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Phosphine-Induced Alterations in Microsomal Enzymes of a Stored Grain Pest *Trogoderma granarium* Collected from Godowns of Punjab, Pakistan

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Authors' Contribution
FRS and SSA designed the research project. TR conducted the experimental work. TR, FRS and ASS analyzed the data and wrote the article.

Key words
***Trogoderma granarium*, Wheat, Phosphine, Microsomal enzymes, Tolerance.**

ABSTRACT

The present study was aimed to determine the toxic effect of phosphine on microsomal enzymes (NADPH-Cytochrome P_{450} reductase (NADPH-CPR), ethylmorphine-N demethylase and aniline 4-hydroxylase) and soluble protein contents of a wheat grain pest, *Trogoderma granarium* collected from godowns of Punjab, Pakistan. Six populations of khpara beetle with different levels of susceptibility to phosphine were used in this study. Based on LC_{50}, five populations (previously exposed to phosphine for 15 years) *viz*., Mandi Bahauddin-I (MBDIN-I), Mandi Bahauddin-II (MBDIN-II), Gujrat, Gujranwala and Sargodha were labeled as phosphine-tolerant populations and one population never exposed to any insecticide/fumigant for 13 years was termed as phosphine-susceptible population. Percent change in the activities of above mentioned microsomal enzymes and soluble protein contents in 4th and 6th instar larvae and adult beetles of phosphine-tolerant populations were evaluated with reference to phosphine-susceptible population as a control. The activities of NADPH-CPR, ethylmorphine-N demethylase, aniline 4-hydroxylase and soluble protein contents were significantly increased in all field collected phosphine-tolerant populations with reference to phosphine-susceptible population. Among developmental stages, the 4th instar larvae possessed higher microsomal enzyme activities than 6th instar larvae and adult beetles in all populations. The increased level of microsomal enzymes in phosphine-tolerant populations as compared to susceptible population has pointed some correlation between microsomal enzymes and phosphine tolerance.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a staple diet in Pakistan and are produced in more quantity as per need (Anon, 2005-2006). Its storage is done in the houses as well as in the large government storage facilities but without any proper arrangement resulting into huge losses. The post-harvest losses of wheat grain have been reported to be 10-15% in Pakistan. Among various contributing factors to these losses, stored grain pest *Trogoderma granarium* (Everts) is considered to be the most destructive pest of wheat (Burges, 2008; Castalanelli *et al*., 2010). The agriculture sector of the economy is facing persistent challenges. By 2020, world demand for wheat is expected to be 40% higher than that of its level in the latter half of the 1990s (Mahroof *et al*., 2005; Fedina and Lewis, 2007).

Various techniques have been applied to reduce the damage caused by these insect pests. Insecticidal approach was the most significant to keep the pests below economic loss level. Organochlorines, organophosphates, carbamates, pyrethroids were used to overcome the problem of pest infestation. Due to impaired ability of these pesticides to control these stored grain pests, the fumigation technique with methyl bromide and phosphine as fumigant is being widely used in godowns (Walter, 2006).

In large stores, pesticides and fumigants are being used from last several years but their doses are not calculated as per resistivity of pest against the pesticides resulting into no control over the pest. The development of tolerance/resistance after exposure to sub-lethal doses of insecticides/fumigants has become universal phenomenon in stored grain pests. The increased tolerance/resistance may be due to the elevated levels of various insecticide hydrolyzing enzymes and other metabolites. This up-regulation is usually attributed to a genetic change in the activities of insecticides degrading enzymes (Riaz *et al*.,

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2017). There is variety of enzyme systems that could be affected by chronic doses of insecticides. The important among these resistance indicators are microsomal mixed-function oxygenase, cytochrome P-450 dependent mono-oxygenase and esterase systems (Vontas *et al*., 2002). This study was aimed to evaluate the direct/indirect effect of phosphine on various microsomal enzymes in *T. granarium* collected from different godowns of Punjab province having regular long exposure to phosphine and to evaluate their role in the development of tolerance against phosphine in *T. granarium*. There is no report on toxic effects of phosphine on microsomal enzymes of *T. granarium.*

