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Original Research Paper

Oxidative Stress Biomarkers in *Helicoverpa armigera* (Lepidoptera: Noctuidae) Exposed to Heavy Metal Contamination

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Key Words

Oxidative stress, Heavy metals, *Helicoverpa armigera*, Antioxidant enzymes, Biomarkers.

Abstract

The concentration of heavy metal is quite hazardous to the life of people and the activities of insects. The study of the biomarkers of oxidative stress in the larvae of the *Helicoverpa armigera* was conducted in this experiment under the dietary unnatural conditions of cadmium (Cd), lead (Pb) and mercury (Hg) toxicity. Third larvae were fed 0, 5, 10, 25 and 50mg kg $^{-1}$ of metal diets respectively during the 7 days. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and malondialdehyde (MDA) are some of the biomarkers assessed in larval tissues. The dose-dependent accumulation of metals reached a maximum with Hg with the highest bioaccumulation factor of 4.8 0.3 at 50mg kg^{-1}. The enzyme activity of the antioxidants was biphasic, SOD increased to 25mg kg^{-1} (Cd: 48.63.2μMg^{-1} protein, 2.1folds over control) and then decreased at 50 mg kg^{-1}. The same case happened with CAT and GPx where the max activities were 10-25mg kg^{-1}. At 50mg kg^{-1} Cd, there was statistically significant ($p< 0.05$) increase in MDA of all metals to 3.4-0.2nmol mg^{-1} protein (3.8-fold increase). The GSH loss was metal selective and Cd elicited highest loss (62.3±4.1% at 50mg kg^{-1}). The principal component analysis revealed that there was a set of biomarkers of each metal. The findings show that the biomarkers of oxidative stress prove useful in differentiating between heavy metals exposures and provide early alerts as to environmental contamination of insects. These results indicate the possibility of using H. armigera as a bioindicator species to detect heavy metal pollution in agroecosystems. The research will have a good contribution to assessment of environmental risks and sustainable management of pests and soil.

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Introduction

One of the environmental issues that is common in agricultural systems the world over is heavy metal contamination. The industrial activities, mining activities, and the careless use of pesticides have led to an increment in cadmium (Cd), lead (Pb) and mercury (Hg) in the soils and crops (Yuan et al., 2016). The insects (more than two-thirds of biodiversity on the land) are relevant bioindicating factors of environmental pollution due to their high sensitivity to contaminants and their position in food webs (Mogren & Trumble, 2010). Lepidopteran species like *Helicoverpa armigera* used in agriculture as agricultural pests are also exposed to the incessant exposure of heavy metals through the contaminated host plant and would serve good model organisms in ecotoxicological research.

The pathophysiology of toxicity induced by the heavy metals takes place through a number of various means and oxidative stress is one of the primary mechanisms of cell damage. Excess metal ions catalyze the Fenton-type reactions to form reactive oxygen species (ROS) including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Jomova & Valko, 2011). These ROS overwhelm cell antioxidant defenses resulting into lipid peroxidation, protein oxidation, and DNA damage. The insects possess a complex antioxidant system comprising of enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (glutathione, ascorbic acid) parts of antioxidants that remove ROS and reestablish the redox balance (Felton & Summers, 1995).

The study has documented oxidative and stress responses of aquatic insects, when exposed to metals but the research has not studied the response of terrestrial lepidopterans (Mann et al., 2012). Most of the experiments focused on single metal exposures under acute levels, which was unrealistic in the real field of multiple metal exposures under sub-lethal levels (Gupta et al., 2024). To determine the comparative sensitivity of different biomarkers on different metals, there is no systematic examination of the sensitivity levels of different biomarkers, as well. This ignorance limits the application of insects in monitoring programs in the environment.

