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Insecticides and Bio-insecticides Modulate the Glutathione-related Antioxidant Defense System of Cowpea Storage Bruchid (*Callosobruchus maculatus*)

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ABSTRACT: The possible cellular involvements of cowpea storage bruchid (*Callosobruchus maculatus* (Fab.) [Coleoptera: Chrysomelidae]) glutathione and its related enzymes system in the cellular defense against insecticides (Cypermethrin and λ -cyhalothrin) and bio-insecticides (ethanolic extract of *Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suaveolens* leaves, and *Jatropha curcas* seed) were investigated. The results showed that the effect of insecticides and bio-insecticides on the *C. maculatus* is a function of oxidative and nitrosative stresses generated in vivo. A significant ($p < 0.05$) increase in carbonyl protein (CP) and lipid peroxidation (LPO) contents in bio-insecticides and insecticides exposed groups compared to the control indicates the extent of vital organs damage. These stresses caused similar and significant increase of glutathione peroxidase and glutathione synthetase in response to insecticides and bio-insecticide exposure in a dose-dependent manner. There was no post-translational modification of glutathione transferases expression induced. The alterations of the insect glutathione-dependent antioxidant enzyme activities reflect the presence of a functional defense mechanism against the oxidative and nitrosative stress and are related firmly to the glutathione demands and metabolism but appear inadequate by the significant reduction in glutathione reductase (GR) activity to prevent the damages. Exogenous application of reduced glutathione (GSH), to complement the in vivo demand, could not protect against the onslaught.

KEYWORDS: antioxidant enzyme, *Callosobruchus maculatus*, glutathione, lipid peroxidation, oxidative stress

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Introduction

Cowpea, *Vigna unguiculata* L. (Walp.), is the most economically and nutritionally important indigenous African grain legume crop in west and central Africa.^{1,2} Cowpea is rich in protein and constitutes a staple food for people in rural and urban areas. Cowpea is also an important cash crop in the region with potential for entering commerce.² Cowpea marketing and trade is severely hampered by storage insects, especially the cowpea weevil.³

The cowpea storage bruchid, *Callosobruchus maculatus* (Fab.) [Coleoptera: Chrysomelidae], a coleopteran storage

pest, is the principal pest of cowpea and chick pea. It plagues cowpea in the storage granary and destroys the harvested grains.⁴ The bruchids lay eggs on the seed surface. The larvae feed and develop inside the seeds and emerge as adults. Even with a minor infestation at harvest, the high reproductive capacity, short life cycle, and continuous generations of the bruchid can lead to complete loss of stored grains in a few months. Traditional breeding to introduce insect resistance into cowpea cultivars has largely failed, mainly because the cowpea gene pool lacks useful resistance genes.⁵ However, successful management of stored grain insects is the final



component of the struggle to limit insect losses in agricultural production. To protect the stored beans against *C. maculatus*, many methods can be used. There has been reported usage of synthetic insecticides and plant materials as bio-insecticides, in the quest to protect this agricultural product against this insect pest.^{6,7} These commercial and traditional methods to control the bruchid have restricted value because of cost, labor, and potential toxicity. Although diverse measures have been employed to control these species, the use of insecticide remains the main and most effective means of controlling them in large-scale storage^{8–10} but it is difficult to design chemicals, which act specifically toward a given group of target insects.¹¹ Synthetic insecticides have been used for the control of *Callosobruchus* species for over four decades, with little concern about the development of resistance.⁸

However, understanding the diversity of cowpea storage bruchid family (*C. maculatus*) responses to chemical pressures (eg, bio-insecticides and insecticides) in their ecological context would represent a key challenge in developing durable pest-control strategies.^{12,13} The responses (oviposition, adult emergence, and growth performances) of the insects to the bio-insecticides and insecticides exposure earlier reported by various authors^{5,7,11} could be informative models for studying molecular mechanism of toxicity and possible and eventual adaptation and resistance. Hypothetically, accumulated physical and chemical stress acts first at the biochemical level, and that linked responses are later reflected at higher levels of biological organization.¹⁴

With this, knowledge of the molecular mechanism of toxicity to storage pest in response to various stresses becomes imperative and urgent. Despite the notable plant protective role, many herbivorous insects have evolved various resistance strategies to evade plant defense and synthetic chemicals. The chemical structure of some synthetic insecticides is comparable to that of some plant-produced compounds; the intensity of selection and the nature of insect resistance traits are likely to differ between these two types of selection pressures.¹² These pressures are heterogeneously distributed through time and space. Insects under these abiotic stresses produce some defense mechanisms to protect themselves from the harmful effect of oxidative stress generated through cytochrome P-450 metabolism of these xenobiotics.¹⁵ Reactive oxygen species (ROS) scavenging is one of the common defense responses against abiotic stresses.¹⁶ ROS scavenging depends on the detoxification mechanism provided by an integrated system of glutathione metabolism and enzymatic antioxidants.¹⁷ The major ROS scavenging activities include complex glutathione system—glutathione reductase (GR), glutathione peroxidase, glutathione transferases, and glutathione synthetase.^{18,19} Glutathione levels are regulated by several enzymes,²⁰ but mainly depend on the balance between glutathione synthesis rate, conjugation rate, oxidation rate, and reduction rate.

