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Varied response of detoxification enzyme activities against lethal and sublethal exposures of phosphine in *Tribolium castaneum* populations

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ABSTRACT

Resistance to phosphine fumigation in stored insects continues to be challenging in managing these pests worldwide. The role of detoxification enzymes in metabolizing phosphine is yet to be explored fully. The dose-response mortality against phosphine was assessed in this study from six field populations of *Tribolium castaneum* (Herbst) collected across India during 2021–23 at the Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi. Further, the strength of Glutathione-S-Transferase (GST) and Cytochrome P450 monooxygenase (CYPs) was estimated at sublethal (LC_{30}) and median lethal concentration (LC_{50}). Dose-response probit assay found that the LC_{30} and LC_{50} values ranged from 0.018 to 0.363 and 0.038 to 1.277 mg/L, respectively. Our result showed that MZ (Mirzapur) and KA (Kailashahar) were the high and least resistant phosphine populations compared to the susceptible reference LS (lab population). The magnitude of GST and CYPs activities in resistant populations were more elevated than in susceptible populations. Our study found that the enhancement of GST and CYPs activity was eight and nine-fold and two and three-fold at LC_{30} and LC_{50} concentrations, respectively. Correlation analysis revealed a significant positive association between GST (r=0.94, P<0.01; r=0.98, P<0.001) and CYPs (r=0.94, P<0.01; r=0.97, P<0.001) to both lethal and sublethal concentrations, respectively. Our findings implied that GST and CYPs activities escalated with increased resistance against phosphine exposure in field populations of *T. castaneum*.

Keywords: Cytochrome P450 monooxygenase, Detoxification, Glutathione-S-Transferase, Lethal concentration, Metabolism, Phosphine resistance

Damage by insect pests resulting in substantial economic losses is a significant concern for quality assurance in stored food grains (Huang *et al*. 2018). Phosphine fumigation safeguards the storage of food grains worldwide because it kills pests quickly at an affordable cost with minimal residue (Jagadeesan *et al*. 2015). However, its widespread and inadequate use has developed resistance in many storage pests, including the red flour beetle, *Tribolium castaneum* (Coleoptera; Tenebrionidae) (Nayak *et al*. 2017). *Tribolium castaneum* is a cosmopolitan, polyphagous pest on various commodities and value-added products (Richards *et al*. 2008).

Detoxification is a vital physiological process for insects' survival in an environment with toxic compounds. The increased synthesis of detoxification-related enzymes and their more significant activity through transcriptional gene upregulation was thought to be connected to the detoxification of pesticides (Zhu *et al*. 2010). Increased activity of cytochrome P450 monooxygenase (CYPS) is likely linked with phosphine resistance (Oppert *et al*. 2015). In phase I, this enzyme catalyzes an array of oxidative processes and helps form more water-soluble compounds, detoxifying different xenobiotics, including phosphine (Liu *et al*. 2015). The crucial involvement of Glutathione-S-Transferase (GST) in the resistance to pesticides had earlier been reported in many insect pests (Muthusamy *et al*. 2014). Reduced glutathione (GSH) is conjugated with various electrophilic metabolites by glutathione-S transferase, critical for detoxifying reactive intermediates and oxygen radicals created by reactive oxygen species (Monteiro *et al*. 2006).

The effect of phosphine on the detoxification enzymes' activities is considered significant. Variability in GST and CYPS activities could alter detoxification processes to phosphine in *T. castaneum* (Zhang *et al*. 2022). However, very little information is available on biochemical alternation in the detoxification processes of phosphine in global

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populations of *T. castaneum*. Hence, the present study focused on investigating the effect of phosphine exposure on GST and CYPS enzyme activities *vis a vis* dose response mortality. An earlier study enumerated that approximately 40% of the weight loss of wheat flour is caused by *T. castaneum* (Ajayi and Rahman 2006). Apart from direct losses, this beetle secretes benzoquinones that influence characteristic colour change of the flour and cause severe health risks to human consumption. The burgeoning issues of phosphine resistance in *T. castaneum* threaten the global prospect-harvest storage sustainability. Henceforth, our study will aid in understanding the differential activity of detoxifying enzymes to phosphine toxicity which may help to evolve better management strategies for stored product insect pests.

