



## SUBLETHAL EXPOSURE ENHANCES SUSCEPTIBILITY TO PHOSPHINE AND AFFECTS CERTAIN FITNESS TRAITS IN RED FLOUR BEETLE *TRIBOLIUM CASTANEUM* (HERBST)

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### ABSTRACT

The insecticidal gaseous phosphine would fail to meet the required concentration owing to improper sealing in a majority of bulk storage units. The resulting sublethal concentration has toxicological and biological implications for the target pests. The red flour beetle *Tribolium castaneum* has developed considerable resistance to phosphine. In this study, the effect of sublethal phosphine (LC<sub>25</sub> and LC<sub>40</sub>) dose exposure is seen in the first filial generation of *T. castaneum*, where it was found to increase the susceptibility of the F<sub>1</sub> generation. The lethal concentration (LC<sub>50</sub>) of parent stock (0.076 mg/ l) was reduced to 0.059 mg/ l and 0.052 mg/ l in the F<sub>1</sub> generation, respectively in the sample prior exposed to sublethal doses of LC<sub>25</sub> and LC<sub>40</sub>. Surviving females of sublethal exposures (LC<sub>40</sub> and LC<sub>25</sub>) produced fewer offspring with a cumulative fecundity reduction of 21 and 84% respectively, compared to parent stock. The variables tested in PCA highlight the negative effect of sublethal dose on the fecundity and fitness of female adults.

**Key words:** Bioassay, biology, F<sub>1</sub> generation, fecundity, fitness trait, fumigation, median lethal dose, phosphine resistance, principal component analysis, probit, sublethal dose, *Tribolium castaneum*

Red flour beetle *Tribolium castaneum* (Herbst), is a pest of stored grains and grain products in both tropical and sub-tropical regions of the world (Bell, 2000). It is known to infest more than 250 stored food commodities including cereals, pulses, and processed food products (Subramanyam and Hagstrum, 1996). Phosphine is found to be extremely effective at fumigating bulk grain storage units while preserving the viability of the grains (Wang et al., 2006). Phosphine has a conserved mechanism of action in insects, nematodes, and other eukaryotes (Chefurka et al., 1976). It causes inhibition of mitochondrial complex IV and disintegration of the electron transport chain. The absence of optimal airtight conditions during fumigation increases the likelihood of control failures and as well the frequency of fumigation (Chaudhry, 2000). Ever since the first report of phosphine resistance through a global survey (Champ and Dyte, 1976), a large number of stored grain pests have shown resistance to phosphine (Nayak et al., 2020). The major concern over the years for such an increase in resistance is the lack of dependable alternative fumigants (Nayak et al., 2013). Several pests, including *T. castaneum*, have developed substantial levels of resistance to phosphine in various regions of the world (Wang et al., 2006; Attia and Greening, 1981; Tyler et al., 1983; Rajendran, 1999).

Resistance to phosphine in *T. castaneum* has been

well documented in different food grains as revealed by reports across the globe. Other reports of resistance come from Oklahoma in the United States (Zettler and Cupreus, 1990), and Bangladesh (Mills, 1983). The status of phosphine resistance in *T. castaneum* was studied based on the analysis of grain samples from godowns across India (Saxena et al., 1991). Subsequent reports also showed the prevalence of widespread phosphine resistance in Indian populations (Rajendran, 1999; Rajendran and Narasimha, 1994). The sublethal concentration of phosphine creates selection pressure, enabling the treated insects to survive and altering resistance in successive generations. There is an inverse relation between the interval between treatments and the mortality of the sublethal phosphine-exposed red flour beetle (Hobbs and Bond, 1989). Sublethal doses of insecticides/ fumigants exhibit distinct physiological, developmental, and behavioral characteristics when compared to the initial population in terms of toxicity with the same pesticides (Cutler et al., 2009). Saxena and Bhatiya (1980) had earlier observed that sublethal exposure led to a decrease in egg laying by *T. castaneum*. Studies by Ridley et al. (2012) further revealed that sublethal phosphine exposure reduced the offspring production of strongly resistant *T. castaneum*. Most offspring suppression was observed when both sexes were subjected to phosphine, and the least suppression was observed when only the

males were fumigated. Similar studies on the effect of sublethal concentration of phosphine on a stored grain pest in general or *T. castaneum* in particular is lacking in India in recent years. Therefore, the current study examines the variation in susceptibility and biological parameters to phosphine in parent and  $F_1$  generation vis-à-vis exposure to phosphine at two sublethal levels i.e.,  $LC_{25}$  and  $LC_{40}$ .