MATERIALS AND METHODS

Rearing and maintenance of pest culture

A notorious stored grain pest, *T. granarium* (Everts) with common name khapra beetle was used in current investigation. Master cultures of five populations of khapra beetle tolerant to phosphine were collected from different godowns of Punjab province *viz*., Gujrat, Mandi Bahauddin-I (MBDIN-I), Gujranwala, Mandi Bahauddin-II (MBDIN-II), and Sargodha. These godowns have more than 15 year history of phosphine fumigation to wheat. The wheat samples containing *T. granarium* were collected in sterilized plastic bags and brought to laboratory for study. A phosphine-susceptible (population never exposed to phosphine previously) were taken from thirteen years old culture maintained in the culture room of department of Zoology, University of the Punjab, Lahore. The master cultures of *T. granarium* (six populations) were reared and maintained in temperature and humidity controlled room at 30±1°C and 65±5% RH. The culture was fed on sterilized broken wheat and flour in 300 ml glass jar tightened with rubber band (Riaz *et al*., 2014; Shakoori *et al*., 2016). Adult beetles (24 h old) were kept in the medium for 5-6 days for egg laying and then dead beetles were discarded form the jars. Eggs in the floor medium were allowed to develop into next adult beetles through various larval and pula stages and it was repeated for 4-5 generations to collect age wise homogenous stock of each population. The larvae were separated on the basis of size, the 4th instar larvae were about 3 mm in length and 6th instar larvae measured 5 mm.

Generation and administration of phosphine

Gaseous Phosphine was generated from commercially available aluminum phosphide (AlP) pellets comprising (approximately 0.2g) in the laboratory fume hood. Different doses of phosphine were calculated according to the formula given in FAO plant protection bulletin (FAO, 1975). Twenty insects of each 4th, 6th instar larvae and adult beetles (24 h old) were introduced for fumigation in each vial with three replicates for each dose. The insect containing vials were placed in vacuumed desiccators and calculated volume of phosphine for each dose was injected through sterilized Hamilton syringe in their respective desiccator. These desiccators were placed in temperature and humidity controlled room for 20 h exposure period. The LC_{50} of phosphine for each developmental stage was calculated according to Riaz *et al*. (2016).

Biochemical parameters (NADPH-CPR, ethylmorphine-N demethylase, aniline 4-hydroxylase and soluble protein contents) of five tolerant populations (Gujrat, MBDIN-I, Gujranwala, MBDIN-II and Sargodha) collected from godowns of Punjab were compared with biochemical parameters of phosphine-susceptible population.

Biochemical analysis of microsomal enzyme activities

The microsomal fraction was prepared by the method of Schenkman *et al*. (1981) described in steps as follow:

Preparation of microsomal fraction

For the preparation of whole homogenate, homogenizing medium, homogenizing tubes, cylinders and centrifuge tubes were kept in ice. Beetles (1g) were homogenized in 3ml of homogenizing medium (0.154M KCl) with five return stroke of motor driven Teflon glass homogenizer with constant cooling in crushed ice. The homogenate was diluted 25% *i.e*., 0.25 g homogenate/ml so, 3 ml of homogenate was diluted with 1ml of homogenizing medium (0.154M KCl). The 4 ml homogenate was covered with parafilm and mixed by gentle inversion. The homogenate was centrifuged at 10,000 g for 20 min at 4ºC. A thin layer of floating lipids were removed with pasture pipette. The supernatant was transferred to other precooled tube and were ultra-centrifuged at 105,000 g for 1 h at 2-4ºC. The cytosol fraction and lipid layer were removed by pasture pipette. Pellet was washed with homogenizing medium (0.154M KCl) and followed by ultra-centrifugation. Supernatants were discarded and microsomal pellet was diluted further and used for biochemical analysis of various enzymes.

Determination of microsomal enzymes activities

The activity of NADPH-CPR was determined spectrophotometrically by the method of Peterson *et al*. (1978) using cytochrome, potassium cyanide and NADPH.

The N-demethylation of ethylmorphine results in the formation of formaldehyde which is trapped in the incubation medium by the presence of semicarbazide and estimated spectrophotometrically by means of Hantysch reaction (Nash, 1953) and modified procedure of Alvares

and Mannering (1970).

The 4-hydroxylation of aniline hydrochloride results in the formation of 4-aminophenol, which is determined spectorphotometrically by indophenol reaction. Aniline 4-hydroxylase was determined by method of Nakanishi *et al.* (1971) with a modification that NADPH is added directly into the incubation medium instead of generating NADPH from NADP, isocitric acid, isocitric dehydrogenase and magnesium ions.

The total soluble proteins were estimated according to Lowry *et al.* (1951). Soluble protein contents were calculated as µg/mg of tissue from standard curve prepared by using bovine serum albumin (BSA).

Analysis of variance (ANOVA) followed by tukey's Post Hoc test was applied to all the data of biochemical parameters to compare pair wise means of various populations to determine the significant difference at P<0.05 using SPSS software.