MATERIALS AND METHODS

Insects and phosphine toxicity: Present experiment was conducted at ICAR-Indian Agricultural Research Institute, New Delhi. Adult beetles of *Tribolium castaneum* (Herbst) were collected from 6 locations (Table 1) in India during 2021–23 and a lab population (New Delhi) was taken as control. These populations were reared on whole wheat flour containing 5% yeast in a growth chamber at a temperature of 30 ± 1°C and with 65±5% relative humidity following a photoperiod regimen of 16:8 h (L:D). The desired amount of phosphine was administered by a gas-tight Hamilton syringe through septa in the lid of the desiccator. All populations were treated for 20 h duration. The experiment was maintained at three replicates. After exposure, the insects were removed from the desiccators and transferred to a fresh vial with food. For each treatment, 90 insects were placed in three plastic vials (4.5 × 1.2 cm). The LC_{50} and LC_{30} values for respective populations were assessed in 2021–23 with repetitive measures by following the standard protocol (FAO 1975). All six populations and lab susceptible were considered for phosphine induction to evaluate the detoxification enzyme activity. The populations were exposed to LC_{50} and LC_{30} concentrations for 20 h at 30 ± 1^0C. The treatments were performed three times independently. After exposure to the phosphine, the surviving adults were collected and used for enzyme extraction.

Biochemical assessment

Tissue processing: The whole tissue of *T. castaneum* was taken for homogenization using buffer (0.1 M sodium phosphate) with a motorized homogenizer. This was conducted at 4°C conditions using a mini cooler. The homogenized content was centrifuged at 10,000 rpm for 20 min at 4°C (Eppendorf, Centrifuge 58101 R, Germany). The supernatant was taken for biochemical analysis. The microplate reader was used for this analysis in triplicate for each population. A blank was maintained for each study in which the same volume of phosphate buffer replaced the volume of the enzymatic source. The protein content in all the tissue was determined spectrophotometrically following the method of Bradford (1976).

Table 1 Information about collected field populations of *Tribolium castaneum*

Population	Collection place	Facility	Host
GR	Gurgaon, Haryana	Flour mill	Wheat
MS	Malwinder Singh, Punjab	Grain storage	Rice
EA	East Kameng, Arunachal Pradesh	Grain storage	Rice
KA	Kailashahar, Tripura	Flour mill	Wheat
MB	Mayurbhanj, Odisha	Flour mill	Wheat
MZ	Mirzapur, Uttar Pradesh	Grain storage	Rice
LS	Lab susceptible, New Delhi	Flour mill	Wheat

GST assay: The activity of GST was determined spectrophotometrically at 25°C following the principle of glutathione (GSH) conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm using the extinction coefficient of 9.6 mM-1 cm-1 (Habig *et al.* 1974). Briefly, 100 µL substrate was added to the total reaction mixture containing 100 µL of reduced glutathione and 100 µL of supernatant. The enzyme activity was expressed as µmoles of CDNB conjugates/min/mg protein. The activity was examined at both LC_{50} and LC_{30} phosphine concentrations.

Cytochrome P450 monooxygenase assay: Cytochrome P450 activity was quantified using p-nitroanisole as a substrate (Yu *et al.* 1992). 2 mM p-nitroanisole, 9.6 mM NADH, and enzyme extract were used and the absorbance was recorded at 405 nm at 30°C. The activity was expressed as nanomoles of p-nitroanisole/min/ng protein for both LC_{50} and LC_{30} exposures of phosphine.

Statistical analysis: The bioassay data were subjected to probit analysis using the POLO program (Polo Plus, Ver. 2.0 Leora Software 2002) following the principle of Finney (1971) to determine the LC_{50} and LC_{30} values and fiducial limit. Further, the Chi-square test was performed to ascertain the goodness of fit. The ANOVA for enzyme activity was performed with an "R" statistical package of version 4.2.3. Values are presented in the table as the means of enzyme activities of the different locations with standard deviation. The T-test function was used for the mean comparison for LC_{50} and LC_{30} and was displayed in the boxplot showing the median value. Visualization of correlation between variables was displaced using Metan Package. Regression analysis was performed using R's linear model (lm) function to predict the dependent variable.