Given the salient roles of glutathione and glutathione systems in cellular defense against oxidative stress and its

potential utility to provide the evidence and mechanism of toxicity to xenobiotics, involvement and response of the GSH and its associated enzymes may underscore selective efficacy and possible and consequent adaptation to chemical stress. Lately, no unarguable direct evidence linking the glutathione antioxidant defense system and free radicals of cowpea storage bruchid has been provided. Therefore, good knowledge of status and response of glutathione antioxidant defense system of cowpea storage bruchid (*C. maculatus*) to chemical pressures (eg, bio-insecticides and insecticides) in their local ecological context would be needful. This would represent a key challenge in developing durable pest-control strategies and therefore add to the growing body of literature addressing the central questions of how and to what extent the glutathione antioxidant enzymatic defense system is altered in response to the insecticides and bio-insecticides exposure in *C. maculatus*.

Materials and Methods

Materials. 2,4-Dinitrophenylhydrazine (DNPH), reduced glutathione (GSH), oxidized glutathione (GSSG), *N*-ethylmaleimide (NEM), *N*-(1-naphthyl) ethylenediamine dihydrochloride, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), metaphosphoric acid, NADPH, nitroblue tetrazolium salt (NBT), and *O*-phthaldehyde (OPT) were purchased from Sigma-Aldrich, Chemie GmbH, Germany. Bradford reagent and BSA as a protein standard were obtained from Bio-Rad Laboratories, Hercules, CA, USA. Cyperforce (Cypermethrin) was a product of Ghada Chemical Ltd, China and Masters (λ -cyhalothrin) was purchased from Sinochem Ningbo Ltd, China. All other chemicals were of analytical grade. Absolute ethanol was used as a solvent in the preparation of the insecticides and bio-insecticides samples.

Insect culture and maintenance. The storage bruchid used in this study was derived from field infested cowpeas bought from Oba Market in Akure, Nigeria in September 2012. This was reared in the laboratory in clean uninfected Kilner jars containing uninfected Sokoto white cultivars of cowpeas at $28 \pm 2^\circ\text{C}$ with ambient relative humidity (50–80%) and a 12-hour photoperiod for two months. The Kilner jars were covered with muslin cloth, held tightly in place for adequate aeration of the culture, and precluded entry or exit of insects. New generations of bruchids were derived from this stock culture by infesting clean uninfested beans with 10 pairs of teneral adult bruchids.

Plant extract preparation and biological testing. The matured plant materials (*Tithonia diversifolia* leaves, *Cyperus rotundus* leaves, *Hyptis suaveolens* leaves and *Jatropha curcas* seed) were collected from the school farm of The Federal University of Technology, Akure, Nigeria in June 2012. They were properly identified, cleaned, and air-dried, and ethanolic extracts were prepared as described elsewhere.²¹ The distillate was stored at 4°C in a closed vials until used. It should be noted that ethanol used as solvent did not show any effect on insect development at the dose applied (control group).



Here, all the ethanolic extracts were conveniently and jointly referred to as bio-insecticides.

Disinfested cowpea seeds weighing 100 g, initially kept at -20°C for 72 hour later cooled to room temperature, were serially treated with various distillates of plant extracts (0, 5,000, 10,000, 15,000, and 20,000 ppm). Cypermethrin and λ -cyhalothrin concentrations used were 50, 100, 150, and 200 ppm. The cowpeas were thoroughly coated with the extract using a glass rod and then allowed to air-dry for about three hours prior to infestation with a-day-old 40 adult bruchids. Each experimental plate was prepared in triplicate. The response of the bruchid was observed for four days.

Enzyme extractions and assays. Frozen insects were ground in liquid nitrogen with a mortar and pestle. Crude enzyme extract for assay was prepared by the method of Hwang et al.²² Whole insect was homogenized in three volumes of cold 100 mM sodium acetate, pH 5.5 containing 1 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 minutes at 4°C . The supernatant was used for various assays. Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA,) based on the method of Bradford²³ with BSA as a standard. All spectrophotometric assays were monitored using Shimadzu UV 1800 double beam UV-visible spectrophotometer.