### MATERIALS AND METHODS

The investigations were carried out at the Division of Entomology, Indian Agricultural Research Institute, New Delhi using the parent stock of *T. castaneum*. A laboratory population of red flour beetle *T. castaneum* was maintained as parent stock on wheat flour added with baker's yeast (5%). The wheat flour used for rearing was sterilized in an oven at 60°C for two hours. This sterilized wheat flour was used to raise the test insects in muslin-covered glass jars (15 x 10 cm). The insect cultures were maintained in insect growth chambers at temperatures of 27±1°C and 75±5% RH. Adult beetles of *T. castaneum* (about 200-250) were allowed to lay eggs in glass jars containing wheat flour. The eggs were separated after five days from the date of release, and a fresh batch of adult insects was grown in a glass jar until the pupa stage. Phosphine bioassay on the laboratory population of *T. castaneum* was conducted as per FAO protocol using aluminum phosphide- 3 g tablet containing 56% (F) a.i. (Anonymous, 1975). The gas was captured in the collecting tube over 12-24 hr, and its concentration was quantified in a gas chromatograph using the procedure described by Daghlish et al. (2002). Phosphine bioassays were performed in gas-tight glass desiccators (as the fumigation chamber) fitted with a rubber septum in the lid. Two-week-old adult beetles of *T. castaneum* (30 for each concentration replicated thrice) were put in plastic cups and placed inside the fumigation chambers). A gas-tight Hamilton syringe was used to inject the required amount of phosphine. A glass desiccator containing plastic cups of beetles without exposure to phosphine was maintained as an untreated control. After the required exposure period (24 hr), the plastic cups were extracted from the desiccators under a fume hood cupboard. The beetles were fed with a small quantity of culture medium in a plastic cup and were maintained for 7 days at 27°C and 75% RH and the mortality was assessed (Jagadeesan and Nayak, 2017).

The  $LC_{50}$  was calculated using the log concentration-probit mortality curves generated for the parent stock population, and the  $LC_{25}$  and  $LC_{40}$  concentrations were

deduced from the log concentration-probit mortality curves generated for the parent stock population. Two sublethal doses viz.,  $LC_{40}$  (0.055 mg/l) and  $LC_{25}$  (0.033 mg/l) were chosen for this study. A batch of 200 adult beetles of *T. castaneum* was exposed to  $LC_{40}$  and  $LC_{25}$  dosages separately. Survivors of these treatments and were designated as  $LC_{40}$  and  $LC_{25}$  batches, respectively and were maintained separately for further analysis. The following crosses were done between parent stock,  $LC_{40}$ , and  $LC_{25}$  batches: Parent stock x Parent stock,  $LC_{40}$  x  $LC_{40}$ ,  $LC_{40}$  x  $LC_{25}$ ,  $LC_{25}$  x  $LC_{25}$ ,  $LC_{40}$  x parent stock,  $LC_{40}$  x parent stock, Parent stock x  $LC_{40}$ ,  $LC_{25}$  x parent stock, Parent stock x  $LC_{25}$ . The investigation of biological parameters was carried out in the  $F_1$  progenies of the crosses: Parent stock x Parent stock,  $LC_{40}$  x  $LC_{40}$ ,  $LC_{25}$  x  $LC_{25}$ . The biological parameters such as number of eggs laid (E), number of larvae (NL), number of pupae (NP), larval period (LD), pupal period (PD), pupation % (P%), adult emergence (%), and number of emerged adult males (ME) and females (FE) were recorded for the three cross combinations: parent stock x parent stock,  $LC_{25}$  x  $LC_{25}$ , and  $LC_{40}$  x  $LC_{40}$ . Thirty newly emerged beetles of *T. castaneum* were placed in plastic cups separately facilitating them to mate and lay eggs. With a stereozoom microscope, the total number of eggs ( $F_1$ ) laid by each female was recorded once every five days after the commencement of oviposition. The eggs were sieved using 80 mesh sieves and allowed to hatch in the same plastic cups containing culture medium. Subsequently, after the hatching, the number of larvae and the pupa were counted; the number of days taken for larval development and pupation was recorded. Adult emergence sex-wise was recorded and % was calculated. For detailed bioassay, five or more doses were used for each population, and mortality data for all crossed and the parent populations were recorded for further calculation of  $LC_{50}$  value. Bioassay data was analyzed for estimation of  $LC_{50}$  value using log-dose probit analysis (Finney, 1971) using Polo Plus 2.0 (Leora Software, Petaluma, CA). Data were analyzed by SPSS 2.0 software and the Duncan test ( $p < 0.05$ ) was used. PCA analysis ('R' software, R studio) was used for the analysis of biological parameters vis-à-vis phosphine susceptibility.