RESULTS AND DISCUSSION

Phosphine exposure in different T. castaneum populations: Six populations of *T. castaneum* were collected from different locations distributed across the Northern and North-eastern regions of India (Table 1). The bioassay was conducted with varying concentrations of phosphine based on range-finding tests. The LC_{50} and LC_{30} estimates were determined with their fiducial limits. The Lab population (LS) was highly susceptible to phosphine exposure and

was regarded as a susceptibility check with an LC_{50} value (0.018 mg/L). A perusal of the dose-response data revealed that all the field populations of *T. castaneum* differed in their susceptibility to phosphine, with their LC_{50} values ranging from 0.038 to 1.277 mg/L. The variability in the resistance among the different populations within the same species, as observed in the study, might be due to their genetic diversity and/or distribution in distinct geographical territories (Nguyen *et al.* 2016). Results of our study revealed that Mirzapur (MZ) was found to resist the phosphine fumigation compared to the susceptibility check. On the contrary, the Kailashahar (KA) was the most susceptible population. Based on the dose-response probit fit, estimates of LC_{30} for all the field populations ranged from 0.018 mg/L (KA) to 0.363 mg/L (MZ). Our findings are consistent with Ramya *et al.* (2018), who reported variability in contemporary resistance among the field-collected population of *T. castaneum,* having the lowest median dose at 0.358 and 1.901 mg/L in the Gohana and Ajmeer locations in India. Our results are also corroborated by the findings of Agrafioti *et al.* (2019), who reportedly found the variability of resistance across collected populations of *T. castaneum* in Greece. Similar findings were also reported in *T. castaneum* from Australia (Jagadeesan *et al.* 2012), USA (Opit *et al.* 2012) and Turkey (Kocak *et al.* 2018).

Specific activities of detoxification enzymes in different field populations of *T. castaneum*. The mean ± standard deviation values followed by distinct letters are significantly different ($p < 0.05$). Tukey's HSD test separated the treatment means. The ratio value represents the specific activity of the test population upon lab susceptibility (A symbolized for LC_{50} ratio, and B symbolizes for LC_{30} ratio). Three replications of each enzyme (Glutathione-S-Transferase, and Cytochrome P450 monooxygenase) were carried out to determine the activity presented in the table.

The specific activity of detoxification enzymes

GST activity*:* The mean specific activity of GST differed significantly among populations of *T. castaneum* at LC_{50} (F value = 277.20, df = 6, P<0.001) and LC_{30} (F value = 558.13, df = 6, P<0.001) concentrations (Table 2). The least susceptible population, viz. MZ, exhibited higher activity of GST 0.165 µM/min/mg with eight-fold enhancements compared to the susceptible check. Khan *et al.* (2020) reported the increased activity of GST to a lethal concentration of neonicotinoids in *Nilaparvata lugens*. A similar trend was recorded for LC_{30} exposure (0.110 µM /min/mg) exhibiting nine-fold elevation compared to the susceptible one. A report from Isamail (2020) revealed that higher activity of GST was noticed in *Spodoptera littoralis* to sublethal doses of spinetoram. Inadequate fumigation (due to leakages in fumigation covers or inadequate exposure periods) may result in sublethal exposure. Although sublethal exposure may not kill the populations, the elevated activity of detoxification enzyme activities may act as priming to resist the phosphine during later fumigations (Isamail 2020). Our data generally implied that the GST activity was escalated with increased resistance in field-collected *T. castaneum* populations. Studies by Zhang *et al.* (2022) found that GST activity was amplified to detoxify xenobiotics in *T. castaneum.* The upregulation of the GST gene contributes to resistance development in *T. castaneum* to phosphine exposure. Hu *et al.* (2018) supports our findings.

