction between Phosphine and CNS neurotransmitter involved in Phosphine tolerance or dependence (Dahchour and White, 2000; Mayas *et al.,* 2000b, 2000c).

This study reveals that in 6th instar larvae aminopeptidase activity increase only in resistant population. Ahmed *et al.* (1998) determined the proteolytic activity in *Musca domestica* and in DDT- resistant and suggested the involvement of these enzymes in the resistant mechanism.Protease activity following Phosphine exposure increased in susceptible population of 6th instar larvae. Change in protease activity at sub lethal doses 20 ppm, 30 ppm of Phosphine could be due to their enhanced synthesis generally termed as enzyme induction which may be involved in the defense mechanism of the target organism. As proteases are involved in breakdown of proteins, enhanced activity indicate their higher need for protein degradation for energy production. This may provide more aminoacids to be used as fuel in the following absorption. In order to counter the stress conditions, animals had to utilize more energy (Ahmad *et al.,* 2002; Raina and Kiran, 2004; Ahmed *et al.,* 2006; Nath *et al.,* 2011).

Amylases are the hydrolytic polysaccharidases utilized to degrade the dietary starch and glycogen in digestive tracts to provide glucose to the cells. In susceptible population the raised activities of amylaseat higher concentration indicated that the polysaccharides were being preferentially utilized by the insect after Phosphine treatment. In resistant population however, the amylase activity was decreased in 6th instar resistant larvae, indicated that the polysaccharides were not being utilized rapidly to provide glucose for the sake of energy production in other words respiratory rate is slow in resistant population (Lhr). Saleem and Shakoori, 1990) demonstrated that malathion + carbohydrases (amylase, trehalase, lactase, maltase) in 6th instar larvae of T. castaneum. In contrast to these results, Saleem and Shakoori (1986) reported significantly lowered amylase activity at 2 and 4 ppm dose levels of cypermethrin in sixth instar *T. castaneum*. Similar type of results described by (Shadnia *et al.,* 2009; Bumbrah *et al.,* 2012; Pereira *et al.,* 1999; Yang and Wang, 1999; Rosell *et al.,* 2002; Itho *et al.,* 1988; Nisa *et al.,* 2011; Mehrpour *et al.,* 2012).

Trehalaseactivity reduced in susceptible population of 6th instar larvae of *Trogoderma granarium* while in resistant population trehalase activity elevated. Saleem and Shakoori (1987a) described significantly depleted levels of trehalase activity in *Tribolium* adults after administration of sub lethal doses of permethrin and Malathion whether used alone or in combination. Increased amount of trehalase indicated the elevation of trehalose, so trehalose degrading enzyme increased to reduces the carbohydrate metabolism (Nighat *et al.,* 2011; Mujeeb *et al.,* 2011). This enzyme showed significant role in resistance.Moreover, Phosphine can be used for the effective control of these populations of stored grain pests. This study will help in understanding the biochemical basis of Phosphine resistance in *T. granarium* andprovide little information for improving pests control programs.

CONCLUSION

Increased level of ASAT, Aminopeptidase and Trehalase While decreased level of AcP, AkP, LDH, ICDH, amylase, ALAT and protease indicated the role of these enzymes in development of resistance against phosphine. This study will help in understanding the biochemical basis of Phosphine resistance in *T. granarium* andprovide information for improving pests control programs by phosphine.

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