RESULTS

The activity of three microsomal enzymes (NADPH-CPR, ethylmorphine N-demethylase and aniline 4-hydroxylase) in three different developmental stages of phosphine-susceptible and tolerant populations (Gujrat, MBDIN-I, Gujranwala, MBDIN-II and Sargodha) of *T. granarium* was described in Table I.

NADPH-CPR, ethylmorphine N-demethylase and aniline 4-hydroxylase activities were significantly increased in all phosphine-tolerant populations when compared to susceptible population at P<0.05. In 4th instar larvae NADPH-CPR activity was significantly different from each other in all tolerant populations likewise in adult beetles significant difference among tolerant populations was observed, whereas in 6th instar larvae, MBDIN-II and Gujranwala had non-significant difference in NADPH-CPR activity at P<0.05. Percent increase in NADPH-CPR activity of tolerant populations in comparison with susceptible population is shown in Figure 1. Based on increased level of NADPH-CPR activity, tolerant populations can be graded as: MBDIN-II > Gujranwala > MBDIN-I > Sargodha > Gujrat. Adult and 6th instar larvae possessed less NADPH-CPR activity than 4th instar larvae in all populations.

Table I.- Activities of various microsomal enzymes (nmol/min/ml)) and soluble protein contents (mg/mg of body weight) of 4th instar larvae, 6th instar larvae and adult beetles of susceptible and five tolerant populations of *T. granarium*.

Populations	NADPH-CPR	Ethylmorphine N-demethylase	Aniline 4-hydroxylase	Total soluble protein contents
4th instar larvae				
Susceptible	428.57 ± 0.218^{a*}	982.5 ±0.290^{a}	39.48 ± 0.144^{a*}	199.61 ± 4. 26^{ad*}
MBDIN-I	517.85 ± 0.248^{a}	1095.01 ±0.350^{a}	45.36 ± 0.197^{a}	213.64 ± 2.21^{ad}
MBDIN-II	535.70 ± 0.244^{a}	1310.21 ±0.290^{a}	47.04 ± 0.375^{a}	210.12 ± 3.13^{a}
Gujrat	494.04 ± 0.234^{a}	1280.13 ±0.283^{a}	42.56 ± 0.233^{a}	229.35 ± 2.39^{ad}
Gujranwala	523.80 ± 0.203^{a}	1350.34 ±0.401^{a}	49.84 ± 0.291^{a}	231.1 ± 3.44^{ad}
Sargodha	470.23 ± 0.272^{a}	1120.47 ±0.266^{a}	43.63 ± 0.175^{a}	211. 34 ± 4.31^{a}
6th instar larvae				
Susceptible	410.70 ± 0.206^{bd}	967.5 ±0.31^{a}	38.92 ± 0.117^{b}	195.21 ± 3.79^{b}
MBDIN-I	499.99 ± 0.123^{bd}	1050.11 ±0.364^{a}	44.24 ± 0.277^{b}	209.69 ± 3.24^{b}
MBDIN-II	511.89 ± 0.169^{b}	1290.04 ±0.306^{a}	46.2 ± 0.145^{b}	207.01 ± 3.93
Gujrat	476.18 ± 0.353^{bd}	1220.39 ±0.277^{a}	42.01 ± 0.116^{b}	225.78 ± 3.41^{b}
Gujranwala	510.89 ± 0.334^{b}	1320.14 ±0.285^{a}	47.88 ± 0.189^{b}	227.35 ± 3.71^{b}
Sargodha	452.37 ± 0.244^{bd}	1100.42±0.321^{a}	43.12 ± 0.177^{b}	226.15 ± 3.06^{b}
Adult beetles				
Susceptible	374.99 ± 0.282^{c}	938.03±0.254^{a}	37.8 ± 0.163^{cd}	189.42 ± 3.51^{c}
MBDIN-I	464.27 ± 0.301^{c}	1020.16 ±0.347^{a}	43.4 ± 0.228^{cd}	206.37 ± 3.86^{c}
MBDIN-II	505.94 ± 0.245^{c}	1207.14 ±0.276^{a}	44.8 ± 0.317^{cd}	205.61 ± 2.91^{c}
Gujrat	434.51 ± 0.227^{c}	1200.26 ±0.258^{a}	40.6 ± 0.239^{c}	221.98 ± 3.20^{c}
Gujranwala	488.08 ± 0.212^{c}	1300.19 ±0.307^{a}	47.32 ± 0.273^{cd}	224.54 ± 3.75^{c}
Sargodha	446.42 ± 0.362^{c}	1070.13 ±0.317^{a}	41.44 ± 0.190^{c}	222.11 ± 3.38^{c}

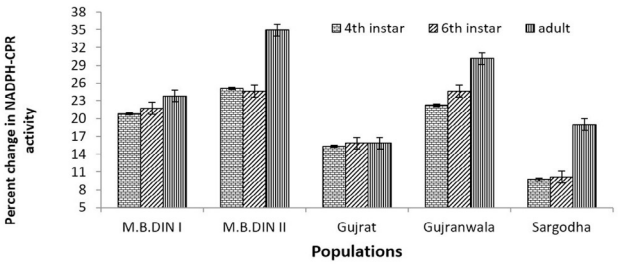

Fig. 1. Percent increase in NADPH-CPR activity of 4th, 6th instar larvae and adult beetles in tolerant populations of *T. granarium* with reference to susceptible population.