Cytochrome P450 monooxygenase (CYPs) activity: The CYPs activity was determined for both concentrations and presented in Table 2. The result showed that the mean CYPs activity significantly differed among the field-collected populations. The activity ranged from 1.22 to 2.18 nano M/min/mg of protein, with a significant level of variation at LC_{50} concentration (F value = 45.58, df = 6, P<0.001). Similar results were found for the LC_{30} concentration (F

Table 2 Specific activities of detoxification enzymes in different *T. castaneum* populations exposed to lethal (LC_{50}) and sublethal (LC_{30}) exposures of phosphine

Population	Glutathione-S-Transferase (µM/min/mg of protein)				Cytochrome P450 monooxygenase (nano M/min/mg of protein)			
	LC_{50}	Ratio[A]	LC_{30}	Ratio[B]	LC_{50}	Ratio[A]	LC_{30}	Ratio[B]
GR	0.088 ± 0.004 [d]	4.40	0.075 ± 0.001 [e]	6.16	1.914 ± 0.018 [ef]	1.83	1.348 ± 0.023 [cd]	2.68
MS	0.085 ± 0.003 [d]	4.27	0.073 ± 0.002 [e]	5.97	1.817 ± 0.059 [de]	1.74	1.248 ± 0.062 [bcd]	2.48
EA	0.038 ± 0.006 [b]	1.91	0.033 ± 0.001 [c]	2.72	1.475 ± 0.058 [bc]	1.41	0.835 ± 0.030 [ab]	1.66
KA	0.046 ± 0.008 [b]	2.34	0.025 ± 0.003 [b]	2.11	1.224 ± 0.162 [ab]	1.17	0.705 ± 0.069 [b]	1.40
MB	0.068 ± 0.001 [c]	3.39	0.052 ± 0.003 [d]	4.29	1.614 ± 0.110 [cd]	1.55	0.944 ± 0.019 [abc]	1.88
MZ	0.165 ± 0.002 [e]	8.23	0.110 ± 0.001 [f]	9.01	2.186 ± 0.151 [f]	2.09	1.517 ± 0.029 [d]	3.02
LS	0.019 ± 0.004 [a]	1.00	0.012 ± 0.001 [a]	1.00	1.044 ± 0.066 [a]	1.00	0.503 ± 0.230 [a]	1.00
df	6		6		6		6	
F value	277.20		558.13		45.58		14.72	
P value	P<0.001		P<0.001		P<0.001		P<0.001	

Refer to the Table 1 for population details.