The present study was predetermined by the purpose to evaluate the profile of oxidative stress biomarkers in the larvae of H. armigera, which were exposed to three heavy metals that were of environmental significance at sub-lethal concentrations. Included: (i) determining dose-dependent accumulation of Cd, Pb and Hg in larval tissues; (ii) determining activities of major antioxidant enzymes (SOD, CAT, GPx) and levels of oxidative damage endogenous (MDA, GSH); (iii) the most sensitive biomarkers in recognizing a metal-specific response. The paper provides a baseline of information on insect-based biomonitoring protocol, mechanistic basis of metal toxicity of terrestrial insects. Environmental stressors, particularly heavy metal contamination, have been shown to significantly alter biological systems and physiological processes in living organisms (Kushwaha et al., 2025), suggesting potential

impacts on endocrine regulation and reproductive functions in sensitive species such as social insects. The ecotoxicology of heavy metal is important, in addition to measuring ecosystem health and agricultural sustainability. Since *Helicoverpa armigera* is one of the principal pest species in contact with the crop systems, its physiological behavior in pollutants can be used to indicate the level of environmental stress in agroecosystems.

Materials and Methods

Rearing Conditions and Experimental Insects

The eggs of *Helicoverpa armigera* were acquired at the National Centre of Integrated Pest Management, New Delhi, and were kept in the insectary at 27 ± 2°C, 65 ± 5°C relative humidity and 14:10 L:D photoperiod. Third instar larvae (body weight 30-35 mg) were raised on a normal artificial diet (Spehar et al., 1978). When selecting uniform-sized third instar larvae to use in experiments, the size of the larvae was standardized to avoid differences in developmental biomarker responses.

Design and Metal Exposure

The study was carried out in 2023-24, and the study design was entirely randomized. Three heavy metals (cadmium chloride, lead acetate and mercuric chloride) were added separately to the artificial diet (0 (control), 5, 10, 25, and 50 mg kg^{-1} fresh diet). The chosen levels of metal were to reproduce the levels of contamination that would be environmentally relevant in agricultural soils that are in most cases due to industrial and agrochemical activities. These concentrations mimicked environmentally relevant levels of contamination at agricultural soils (Nagajyoti et al., 2010). Every treatment had five replications containing 20 larvae in each replication (n=100). The larvae were left to feed ad libitum over a 7-day period and fresh diet supplied on daily basis.

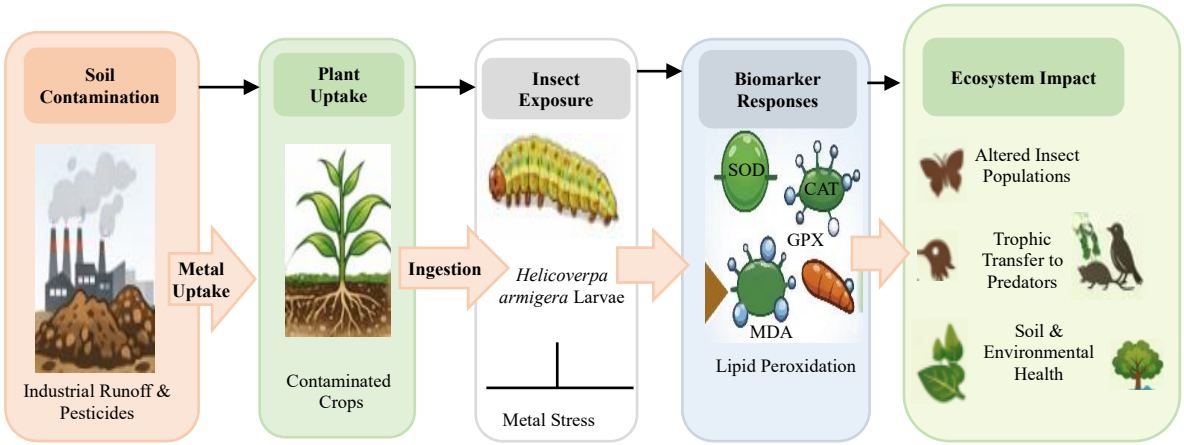

Figure 1: Environmental Biomonitoring Framework of Heavy Metal Contamination

Figure 1 shows the conceptual model of environmental biomonitoring of the bioindicator species *Helicoverpa armigera*. The illustration shows the route of the heavy metal pollution caused by the industrial discharge and agricultural chemicals to soils, which is then taken up by plants and finally consumed by the insect larvae. Physiological stress is caused by