Oxidative stress and redox potential. The GSH and GSSG status was assessed fluorimetrically using OPT as a fluorophore. For GSH, 100 μL of supernatant was incubated with 100 μL of the fluorophore (0.1% in methanol) and 800 μL of 0.1 M phosphate buffer (pH 8.0) for 15 minutes at room temperature in dark. A spectrofluorometer (Hitachi F-4500) was used to perform the fluorescence measurements. For the assay, the excitation wavelength was set at 350 nm and fluorescence data was collected at 420 nm at 5 nm bandwidth. For GSSG estimation, it was measured the same way except at pH 12 NEM. NEM was added to complex with GSSG to prevent interference of GSH with the measurement of GSSG. The values were expressed as nmol/mg of protein.

Putative oxidative stress was calculated as described by Boehme et al.²⁴

$$\text{Oxidative stress index} = (2 \times \text{GSSG})/t\text{GSH} \times 100$$

where $t\text{GSH}$ is the sum of GSSG and GSH.

Redox potentials (E_{h}) of the glutathione redox couple were calculated using Nernst equation from experimentally determined concentrations of GSH and GSSG.²⁵ Insect homogenate nitrite and nitrate were estimated by the method of Yokoi et al.²⁶ with slight modifications using Griess reagent, which relies on a colorimetric reaction between nitrite, sulphanilamide, and *N*-(1-naphthyl) ethylene diamine dihydrochloride to produce a pink azo product, which was measured at 520 nm. For the nitrate estimation, prior to the addition of Griess reagent, all nitrates were converted to nitrite using cadmium sulfate and copper sulfate. Concentrations were deter-

mined by comparison to a standard solution of sodium nitrite. The levels of nitrate were obtained by subtracting the value of nitrite (nitrate converted to nitrite) from total nitrite content. The levels of nitrite and nitrate were expressed as $\mu\text{mol/mg}$ protein.

Lipid peroxidation and protein carbonyl content. The lipid hydroperoxides assay kit (Cayman Chemical, Ann Arbor, MI, USA, Cat No 705002) was used to measure lipid hydroperoxide directly utilizing the redox reactions with ferrous ions to produce ferric ions, which are detected with the aid of chromogen at 500 nm. Lipid hydroperoxide concentration was calculated as described in the kit protocol manual and expressed as nmol/mg protein.

The protein carbonyl content was measured by the amount of 2,4-dinitrophenylhydrazone formed from reactions of proteins with 2,4-dinitrophenylhydrazine (DNPH) as described by Lushchak et al.²⁷ A molar extinction coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the change in absorbance at 370 nm.

Glutathione-dependent antioxidant enzymes assay. Glutathione peroxidase was monitored based on the rate of NADPH oxidation at 340 nm according to the method of Lawrence and Burk.²⁸ Briefly, in the glutathione peroxidase assay, the reaction mixture of 3 mL contained in the final concentration 0.2 mM NADPH, 1 mM GSH, GR, and 0.25 mM H_2O_2 . The amount of GSSG generated was measured as a change in absorbance at 340 nm caused by the GR-dependent oxidation of NADPH coupled to GSSG reduction. All reactions proceeded at 25°C . The results were expressed as $\mu\text{mol/minute/mg}$ protein.

GR was assayed as reported elsewhere.²⁹ The assay mixture (3.0 mL) consisted of final concentration of 0.1 M potassium phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM GSSG, 0.16 mM NADPH, and 30 mL of the enzyme source. NADPH oxidation was monitored at 340 nm for three minutes at 25°C and the enzyme activity was expressed as nmol/minute/mg protein. Activity was read from a standard curve constructed from pure GR.

Glutathione synthetase activity was monitored spectrophotometrically by measuring the formation of ADP at 340 nm for one hour at room temperature.³⁰ One unit of enzyme is defined, after calculation, as the amount that catalyzes the formation of 1 μmol of product/minute/mg protein ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

Glutathione transferase activities were determined as described by Habig and Jakoby.³¹ The reaction volume of 3.0 mL contained a final concentration of 100 mM sodium phosphate buffer (pH 6.5), 1 mM GSH, and 1.0 mM 1-chloro-2,4-dinitrobenzene (CDNB) (dissolve in ethanol to a final concentration less than 4%). Glutathione transferase (GST) activity was initiated by the addition of 30 μL of enzyme source. The appearance of thioether was monitored at 340 nm for three minutes. An extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used in all calculations.