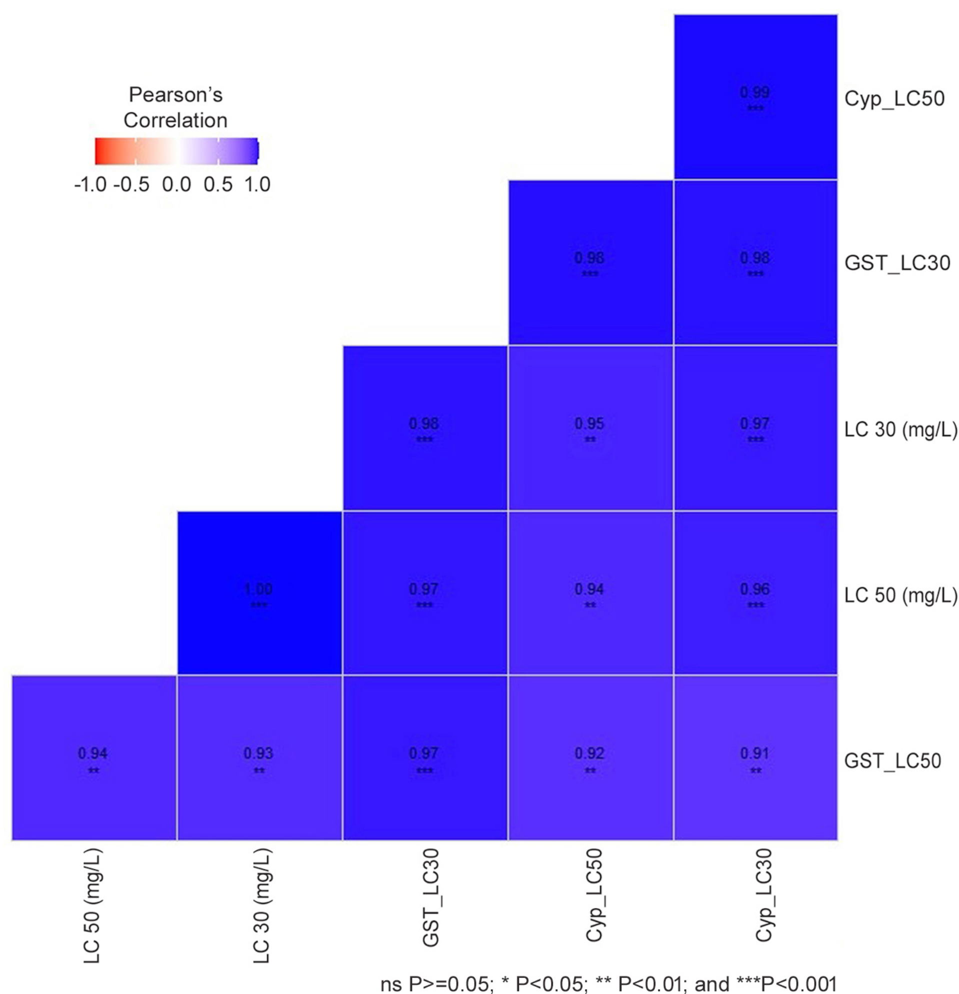

Fig 1 Correlation analysis of phosphine exposure with the detoxification enzymes: GST and CYPs in *T. castaneum.*
Correlation of LC_{50} and LC_{30} with GST and CYPs; Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

value = 14.72, df = 6, P<0.001). The MZ population showing higher resistance exhibited an elevated level of CYPs at both doses compared to the LS population (Table 2). We assumed the enhanced activity of CYPs in resistant populations to a sub-lethal and median lethal concentration of phosphine exposure due to the upregulation of the functional gene responsible for the CYPs activity (Wang *et al.* 2020). On the contrary, the KA population having the least resistance expressed lower activity for CYPs (1.22 and 0.70 nano M/min/mg at LC_{50} and LC_{30}, respectively). Our results revealed that the CYPs concentration was two and 3-fold higher than the susceptible population at LC_{50} and LC_{30}, respectively (Table 2). Similar GST, the CYPs activity escalated with enhanced resistance across collected populations of *T. castaneum.* The higher activity of the P450 enzyme and over-expression of genes of family CYPs likely associate with phosphine resistance, consistent with our study (Huang *et al.* 2019).

Correlation of phosphine toxicity with detoxification enzymes: The mean specific activity of detoxification enzyme and respective phosphine concentrations were analyzed using Pearson's correlation model (Fig 1). Our result found that the values of correlation coefficients are positively associated with GST in all *T. castaneum* populations to LC_{50} (r=0.94, P<0.01) and LC_{30} (r=0.98, P<0.001), respectively. A similar trend was observed for CYPs activity (Fig 1). Moreover, a significant variation was noticed in detoxification enzyme activities to varied lethal concentrations. The regression analysis used the lethal concentrations as an independent variable and the detoxification enzymes as the dependent variable. The R^2 values were found for GST (0.96 & 0.88) and CYPs (0.94 & 0.88) at LC_{50} and $LC_{30,}$ respectively (Fig 2). In addition, the slopes are positively associated with the GST and CYPs with different toxicity concentrations. Our correlation analysis revealed that GST and CYP enzymes were associated with the detoxification processes to phosphine exposure at lethal and sub-lethal concentrations resulting in resistance build-up in *T. castaneum*. Positive association and upregulation of GST and CYPs activities to xenobiotic exposure resulting in detoxification in *T. castaneum* are reported by Zhang *et al.* (2022). Hu *et al.* (2018) and Wang *et al.* (2020) also demonstrated that CYPs and GST levels were strongly associated with phosphine resistance.