### RESULTS AND DISCUSSION

The log concentration-probit mortality curve was generated for the parent stock population and the  $LC_{50}$  of the parent population was estimated to be 0.076 mg/l. Similarly, the  $LC_{25}$  and  $LC_{40}$  concentrations were computed as 0.033 mg/l and 0.055 mg/l, respectively

(Table 1). The  $F_1$  population of  $LC_{40}$  exposure when exposed to phosphine showed a reduction in  $LC_{50}$  by 77.6% (0.059 mg/ l) than the parent population. Similarly, the  $F_1$  of the  $LC_{25}$  exposed population showed a 68% reduction in  $LC_{50}$  value (0.052 mg/ l) compared to the parent population (Fig. 1A). The survivors of all the crosses involving sublethal (either  $LC_{25}$  or  $LC_{40}$ ) with parent stock were found to be more susceptible to phosphine as revealed by significantly lower  $LC_{50}$  values (Fig. 1B). Sublethal exposure was found to increase the in *T. castaneum* susceptibility to phosphine significantly in the  $F_1$  generation and was found significantly different in many of the crosses. There was a marginal decrease in susceptibility to phosphine in all the filial generations of all crosses involving sublethal-exposed

individuals as either of the parents. This increased susceptibility could be owing to the suppression of energy-metabolizing respiratory enzymes. The in-vitro treatment of phosphine with mitochondria derived from rat liver and insects retarded the respiration rate (Chefurka et al., 1970; Price, 1980). Hobbs and Bond (1989) observed that a sublethal dosage of phosphine caused a metabolic lesion which enhanced sensitivity to future phosphine exposures. This hypothesis could assist to explain the cumulative nature of phosphine toxicity, which is unlike that of other fumigants. When compared to the control, respiration remained lowered for around four days in those insects treated once with the sublethal dose, and this time period coincided roughly with the interval during which flies were most

Table 1. Susceptibility of *T. castaneum* to sublethal doses of phosphine

Treatments	$LC_{50}$ value (mg/ l)	N	Fiducial limit (mg/ l)	Slope± S.E.	$\chi^2$	d. f	'P' value
Parent stock	0.076	315	0.06-0.096	1.585± 0.167	10.784	5	0.0558
$LC_{40} \times LC_{40}$	0.059	270	0.048-0.072	2.041± 0.2256	3.355	4	0.5002*
$LC_{25} \times LC_{25}$	0.052	270	0.043-0.063	2.176± 0.237	2.754	4	0.5997*
$LC_{25} \times P$	0.063	270	0.05-0.079	1.746± 0.211	5.066	4	0.2805*
$P \times LC_{25}$	0.065	270	0.050-0.083	1.576± 0.224	2.879	4	0.3986*
$LC_{40} \times P$	0.061	270	0.05-0.073	2.108± 0.228	4.441	4	0.3496*
$P \times LC_{40}$	0.064	270	0.052-0.079	1.868± 0.216	1.916	4	0.7512*

P-Parent stock; N-Number of insects exposed to phosphine; Crosses represent (Female  $\times$  Male). S.E- represents standard error of mean; \*Significant at  $p > 0.05$ .

