Metabolic profile of phosphine resistant *Trogoderma granarium* collected from different godowns of Punjab

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Abstract-*Trogoderma granarium* is a menacing pest of stored grain products. Phosphine is an effective insecticide against stored-product pests, but over time, the pest has developed resistance to phosphine due to its continuous use. The present study investigates phosphine resistance levels in insect pests across five districts of Punjab. The metabolic profile of resistant populations of the beetle was analyzed. The present investigation revealed that Layyah was the most resistant population to phosphine among all collections. Metabolic analysis showed that contents of glycogen, lipids, and trehalose reduced significantly whereas glucose, soluble proteins, and free amino acids increased remarkably in resistant populations. The insect used its energy reserves to satisfy the higher metabolic needs to cope with the stress caused by exposure to phosphine. It suggests that calculating the lethal dose of insecticide could overcome the infestation caused by the stored grain pests.

Keywords- insecticide, metabolic profile, phosphine resistance, *Trogoderma granarium*

I. INTRODUCTION

Over 20,000 species of insect damage agricultural and animal storage products, affecting both quantity and quality (Rajendran and Sriranjini 2008). The infestation of stored grain insects not only damages storage products and poses a threat to human health, but also results in significant financial losses for the national treasury (Wakil et al. 2019). *Dermestes*, *Trogoderma*, and *Attagenus* are three genera of the dermestid family that are well-known for being storage pests. One of the 100 worst invasive species worldwide is *Trogoderma granarium* (Everts) and it is often in quarantine in multiple countries (Schef et al. 2020). The infestation of *T. granarium* occurs when the environmental conditions are favorable (35°C, 60% RH) (Riaz et al. 2014). Under unsuitable conditions, the species undergoes diapause (Wakil et al. 2019), which is one of the crucial aspects of *T. granarium* biology. The larvae in diapause can survive in conditions of extreme temperatures and starvation (Shivananjappa et al. 2020). *T. granarium* primarily feeds on dried plant and animal matter, with a preference for cereals such as wheat, barley, oats, rye, maize, and rice (Harris 2006). It is a destructive feeder that can cause weight loss of up to 30% of the stored product, and in extreme cases, up to 70% (Szito 2007). Significant losses occur in developing nations due to conventional storage methods, unsanitary transportation practices, and unhygienic conditions during the processing of commodities. (Singh et al. 2017).

To control this species, several studies have been conducted using different methods *i.e.*, control by using plant extracts (Ali et al. 2012; Derbalah 2012; Sultana et al. 2019; Kteo and Mohammed 2022), chemical control by using pesticides (Kavallieratos et al. 2019; Ali et al. 2022) and fumigation with phosphine, sulfuryl fluoride and propylene oxide (Gourgouta et al. 2021; Lampiri and Athanassiou 2021; Myers et al. 2021), physical control (Wilches et al. 2019) and biological control (Ali et al. 2011; Iqbal et al. 2021; Karanastasi et al. 2020).

For control of stored grain pests, phosphine is commonly used as a fumigant as it is considered an essential method of conservation of reserved commodities. The properties of phosphine such as less residual impact than the contact pesticides, inexpensiveness, and ease of application to a broad spectrum of stored reserves and structures (e.g., cereal mills, bunkers, soils, ships during transport, bag stacks, and warehouses) make it an ideal fumigant to control stored grain pests (Bell 200; Chaudhry 2000; Phillips and Throne 2010; Kaur and Nayak 2015; Holloway et al. 2016). Pakistan also relies on phosphine for stored commodities protection against stored grain insect pests (Wakil et al. 2021)

The overuse of phosphine has led to the development of resistant strains. Phosphine resistance in insects was initially identified in the 1970s during a global survey on pesticide resistance, where insects collected in laboratories were examined. Reports of field resistance to phosphine were later confirmed in Bangladesh in 1982, and subsequently in other countries such as Pakistan, India, Africa, and Southeast Asia (Taylor 2002). Enzymes aid insects in developing xenobiotic resistance through metabolic pathways, alongside biochemical and regulatory molecules involved in detoxification. Spectrophotometric analysis of total protein, lipids, and sugar contents can be used to measure energy consumption while electron transport activity at the mitochondrial level can be used to measure reserve energy for metabolism. In resistant populations, the discrepancy between energy reserves and consumption can be used as a biomarker for the fitness cost and to determine how much energy is available for growth (Guedes et al. 2006; Araujo et al. 2008a, b; Lopes et al. 2010).