Differential activity of detoxification enzymes to phosphine concentration: Two sample t-test was performed to assess the variability of detoxification enzymes at different concentrations. Our data implied that a significant association prevailed in CYPS activity against LC_{50} and LC_{30} (t = 5.05, df = 40, P<0.001) across the collected *T. castaneum* populations (Fig 3). On the contrary, the GST activity was non-significantly linked (t = 1.5, df = 40, P>0.05). Nevertheless, both the GST and CYPs activities were enhanced in the resistance population. Our findings are parallel with the observation of Wang *et al.* (2020).

However, a detailed understanding of the increased activity of detoxification enzymes to phosphine exposure in resistant populations must be explored. In addition,

y = 0.02 + 0.23 x
R^2 = 0.96
GST_LC30
LC30
a

y = 0.031 + 0.088 x
R^2 = 0.88
GST_LC50
LC50
b

y = 0.64 + 2.5 x
R^2 = 0.94
CYP_LC30
LC30
c

y = 1.3 + 0.74 x
R^2 = 0.88
CYP_LC50
LC50
d

Fig 2 Regression analysis of phosphine exposure with the detoxification enzymes: GST and CYPs in *T. castaneum*.
The figure depicts the regression equation (Y=MX+C) with an R^2 value. The shaded area represents the confidence interval. The black dots (●) gives the distribution of data. a, GST_ LC_{30}; b, GST_ LC_{50}; c, CYP_ LC_{30}; d, CYP_ LC_{50}.

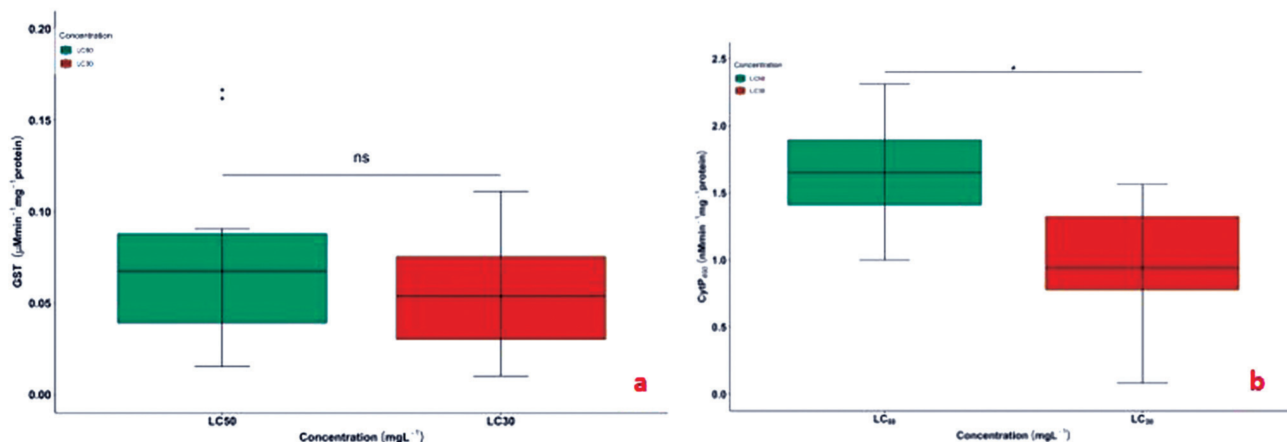

Fig 3 Differential activity of detoxification enzymes to phosphine concentration in *T. castaneum*.
The figure depicts the box plot illustration. The middle line is the median value. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. a, GST_ LC_{30}; and GST_ LC_{50}; b, CYP_ LC_{30}; and CYP_ LC_{50}.

molecular and genomic perspectives would unravel the mechanism insight into this cause.

In conclusion, six field populations of *T. castaneum* had varied levels of resistance observed in this study. Our result demonstrated that the increased activity of GST and CYPs is associated with phosphine-resistant populations. This finding strongly suggests that the detoxification enzyme plays an essential role in phosphine resistance and further

sheds light upon the intriguing approaches for effectively managing *T. castaneum* in stored commodities.

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