Percent increase in ethylmorphine N-demethylase activity of tolerant populations in comparison with susceptible is shown in Figure 2. Based on increased level of ethylmorphine N-demethylase activity, tolerant populations can be graded as: Gujranwala > MBDIN-II > Gujrat > Sargodha > MBDIN-I. Ethylmorphine N-demethylase activity of adult is found less than that of 6th instar larvae and likewise ethylmorphine N-demethylase activity of 6th instar larvae is also less compared to 4th instar larvae in all populations.

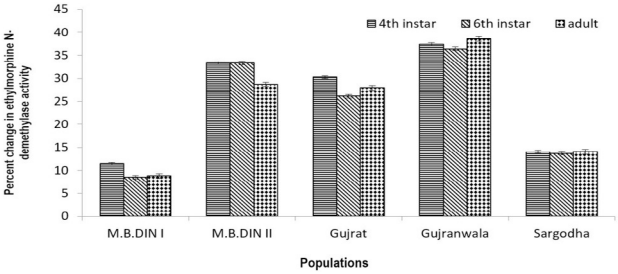

Fig. 2. Percent increase in ethylmorphine N-demethylase activity of 4th, 6th instar larvae and adult beetles in tolerant populations of *T. granarium* with reference to susceptible population.

Aniline 4-hydroxylase activity was significantly different in 4th instar larvae of all tolerant populations likewise 6th instar larvae also possessed significant difference among all tolerant populations, whereas adult beetle of Gujrat and Sargodha population had non-significant difference in aniline 4-hydroxylase activity at $P<0.05$. Percent increase in aniline 4-hydroxylase activity of tolerant populations in comparison with susceptible population is shown in Figure 3. Based on increased level of aniline 4-hydroxylase activity, tolerant populations can be graded as: Gujranwala > MBDIN-II > MBDIN-I > Sargodha > Gujrat. Adult beetles and 6th instar larvae possessed less aniline 4-hydroxylase activity compared to 4th instar larvae in all populations.

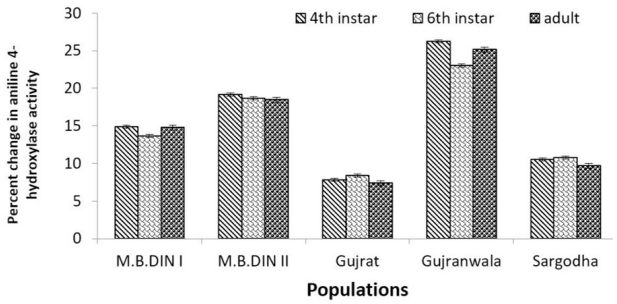

Fig. 3. Percent increase in aniline 4-hydroxylase activity of 4th, 6th instar larvae and adult beetles of five tolerant populations of *T. granarium* as compared to susceptible population.

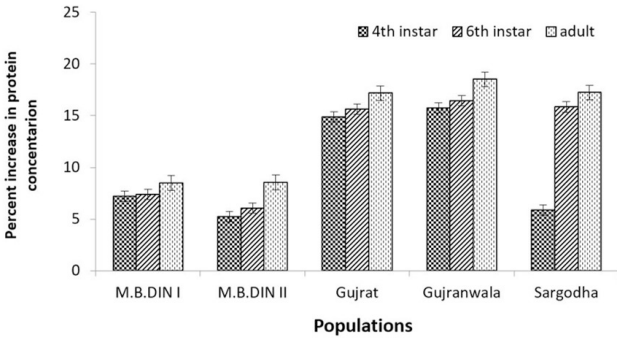

Fig. 4. Percent increase in total soluble proteins of 4th, 6th instar larvae and adult beetles in tolerant populations of *T. granarium* with reference to susceptible population.

Total soluble proteins of adult is less than that of 6th instar larvae and likewise total soluble proteins of 6th instar larvae is less compared to 4th instar larvae in all populations except for Sargodha population. In Sargodha population 6th instar larvae exhibited higher total soluble proteins activity compared to 4th instar larvae. Percent increase in total soluble proteins of tolerant populations in comparison with susceptible is shown in Figure 4.