Agriculture plays a vital role in the economy of Pakistan, as the country is predominantly dependent on the agricultural sector. Wheat is the most important agricultural commodity in Pakistan, but storage losses of wheat caused by the khapra beetle range between 3.5 to 25%. To control this loss, phosphine is used universally because it is highly effective in fumigating bulk grain, without compromising grain viability (Opit et al. 2012). The exact mechanism of phosphine toxicity is not yet well comprehended. However, neurological, metabolic, and redox-related reactions are the three categories into which phosphine-induced biochemical and physiological changes fall (Nath et al. 2011). The fumigant phosphine becomes effective based on the concentration and duration of exposure. When the concentration of the fumigant is higher, it requires less exposure time to control pests.The relationship between time and concentration can be linear when plotted on a log-log scale, within certain concentration ranges (Nayak et al. 2020).

The objective of this study was to determine the lethal concentration (LC_{50}) of phosphine for the 4th instar larvae of *T. granarium* over a wide range of exposure periods and to evaluate its effect on various metabolites. The impact of phosphine's lethal concentration (LC_{50}) on the activity and metabolite levels of various *T. granarium* populations has not been reported. The purpose of this work is to improve our knowledge of the biochemical reaction that occurs in khapra beetle larvae when they come into contact with lethal insecticide concentrations. The findings of this research can serve as a marker to determine insecticide exposure. Furthermore, it can aid in devising effective control strategies for the management of this pest.

II. MATERIALS AND METHODS

A. Collection and maintenance of insect culture

The *T. granarium* containing wheat samples were collected from different godowns of Gujranwala, Layyah, Gujrat, Sahiwal, and Kasur of Punjab in sterile plastic bags and carried to the laboratory for assessment. The *T. granarium* culture was maintained at 35±2°C and 60±5% RH (Riaz et al. 2014). From the 12-year-old culture, the phosphine-susceptible population (never brought into contact with phosphine) was taken from the insectary of the Institute of Zoology, University of the Punjab, Lahore.

B. Determination of LC_{50} of phosphine against T. granarium

The LC_{50} against *T. granarium* larvae in their 4th instar that were collected from several locations in Punjab was determined using different phosphine concentrations. Phosphine was produced in the laboratory by dissolving 0.2g of commercially available aluminum phosphide tablets in a 5L gas-tight glass container containing 10% (v/v) sulfuric acid solution. The formula in the FAO Plant Protection Bulletin (1975) was used to compute various doses of phosphine. Twenty insects (4th instar larvae) for each population were introduced in the airtight glass vacuum desiccators. Phosphine doses 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110ppm were taken from freshly generated gas source with a Hamilton microsyringe and injected through a gas-tight rubber septum of the vacuum desiccator. The larvae were exposed to phosphine for the duration of 24, 48, and 72 hours for the evaluation of LC_{50} of each population. After the ending of this period, the larvae were shifted to an untreated petri dish, with wheat grains for 48 hours, and all dishes were placed in an incubator set at 35°C and 60% RH. Beetles were determined to be dead by lack of response to being pressed with a camel hairbrush (Llyod 1969). The LC_{50} was determined by subjecting the data to Probit analysis (Finney 1971). The corrected mortality was determined by using the Abbot Formula (Abbott 1925). The 4th instar larvae exposed to LC_{50} of phosphine for 24 hours were used for biochemical analysis.