Western blotting. 12% SDS-PAGE and western blot analyses were performed as illustrated by De Luca et al.³² Proteins were transferred to immune-blot polyvinylidene fluoride membrane and later probed with primary antibodies followed by anti-mouse immunoglobulins. Immunoreactive bands were detected using ECL reagents (Amersham Biosciences). Densitometry was conducted on glutathione transferase protein bands using Hp scanner Imager and quantified using Total-Lab Software, Germany.

Statistical analysis. Experimental data were expressed as mean \pm SD of the representative experiments; experiments were repeated at least thrice. Statistical analysis was performed using analysis of variance (ANOVA) followed by Duncan's multiple range test. The bivariate correlation was performed, quoting the Pearson correlation coefficient and test of significance.³³ *P* values ≤ 0.05 are to be considered as significant. SPSS 16 (SPSS Inc., USA) software was used to test the significance of the experiment data generated.

Results

Oxidative stress index and redox potential. The results of oxidative stress index, redox potential, and nitrosative imbalance induced by the different bio-insecticides and chemical stresses on the cowpea storage bruchid reared on the cowpea are shown in Table 1. Exposure to different concentrations of insecticides and bio-insecticides brought about the significant increase in the oxidative stress index compared with the control. The values of GSH and GSSG were used in the calculation of the oxidative stress index; its significant changes were the reflection of the oxidative stress index and redox potential. Cypermethrin (at 200 ppm) caused the highest oxidative stress of 36.42%. *H. suarvolens* ethanolic extract among the bio-insecticides extract has the lowest oxidative stress. GSH/GSSG redox homeostasis was calculated using the Nernst equation at 25°C (Table 1). The values are all negative, and corresponding, more negative values indicate a more reducing state. The nitrates and nitrites were found to be higher with insect sample subjected to bio-insecticides and chemical stresses were higher compared to the control (Table 1). However, the nitrite and nitrates values (an indicator of nitric oxide (NO) formation) were higher in chemical insecticides than the bio-insecticides.

Lipid peroxidation and carbonyl proteins. Figures 1 and 2 represent the value of lipid peroxidation (LPO) and formation of carbonyl proteins (CPs) after exposure to different concentrations of bio-insecticides and insecticides, respectively. The values of the LPO and CPs formations were significantly higher than the control. The value varies in a dosage-dependent manner. Generally, the insecticides produced more of the LPO and CPs compared to the bio-insecticides used. The result shows that insecticides' stress was potent that bio-insecticides in causing the oxidative and nitrosative stresses. The result of the oxidative stress from LPO and CPs confirmed the extent of putative oxidative stress index and altered redox potentials.

Glutathione-dependent antioxidant enzymes. The level of glutathione peroxidase activity in the bruchid exposed to different concentrations of bio-insecticides and insecticides along with the control is shown in Figure 3. The results, in general, showed a significant increase in the activity of glutathione peroxidase among the insecticides and bio-insecticides used, in comparison to their respective controls. The result also indicates that the insecticides brought about a more significant increase in the activity of the enzyme. Figure 4 presents the result of the effect of both bio-insecticides and insecticides on GR activity. There was an initial increase in the enzyme activity but the activity of the enzyme gradually decreases as the concentration of the insecticides and bio-insecticides increases. The exogenous application of 2 M reduced GSH to another group of insects exposed to same and different concentrations of the bio-insecticides and insecticides could not complement the reduction GR activity (results not shown; no statistically significant differences). Thinking that compromised membrane integrity would allow unrestricted entry of the GSH, and two GSH would complex with the insecticides and bio-insecticides in vitro and thereby reducing their respective toxic effects. This non-consequential effect showed that the molecular basis of the toxic effect appears more complex than addition of GSH. We came to a conclusion that GSH must be enzymatically thiolated to be effective.