metals in insects with a resulting biomarker response (antioxidant enzyme activity (SOD, CAT, GPx), glutathione depletion (GSH), and lipid peroxidation (MDA)). These biochemical transformations are early solutions to environmental pollution. The framework also emphasizes the wider environmental ecological considerations, such as the change of insects, the possible trophic cascade to higher organisms, and soil and ecosystem health effects, hence, the importance of insects in environmental quality and sustainability.

Metal Accumulation Analysis

Following exposure, larvae were starved 24 h to empty gut contents, washed in deionized water, and blotted. Each replication was pooled with ten larvae which were weighed and digested in 80o C in a mixture of nitric acid and perchloric acid (4:1v/v). The concentrations of metals were analyzed with flame atomic absorption spectrophotometer (Analyst 400, PerkinElmer) according to the standard procedures. Bio accumulation factor (BAF) was determined as metal concentration of the larvae divided by dietary concentration.

Biomarker Assays

Homogenization of larval tissues was done with ice-cold 0.1M phosphate buffer (pH 7.4) to which 0.15M KCl had been added and centrifuged at 10,000g at 4°C. All the assays were performed using the supernatant. The content of protein was estimated using Bradford method in terms of bovine serum albumin as a standard.

SOD (EC 1.15.1.1) activity was determined using its capacity to suppress photochemical dereduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp & Fridovich, 1971). The quantity of enzyme required to inhibit reduction of NBT 50% was considered one unit of SOD. The CAT (EC 1.11.1.6) was measured by observing the degradation of H_2O_2 at 240 nm (Aebi, 1984). The activity of glutathione peroxidase (GPx; EC 1.11.1.9) was determined with the cumene hydroperoxide as a substrate at the wavelength of 340 nm (Flohé and Günzler, 1984).

At 412 nm, reduced glutathione (GSH) was estimated by reaction with 5, 5'- dithiobis (2- nitro benzonic acid) (Ellman, 1959). The diagnosis of lipid peroxidation was evaluated through enhancing the presence of malondialdehyde (MDA) by using thiobarbituric acid reaction at 532 nm (Ohkawa et al., 1979).

Statistical Analysis

Two-way analysis of variance (ANOVA) was used in data analysis where metal type and concentration were used as factors. Tukey honestly significant difference (HSD) test was conducted at p=0.05 to make mean comparisons. All statistical calculations were done in IBM statistics SPSS version 26.0. PAST software version 4.03 was used to perform principal component analysis (PCA) to identify the ability of biomarker patterns to discriminate between treatments.

Results and Discussion

With all the three heavy metals, there was a significant increase in metal accumulation in

larval tissues with dietary concentration ($p<0.01$) (Table 1). Mercury had the highest potential of bioaccumulation with the BAF values of 2.1 ± 0.2 at 5mg kg^{-1} and 4.8 ± 0.3 at 50mg kg^{1}. Cadmium and lead exhibited moderate and low BAF (1.3-2.7 and 0.8-1.6) respectively suggesting a difference in uptake and excretion pathways. These variations are metal-specific physicochemical characteristics and how interact with insect detoxification systems. The accumulated patterns are in line with the earlier reports on Spodoptera litura fed a contaminated diet (Wang et al., 2023). These accumulation patterns show that there may be a transfer of the heavy metals across the trophic levels and therefore cause a threat to high organisms such as predators and birds in agricultural ecosystems.