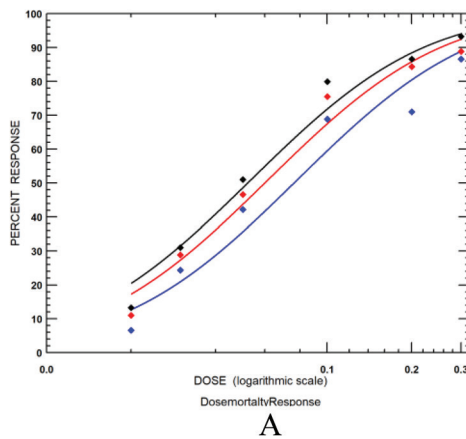


Fig. 1A. Log dose probit- line representing dose mortality response curve of parent stock,  $LC_{25}$  and  $LC_{40}$  crosses. Here, blue line (-) represents parent, red line (-) represents  $LC_{25}$  and black line (-) represents  $LC_{40}$  population's dose-response.

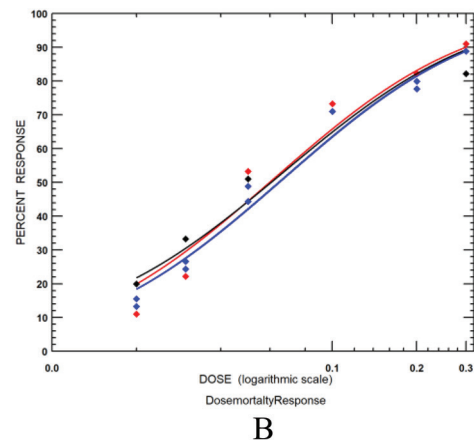


Fig. 1B. Log dose probit- line representing dose mortality response curve of parent  $\times$   $LC_{25}$ , parent  $\times$   $LC_{40}$ ,  $LC_{25}$   $\times$  parent and  $LC_{40}$   $\times$  parent crosses. Here, red line (-) represents  $LC_{40}$   $\times$  parent, blue line (-) represents parent  $\times$   $LC_{40}$  and  $LC_{25}$   $\times$  parent and black line (-) represents parent  $\times$   $LC_{25}$  population. Both these curves were generated by using six different concentrations of phosphine 0.02, 0.03, 0.05, 0.1, 0.2 and 0.3 mg/ l and a set of untreated population (control) was used to correct the mortality using Abbot's formula. This curve was generated by Polo Plus 2.0 software.

sensitive to the retreatment reported previously (Hobbs and Bond, 1989). PCA results in our study also reveal the reduction in  $LC_{50}$  and/or negative effect of sublethal exposure on toxicity in the  $LC_{25} \times LC_{25}$  population followed by  $LC_{40} \times LC_{40}$  (Fig. 2) in comparison to parent  $\times$  parent cross. Thus, it is evident from these studies that sublethal exposures of phosphine leave a transgenerational impact on the susceptibility to phosphine in insects in future exposures.

In each population involving crosses: parent stock  $\times$  parent stock,  $LC_{25} \times LC_{25}$ , and  $LC_{40} \times LC_{40}$ , thirty insects