C. Biochemical analysis

Twenty 4th instar larvae, from both susceptible and resistant *T. granarium* populations were weighed, homogenized in 1.5 mL saline solution (0.89%), and centrifuged for 30 minutes at 4°C at 3000×g. The clear supernatant was utilized to evaluate the levels of soluble proteins, glucose, and trehalose. Estimation of the content of soluble protein, trehalose, and glucose was done by the method outlined by Lowry et al. (1951), Roe and Dailey (1966), and Hartel et al. (1969), respectively. The free amino acid (FAA) content was extracted from 4th instars by macerating them in 80% ethanol, then centrifuged for 10 minutes at 461×g. The FAA content was estimated using the method described by Moore and Stein (1954). The 4th instars were crushed in a 30% potassium hydroxide solution to extract the glycogen content, which was then calculated using the anthrone method developed by Consolazio and Lacono (1963). To estimate the total lipid content, we used the method developed by Zollner and Kirsch (1962). Each experiment was performed in triplicates.

D. Statistical analysis

The statistical analysis for LC_{50} and metabolites was conducted using Minitab 16 and SPSS 20, respectively. To determine the toxic effects of the lethal dose of phosphine (LC_{50}) on metabolites, Tukey's test and one-way ANOVA were used to analyze the data. To find any significant differences, the means of the susceptible and resistant populations were compared at a significance level of $P \leq 0.05$.

III. RESULTS

The LC_{50} values of the 4th instar larvae of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal after 24, 48, and 72 hours of exposure are displayed in Table I.

Table I. LC_{50} values for the populations of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal.

Population locality	n[a]	Time	LC_{50} (ppm)	R. F
Layyah	20	24	100.98	4.17
		48	78.89	3.84
		72	58.11	3.71
Gujranwala	20	24	80.28	3.32
		48	56.09	2.73
		72	31.00	1.98
Kasur	20	24	76.15	3.15
		48	55.31	2.69
		72	26.82	1.71
Gujrat	20	24	41.18	1.70
		48	30.40	1.48
		72	20.22	1.29
Sahiwal	20	24	29.13	1.20
		48	22.60	1.10
		72	8.56	0.50
Susceptible	20	24	24.16	Reference
		48	20.50	
		72	15.65	

The results showed resistance in the following order: Layyah>Gujranwala>Kasur>Gujrat>Sahiwal. Layyah's strain was observed to be the most resistant strain among all of them and the strain from Sahiwal was the least phosphine-resistant (Table I).

E. Effect of LC_{50} on the metabolites of T. granarium

The highest resistance factor in all populations was observed after 24 hours exposure to phosphine as compared to 48- and 72-hour exposure. Thus, 24 hours of exposed 4th instar larvae of phosphine-resistant populations were analyzed for various metabolites. Percent change in the metabolites of the mentioned populations was calculated compared to the susceptible population shown in Fig. 1.

F. Glycogen

The content of glycogen in the 4th instar larvae was noticeably reduced by 81.07, 73.32, 68.46, 68.1 and 67.41% in phosphine-resistant populations of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal, respectively compared to susceptible population (Fig. 1).

G. Glucose contents

After 24 hours of exposure to phosphine, the 4th instar larvae of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal showed a significant rise in glucose contents *i.e.*, 51.67, 54.05, 89.36, 94.72 and 104.83%, respectively compared to susceptible population (Fig. 1).

H. Lipids contents

Lipids contents in 4th instars larvae of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal populations were significantly depleted by 27.60, 22.39, 19.99, 6.72 and 1.18%, respectively compared to the susceptible population after 24 hours of exposure to LC_{50} of phosphine (Fig. 1).

I. Free amino acids

Phosphine-resistant populations showed increased free amino acid contents with reference to the susceptible population. The populations of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal experienced an increase of 165.40, 143.88, 130.57, 74.64, and 66.18% in their 4th instar larvae, respectively. (Fig. 1).

J. Soluble proteins

In comparison to the susceptible population, the soluble protein level was significantly higher in the phosphine-resistant populations of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal *i.e.*, 97.16, 87.11, 79.47, 50.61, and 48.23% respectively (Fig. 1).