The survival of larvae reduced dose-dependently, and high mortality rates were recorded at the 50mg kg^{-1} of all metals (Cd: 28.3±2.5%, Pb: 21.7±1.8%, Hg: 35.0±2.9% mortality). Exposure to metals also lowered the larval development, with the mean weight increment kg^{-1} (50) declined by 41.2%, 33.8%, and 52.4% of Cd, Pb and Hg respectively, relative to controls. This growth retardation is an energy re-distribution towards detoxification and cell repair activities at the cost of biomass increase.

There were metal- and dose-dependent biphasic responses of antioxidant enzyme activities (Table 2). The increase in SOD was significant to 25mg kg^{-1} of all metals, and was found to be optimally induced in Cd-treated larvae (48.6 ± 3.2 U mg^{-1} protein, 2.1 times higher than control). This elevation is a compensative process to eliminate O_2^- radicals formed in the processes involving metal catalysis. Nevertheless, SOD activity decreased drastically at 50mg kg^{-1}, indicating that the activity was inactivated, or that the antioxidant potential was overused. The same trends were observed in Cd-exposed Chilo suppressalis in which the induction of SOD was reached at moderate levels before repressed at potentially lethal levels (Zhu et al., 2021). These physiological disruptions may impair insect fitness, potentially altering pest population dynamics and ecological balance in contaminated environments (Nanaware et al., 2025).

The highest stimulated catalase activities of Cd (34.7 ± 2.4 μmol H_2O_2 min^{-1} mg^{-1} protein) and Pb (31.2 ± 2.4 μmol H_2O_2 min^{-1} mg^{-1} protein) were observed at 10mg kg^{-1}, and then decreased. Exposure to mercury led to progressive inhibiting effects of CAT at a concentration of 10mg kg^{-1} and above which showed direct damage to the enzyme. Similar tendencies occurred with GPx where Cd (12.8 ± 0.9 nmol NADPH min^{-1} mg^{-1} protein) and Pb (11.5%) had a peak activity of 25mg kg^{-1}. The bifurcated response of the GPx in H_2O_2 and lipid hydroperoxide detoxification is indicated by the differential reaction to H_2O_2.

The level of malondialdehyde which is a sign of lipid peroxidation rose steadily in all the treatments (Figure 1). The worst oxidative effects were the result of cadmium, where MDA was 3.4 ± 0.2 nmol mg^{-1} protein at 50mg kg^{-1} (3.8-fold greater than control). Lead and mercury generated moderate but considerable increases

(2.9 ± 0.2 and 3.1 ± 0.2 nmol mg^{-1} protein, respectively, at 50mg kg^{-1}). The accumulation of MDA even at the level at which antioxidant enzymes were induced is evidence of the fact that ROS generation overcame the protective ability. This observation and other works on Bombyx mori regarding Cd levels reported that the pro-oxidant/antioxidant balance was destabilized (Lin et al., 2021; Zizolfi et al., 2024).

There were metal specific depletion patterns observed in reduced glutathione content (Table 3). Exposure to cadmium led to considerable depletion of GSH as at 10mg kg^{-1} and above, with 62.3 ± 4.1% depletion at 50mg kg^{-1}. The effect of mercury was also less damaging to GSH (45.2 ± 3.8% at 50 mg kg^{-1}). Unexpectedly, low concentrations of Pb (5–10mg kg^{-1}) temporarily affected GSH content (12.4 ± 1.1 µg mg^{-1} protein at 10mg kg^{-1} vs. 10.8 ± 0.9 µg mg^{-1} protein in the control) indicating the stimulation of glutathione production pathways. This protective effect however failed in higher concentrations with GSH reducing to 7.6 ± 0.7 µg mg owed down to protein at 50mg kg^{-1}. The high metal loads cause the GSH depletion that indicates that the GSH is consumed during metal chelation and serving as a cofactor in the GPx activity. Persistent oxidative stress may reduce survival and reproductive capacity, thereby influencing species distribution and ecosystem stability.