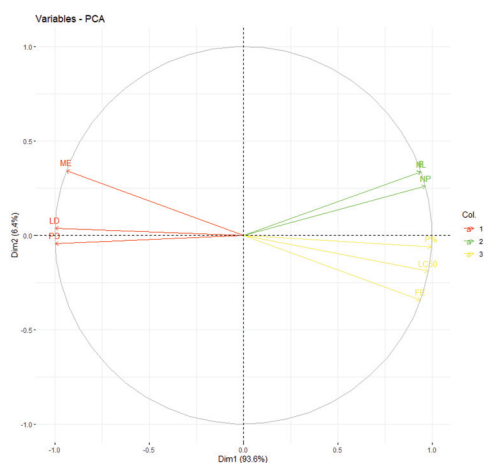


Fig. 2. Principal component analysis (PCA) score plot of data [Median lethal concentration ( $LC_{50}$ ), Fecundity (F), number of larvae (NL), number of pupae (NP), pupation % (P%), male emergence (ME), female emergence (FE), larval duration (LD), and pupal duration (PD)] used to determine the parameters contributing the most for the separation of  $LC_{25} \times LC_{25}$  (Red line-; 1),  $LC_{40} \times LC_{40}$  (Green line-; 2) and Parent stock  $\times$  Parent stock (yellow line-; 3) treatments. A total of 24 data points (8 parameters and 3 replicates) was used for PCA analysis.

were sampled for estimation of biological parameters in the first filial generation ( $F_1$ ). The number of eggs recorded in parent stock (26.61) varied significantly from the  $LC_{25}$ -exposed population (4.35) with  $T$  stat=3.60;  $P < 0.05$ . The number of larvae in the parent stock (14.36) varied significantly from the  $LC_{40}$  (11.85) ( $t$  stat=13.08,  $P < 0.01$ ) and  $LC_{25}$  (3.58) ( $t$  stat=6.95,  $P < 0.05$ ) populations. The larval and pupal phases were found to be significantly longer in the crosses involving sublethal exposures than that of the parent stock and these values were significantly higher than that of the parent population. Fecundity was reduced in  $LC_{25}$  and  $LC_{40}$  exposed populations when compared to the parent stock (Table 2).

Sublethal phosphine exposure reduced the number of offspring produced by *T. castaneum* survivors and this fact has been well documented. It was observed that the number of eggs produced by the population exposed to a sublethal phosphine dose was significantly lower than the parent stock. Fumigation and phosphine resistance reduced fertility in *T. castaneum*, according to Saxena and Bhatiya (1980). Results of the present study show that the fecundity, egg hatchability and pupal emergence were significantly lower in populations exposed to sublethal doses of  $LC_{25}$ , and  $LC_{40}$  (Table 2). When phosphine-exposed males were mated with non-exposed females of *R. dominica*, Ridley et al. (2012) observed a transient reduction in offspring production. There is strong evidence for fitness costs associated with phosphine resistance among the beetle pests in stored grains (Pimentel et al., 2007). Lu et al. (2020) reported that sublethal fumigation with phosphine delayed oviposition and inhibited egg hatching and emergence rates of psocid, *Liposcelis entomophila*. Earlier, studies

Table 2. Biological parameters in  $F_1$  survivors of *T. castaneum* exposed to sublethal doses of phosphine

Parameters	P $\times$ P	$LC_{40} \times LC_{40}$	$LC_{25} \times LC_{25}$
Number of eggs	26.16 $\pm$ 2.34 <sup>a</sup>	21.10 $\pm$ 2.10 <sup>b</sup>	4.35 $\pm$ 1.06 <sup>c</sup>
Number of larvae	14.36 $\pm$ 2.8 <sup>a</sup>	11.85 $\pm$ 2.05 <sup>b</sup>	3.58 $\pm$ 1.8 <sup>c</sup>
Larval period (days)	27.33 $\pm$ 2.4 <sup>b</sup>	31.33 $\pm$ 2.3 <sup>a</sup>	34.33 $\pm$ 3.4 <sup>a</sup>
Number of pupae	13.14 $\pm$ 2.4 <sup>a</sup>	9.99 $\pm$ 2.5 <sup>b</sup>	2.84 $\pm$ 1.0 <sup>c</sup>
Pupation (%)	91.50 $\pm$ 2.015 <sup>a</sup>	84.30 $\pm$ 1.241 <sup>b</sup>	79.32 $\pm$ 1.736 <sup>c</sup>
Pupal period (days)	15.00 $\pm$ 2.07 <sup>c</sup>	17.00 $\pm$ 2.24 <sup>b</sup>	19.00 $\pm$ 2.12 <sup>a</sup>
Adult emergence (%)	76.78 $\pm$ 1.381 <sup>b</sup>	78.67 $\pm$ 1.069 <sup>a</sup>	49.29 $\pm$ 1.077 <sup>c</sup>
Number of adult males	52.13% <sup>a</sup>	56.43% <sup>b</sup>	57.13% <sup>c</sup>
Number of adult females	47.87% <sup>a</sup>	43.57% <sup>b</sup>	42.87% <sup>c</sup>