K. Trehalose

Trehalose content in the 4th instar larvae of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal populations decreased by 13.05, 10.66, 9.40, 5.43, and 3.73% respectively regarding the susceptible population (Fig. 1).

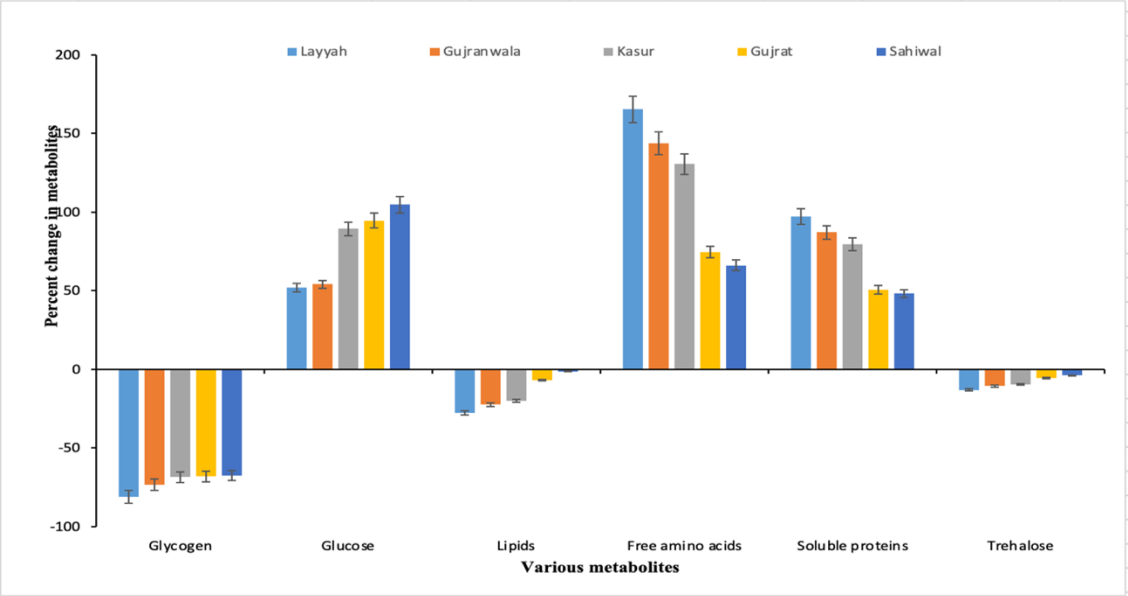

Fig 1. Percent change in different metabolites of 4th instar larvae of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal population, compared with susceptible population.

IV. DISCUSSION

Phosphine-resistant populations *viz.*, Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal had a significantly higher LC_{50} value compared to the phosphine-susceptible population of *T. granarium*. The LC_{50} values for 4th instars of Layyah, Gujranwala, Kasur, Gujrat, and Sahiwal populations for 24, 48, and 72-hour exposure were (4.17, 3.84 and 3.71 ppm), (3.32, 2.73 and 1.98 ppm), (3.15, 2.69 and 1.71 ppm), (1.70, 1.48 and 1.29 ppm) and (1.20, 1.10 and 0.050 ppm) respectively. The Layyah population exhibited greater resistance compared to the susceptible population according to the LC_{50} data. The high resistance was observed after 24 hours of exposure and the least resistance was observed in larvae exposed for 72 hours in all populations.

Results revealed that glycogen, lipids, and trehalose showed a significant decrease in all populations that were resistant to phosphine as compared to the susceptible population. While elevation in the level of glucose, free amino acids, and soluble proteins was noticed in all populations of *T. granarium* after 24 hours of exposure.