The principal component analysis showed that each metal has a specific biomarker signature (Figure 1). The two major components accounted 76.4% of variability and PC1 distinguished high dose treatments and controls and PC2, identified metal types. Treatments where there was clustering along the positive PC1 axis with high MDA and low GSH were cadmium treatments. High levels of metal accumulation and moderate levels of enzyme induction were linked to mercury treatments and intermediate levels were linked to lead exposures. This segregation implies that it is possible to distinguish between types of heavy metal contamination using a multi-biomarker method, using insects collected in the field. The ability to distinguish metal-specific stress signatures supports the use of insects as cost-effective bioindicators for environmental monitoring programs.

The responses of the biomarker observed are of great importance to the population dynamics and health of the insects as well as ecosystem. When the degree of oxidative stress is sub-lethal, it may undermine immune activity, decrease fecundity and expose an organism to pathogen exposure, which may result in population drops despite a low rate of mortality (Galloway & Handy, 2003). The bi-phasic pattern of the enzyme response indicates that middle contamination can be concealed by the adaptive response, and it is difficult to notice the incidence early. Nevertheless, MDA remains high in all metals, and thus lipid peroxidation is a dependable predictor of oxidative damage independent of the type of metal.

Conclusion

The current experiment shows that sub-lethal dietary cadmium, lead, and mercury exposure causes considerable oxidative stress in larvae of *Helicoverpa armigera* in a metal-dependent and

dose-dependent fashion. Of the metals tested, mercury was found to have the greatest potential to bioaccumulate, whereas cadmium had the greatest oxidative potential of all, as indicated by high levels of lipid peroxidation, high levels of reduced glutathione depletion, and high levels of antioxidant enzyme inhibition. The bifaceted reaction of the main antioxidant enzymes (SOD, CAT and GPx) means that at low to moderate doses, there is an initial adaptive mechanism of protection, and subsequently, the defense mechanism of antioxidants is suppressed in higher doses and this shows that there is a collapse of the system of antioxidant defense in excessive exposures. Notably, these results emphasize the ecological and environmental differences of biomarkers of oxidative stress in insects. The physiological disturbances that are observed can impact the survival, fitness, and population of insects and thus the trophic interactions and ecosystem stability in polluted agroecosystems. The biomarker responses and metal-specific signatures are also unique to justify the usefulness of H. armigera as a bioindicator species to detect heavy metals pollution. On the whole, the given study offers essential information on the topic of environmental risk assessment and the necessity of incorporating the use of biomarkers into the sustainable agricultural and environmental management practices. Early warning of the presence of heavy metal contamination with the help of biological indicators can help to intervene in time, minimize risks to the environment, and lead to better soil health and food safety.

Table 1: Heavy Metal Accumulation, Bioaccumulation Factor (BAF), Survival and Weight Gain In *Helicoverpa armigera* Larvae Exposed to Different Metal Concentrations for 7 Days

Metal	Concentration (mg kg^{-1})	Larval Metal Content (µg g^{-1})	BAF	Survival (%)	Weight Gain (mg $larva^{-1}$)
Control	0	0.02±0.01	-	98.3±1.2	52.4±3.8
Cd	5	6.8±0.5	1.36±0.10	95.0±1.8	48.7±3.2
Cd	10	14.2±1.1	1.42±0.11	91.7±2.1	44.3±3.0
Cd	25	33.8±2.4	1.35±0.09	85.0±2.5	38.2±2.8
Cd	50	67.5±4.2	1.35±0.08	71.7±2.8	30.8±2.5
Pb	5	4.2±0.3	0.84±0.06	96.7±1.5	49.5±3.4
Pb	10	8.9±0.7	0.89±0.07	93.3±1.9	46.8±3.1
Pb	25	22.4±1.8	0.90±0.07	88.3±2.3	41.5±2.9
Pb	50	48.2±3.5	0.96±0.07	78.3±2.6	34.7±2.7
Hg	5	10.5±0.8	2.10±0.15	93.3±1.9	47.2±3.3
Hg	10	23.6±1.9	2.36±0.18	88.3±2.3	41.8±3.0
Hg	25	58.2±4.1	2.33±0.16	80.0±2.7	35.5±2.8
Hg	50	120.4±8.6	2.41±0.17	65.0±3.1	24.9±2.3

Values are mean ± SE of five replications (n=100 larvae per treatment). Statistical significance ($p<0.05$) was observed for all parameters across treatments.