Data represents mean value of 15 replications along with its standard error of mean. In row, means followed by different letters significantly different by Duncan test ( $p < 0.05$ ). P- represents 'parent stock'.

had also documented that phosphine fumigation inhibited egg development of psocid *L. bostrychophila* (Nayak et al., 2003) delayed and reduced hatching in *Cryptolestes ferrugineus*, *Lasioderma serricorne* and *Oryzaephilus surinamensis*. Thus, phosphine might cause damage to the reproductive system of adults of *T. castaneum* leading to diminished oviposition.

Principal component analysis (PCA) facilitated reducing the variables and summarizing them into two meaningful dimensions that helped the graphical visualization of the data. PCA was applied to the data set to identify the main determinants of the differences between the three major crosses i.e., parent stock x parent stock,  $LC_{40} \times LC_{40}$ , and  $LC_{25} \times LC_{25}$  (Fig. 2). The different crosses regimes were clearly separated along PC1, with parent x parent samples displaying the highest PC1 score (3.64),  $LC_{25} \times LC_{25}$  population displaying the lowest PC1 scores (-3.46), while  $LC_{40} \times LC_{40}$  populations showed intermediate PC1 score (-0.18) (Fig. 3). The results of PCA in our study confirm the reduced oviposition, and reduced female fitness (less female emergence) along with the increased larval and pupal duration in  $LC_{25} \times LC_{25}$  followed by  $LC_{40} \times LC_{40}$  as compared to parent x parent. Guedes et al. (2017) suggested the need for further investigations on phosphine as they are directly or indirectly involved in oxidative stress in cells and energy transfer systems in insects. The paradoxical phenomenon called “sweet spot” a characteristic of phosphine fumigation has been recorded in several species of stored product insect pests irrespective of the level of population resistance to phosphine (Lampiri et al., 2021). As the elevated concentration of phosphine would result in increased survival at the ‘sweet spot’ of fumigation, sublethal exposure of phosphine in insects holds promise from a management perspective as well. Hence, further investigations are needed to assess the impacts of

sublethal fumigation with the widespread prevalence of phosphine resistance that poses a threat to the sustainable management of stored product insect pests.

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#### AUTHOR CONTRIBUTION STATEMENT

RHV carried out the experiments, and wrote the manuscript; SS, and CS, SMN– conceptualized the research and outlined the objectives and expectations; APS- assisted RHV in carrying out experiments and analyzing the data; SS- corrected the manuscript, reviewed and edited the manuscript.

#### CONFLICT OF INTEREST

No conflict of interest.

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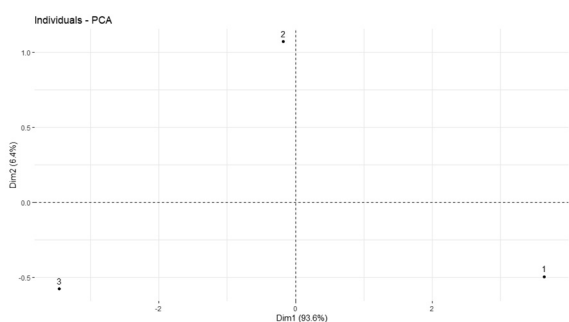


Fig. 3. Different crosses regimes separated under distinct principal components in PCA. The values are denoted with respect to PC1. Here 1-denotes Parent x Parent; 2-denotes  $LC_{40} \times LC_{40}$ ; 3- denotes  $LC_{25} \times LC_{25}$ .

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