The present study suggests that insecticidal exposure induced stress conditions, leading to elevated glucose levels and decreased glycogen content, potentially activating glycolysis for survival (Tufail et al. 1994; Dezwann and Zandee 1972). Insects usually have free glycogen in their hemolymph, which is released to balance glucose levels when the insect experiences stress. This is achieved by the release of catecholamine, glucagon, and corticosteroids, which stimulate glycogenolysis, leading to the release of glucose from the breakdown of glycogen to meet energy requirements (Dezwann and Zandee 1972; Shoba et al. 2011) resulting in increased level of glucose but decreased level of glycogen. Nath (2002) also reported that depletion of glycogen is associated with an increase in glycogenolysis when insects are exposed to insecticidal stress that ultimately leads to increased content of glucose. When the phytopesticide nimbecidine was applied to *Sphaerodema rusticum* Fabricius (Hemiptera: Belostomatidae), Shoba et al. (2011) observed a noteworthy decrease in the amount of glycogen. Shakoori et al. (2016, 2018) observed a decline in lipids and glycogen levels in *T. granarium*, but an increase in glucose levels when exposed to lethal doses of lambda-cyhalothrin and phosphine. According to Hafiz et al. (2017) *T. granarium* exposed to deltamethrin showed an increase in glucose levels but a decrease in lipid and glycogen content during different developmental stages.

Disruptions in lipid metabolism, lipid biosynthesis, and lipid utilization as a stress-reduction energy source could be the cause of the decrease in lipid content (Shaurub and El-Aziz 2015). This also indicated that exposure to pesticides may cause the conversion of lipids to proteins as an additional energy source. This decline may be due to hormonal dysfunction, which disturbs the hormones that control lipid metabolism and prevents their secretion (Yazdani et al. 2013). According to Mulye and Gordon (1993), budworms given juvenile hormone analogs had significantly reduced lipid synthesis and fat body catabolism. Similar effects of different insecticides on glycogen and lipid contents have also been reported by Shakoori et al. (1994), Omar et al. (2005), Ali et al. (2007), Shoba et al. (2011), and Shakoori et al. (2016).

In the present study, the reduced level of trehalose in the 4th instar larvae of *T. granarium* indicates that energy production through the utilization of energy reserves in the body was switched on and accelerated to cope with insecticidal stress. It is also evident from the decrease of trehalose content in all populations that the fumigation caused malfunctioning of hepatic caeca (Nath 2002). Following exposure to esfenvalerate and λ-Cyhalothrin, Shakoori et al. (2018) observed a decrease in lipids, glycogen, and trehalose levels and an increase in glucose levels in 4th and 6th larval instars of *T. granarium*.

Increased FAA levels were noticed among phosphine-resistant populations as compared to susceptible population. The increased content of FAA suggests that the total protein might be converted to amino acids, which can then enter the Krebs cycle as keto acids to provide energy under stress conditions (Nath et al. 1997). Thus, the decrease in total protein content during a stress phase may be a compensatory mechanism to provide the insect with intermediates of the Krebs cycle while maintaining levels of free amino acids (FAAs). Hafiz et al. (2017) observed an increase in FAAs in various developmental stages of *T. granarium* after exposure to deltamethrin. Similarly, Hussain et al. (2012) and Ali et al. (2011) observed an increase in FAA levels in *R. dominica* and *T. castaneum*s respectively, when exposed to malathion and abamectin. After being exposed to phosphine, adult *T. granarium* beetles showed an increase in FAA levels, as reported by Shakoori et al. (2016).

Results showed a significant increase in soluble proteins in 4th instar larvae of the phosphine-resistant populations compared to the susceptible population. The observed increase in protein biosynthesis could be attributed to enzyme induction that counteracts the toxic effects of insecticides (Ali et al. 2011). Shakoori et al. (2016) also noted higher soluble protein contents in adult beetles of *T. granarium* after 24- and 48-hour exposure to phosphine, which then decreased over time. Following exposure to malathion, Ali et al. (2011) also observed elevated protein contents in *R. dominica*.

V. CONCLUSION

This study helps in finding out the exact amount of doses of phosphine that should be given to organisms

of each strain to decontaminate the crop from them. This study also implies that there is a need to find other feasible methods to decontaminate the crops from phosphine-resistant strains of the organism to avoid using great doses of phosphine, which can be harmful to human health.

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