DISCUSSION

Khapra beetle, *Trogoderma granarium* is a serious pest of stored grains all over the world. In present study the increased activities of NADPH-CPR, ethylmorphine N-demethylase and aniline 4-hydroxylase were observed in all phosphine tolerant populations with reference to susceptible population. The MBDIN-II population has a significant percent increase 25, 24.63 and 35% NADPH-CPR activity, Gujranwala population has 37.43, 36.44 and 38.60% increase in ethylmorphine N-demethylase activity and 26.24, 23.02 and 25.18% increase in aniline

4-hydroxylase activity in 4th, 6th instar larvae and adult beetle respectively, with reference to susceptible population. Among developmental stages, the 4th instar larvae possessed higher NADPH-CPR, ethylmorphine N-demethylase and aniline 4-hydroxylase activities than 6th instar larvae and adult beetles revealing that microsomal activities started to decrease as development proceeds.

There was no data available on activities of NADPH-CPR, ethylmorphine N-demethylase and aniline 4-hydroxylase activities in *T. granarium* against phosphine. It was assumed that mortality after phosphine exposure occurred due to disruption of mitochondrial respiration which suggests that energy insufficiency was the cause of mortality after phosphine exposure. It was proposed that there is a relationship between energy metabolism and phosphine resistance and tolerance is associated with degree of oxygen uptake. Under hypoxic conditions, metabolic demand is suppressed and ultimately suppressed respiration occurs so insect remained insensitive to phosphine. Mitochondrial uncouplers, increase metabolic demand and increase toxicity (Valmas *et al*., 2008). The tolerance may be due to non-disruption of electron transport chain and phosphorylation for energy production. So insect survived and developed tolerance.

Mixed function oxidases are very important markers of resistance. High titers of MFOs are associated with resistance (Tomita *et al*., 1995). They are able to convert lipophilic compounds into polar metabolites that can be easily eliminated from the body; for that reason, they are mainly located in the digestive apparatus (Feyereisen, 2005). Cytochrome P-450 dependent mono oxygenases are a very diverse group of heme containing hydrophobic enzymes that involve in detoxification of various exogenous and endogenous compounds. The detoxification mechanism occurs as a substrate metabolism.

NADPH cytochrome P-450 reductase (EC 1.6.2.4) plays very important role in oxidative metabolism of many endogenous and exogenous compounds (Nelson, 2003). During oxidative metabolic reactions, NADPH cytochrome P-450 reductase accepts a hydride ion (one proton and two electrons) from NADPH and donates two electrons, one at a time to P-450. In this way cytochrome P-450 reductase is an enzyme with two substrates, NADPH and artificial electron acceptor cytochrome c (Gigon *et al*., 1969). Due to the large number of enzymes and their substrate specificity, P450s are able to catalyze different reactions like epoxidation, hydroxylation, N-dealkylation, O- dealkylation or desulfurization; for that reason they play an important role in the metabolism of many insecticide classes, including carbamates, organophosphates, pyrethroids and DDT (Simon, 2014). These monooxyganases are involved to oxidize phosphine to its oxons through phosphorus oxidation reaction (Dauterman and Hodgson, 1990).

Monooxygenase mediated resistance is the most prominent type of metabolism based insecticide resistance reported by Oppenoorth, (1984) and Brattsten *et al*. (1986). Vincent *et al*. (1985) reported that in house fly it was first time detected that P-450 reductase level was higher in insecticide resistant population and later on its higher level was also reported by Kotze and Wallbank (1996) in other insect species. Elevated levels of P-450 reductase associated with monooxygenase mediated resistance suggested that there was a change in proteins that could lead to tolerance (Oppenoorth, 1984) proposed that resistance was developed due to increased detoxification, which results from a change in the catalytic activity of the P-450 reductase and also due to change in the level of expression of the P-450.

CONCLUSION

The microsomal enzyme activities were significantly higher in phosphine-tolerant population collected from godowns as compared to phosphine-susceptible population. This increase in microsomal enzyme level is an indication of some indirect effects of phosphine on microsomal enzyme activities and tolerance is not developed at once but it is a gradual process after continuous exposure to sub lethal concentrations of fumigant. So it is recommended that the lethal concentration of phosphine for each population should be calculated periodically to avoid development of tolerance/resistance, so some control could be achieved to eliminate this pest in storage facilities of wheat.

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Ethical standard

This article does not contain any studies with human participants or animals performed by any of the authors.

Statement of conflict of interest

The authors stated no conflicts of interest.

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