Values are mean ± SE of five replications. Enzyme activities are expressed per mg protein. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase.

Table 2: Activities of Antioxidant Enzymes in *Helicoverpa armigera* Larvae Exposed to Heavy Metal Contaminated Diets

Metal	Concentration (mg kg⁻¹)	SOD (U mg⁻¹ protein)	CAT (µmol min⁻¹ mg⁻¹ protein)	GPx (nmol min⁻¹ mg⁻¹ protein)
Control	0	23.2±1.8	18.5±1.4	6.8±0.5
Cd	5	32.4±2.5	26.8±2.1	8.9±0.7
Cd	10	41.6±3.2	34.7±2.8	11.2±0.9
Cd	25	48.6±3.2	29.4±2.3	12.8±0.9
Cd	50	31.2±2.4	22.1±1.8	8.4±0.6
Pb	5	28.7±2.2	24.3±1.9	7.6±0.6
Pb	10	36.2±2.8	31.2±2.4	9.8±0.8
Pb	25	42.8±3.1	27.6±2.2	11.5±0.8
Pb	50	35.4±2.7	21.8±1.7	9.2±0.7
Hg	5	30.5±2.3	21.4±1.7	8.2±0.6
Hg	10	38.2±2.9	19.6±1.5	9.4±0.7
Hg	25	40.8±3.0	17.2±1.4	10.6±0.8
Hg	50	33.6±2.5	15.8±1.2	8.8±0.6

Table 3: Oxidative Damage Markers in *Helicoverpa armigera* Larvae Exposed to Heavy Metal Contaminated Diets

Metal	Concentration (mg kg⁻¹)	MDA (nmol mg⁻¹ protein)	GSH (µg mg⁻¹ protein)	Protein Carbonyls (nmol mg⁻¹ protein)
Control	0	0.9±0.1	10.8±0.9	2.1±0.2
Cd	5	1.4±0.1	9.2±0.7	2.8±0.2
Cd	10	1.9±0.2	7.8±0.6	3.6±0.3
Cd	25	2.6±0.2	6.2±0.5	4.5±0.3
Cd	50	3.4±0.2	4.1±0.3	5.2±0.4
Pb	5	1.2±0.1	11.2±0.9	2.4±0.2
Pb	10	1.6±0.1	12.4±1.1	3.1±0.2
Pb	25	2.1±0.2	9.8±0.8	3.9±0.3
Pb	50	2.9±0.2	7.6±0.7	4.8±0.4
Hg	5	1.3±0.1	10.2±0.8	2.6±0.2
Hg	10	1.8±0.2	9.1±0.7	3.4±0.3
Hg	25	2.4±0.2	7.5±0.6	4.3±0.3
Hg	50	3.1±0.2	5.9±0.5	5.0±0.4

Values are mean ± SE of five replications. MDA: malondialdehyde; GSH: reduced glutathione.

Authors' contribution

Conceptualization of Research

N.K.T., V.K.S., R.T.

Designing of the Experiments

N.K.T., V.K.S., A.L.C.

Contribution of Experimental Materials

A.L.C., P.R.B., M.C.

Execution of Field/Lab Experiments and Data Collection

N.K.T., V.K.S., S.G.

Analysis of Data and Interpretation

N.K.T., M.C., P.R.B.

Preparation of the Manuscript

N.K.T., V.K.S., R.T., S.G.

Declaration

The authors declare that do not have any conflict of interest.

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