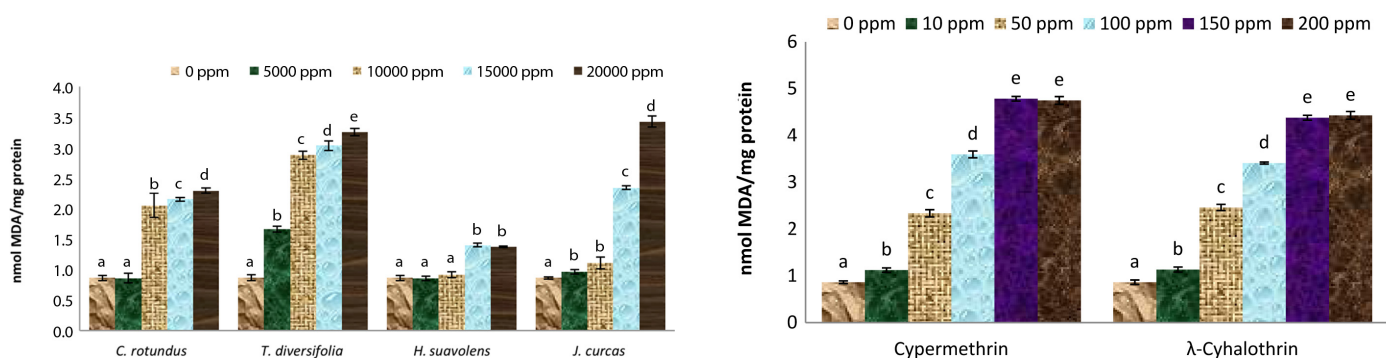
The result of the effect of the insecticides and bio-insecticides on the bruchid glutathione transferases is shown in Figure 5. There was an insignificant increase in the activity of bruchid glutathione transferases as shown by the result when compared with the control. The insect glutathione transferases protein expressions were investigated by western blotting to determine whether the increase in GST was correlated with modulation of GST expression. The polyclonal antibody raised to the bruchid glutathione transferase gave a single band on western blots (Fig. 6). There was an increase in the intensity of the band as the concentration of the insecticides increases. It revealed an upregulation of the enzyme and confirms and amplifies that the change in GST assay was correct. The blot also showed that there was no change in the molecular weight of the bruchid GST, which indicates that induction and expression are not connected to post-translational modification. It appears that the overexpression of GST is an important means of cell protection. It might be plausible to suggest that the bruchid detoxification function in response to the oxidative and nitrosative stress.

The effect on glutathione synthetase activity is shown in Figure 7. There was a significant induction in the glutathione synthetase activity in all bio-insecticides and insecticides used compared with the control. The 200 ppm cypermethrin has the highest glutathione synthetase activity of 5.55 $\mu\text{M}/\text{minute}/\text{mg}$ proteins. The 20,000 ppm *T. diversifolia* has a value of 5.248 $\text{mM}/\text{minute}/\text{mg}$ proteins.

Table 1. Levels of oxidative stress index, redox potentials (E_h), and NO levels in cowpea storage bruchid exposed to different concentrations of bio-pesticides and pesticides.

	CONC. (ppm)	OXIDATIVE STRESS INDEX (%)	E_h (mV)	NITRITE ($\mu\text{M}/\text{mg}$ protein)	NITRATES ($\mu\text{M}/\text{mg}$ proteins)
Control		13.11	-270.13	1.09 \pm 0.02 ^e	2.31 \pm 0.03 ^e
<i>C. rotundus</i>	5,000	16.72	-268.56	1.27 \pm 0.02 ^d	2.48 \pm 0.01 ^d
	10,000	17.41	-268.29	1.35 \pm 0.03 ^c	2.59 \pm 0.12 ^c
	15,000	19.01	-267.69	1.48 \pm 0.01 ^b	2.68 \pm 0.11 ^b
	20,000	19.70	-267.46	1.54 \pm 0.02 ^a	2.87 \pm 0.08 ^a
<i>T. diversifolia</i>	5,000	17.06	-268.43	1.33 \pm 0.06 ^c	2.83 \pm 0.02 ^d
	10,000	18.33	-267.96	1.43 \pm 0.14 ^c	3.05 \pm 0.04 ^c
	15,000	20.02	-267.36	1.57 \pm 0.01 ^b	3.25 \pm 0.1 ^a
	20,000	23.24	-266.39	1.86 \pm 0.03 ^a	3.38 \pm 0.03 ^a
<i>H. suavolens</i>	5,000	14.17	-269.64	1.08 \pm 0.03 ^d	2.21 \pm 0.03 ^c
	10,000	15.12	-269.23	1.16 \pm 0.02 ^c	2.27 \pm 0.02 ^c
	15,000	15.62	-269.01	1.21 \pm 0.03 ^b	2.42 \pm 0.02 ^b
	20,000	16.56	-268.63	1.28 \pm 0.03 ^a	2.64 \pm 0.02 ^a
<i>J. curcas</i>	5,000	15.04	-269.20	1.14 \pm 0.03 ^d	2.35 \pm 0.01 ^d
	10,000	17.72	-268.12	1.29 \pm 0.01 ^c	2.53 \pm 0.01 ^c
	15,000	19.90	-267.16	1.49 \pm 0.03 ^b	2.70 \pm 0.03 ^b
	20,000	22.42	-266.28	1.57 \pm 0.08 ^a	2.97 \pm 0.01 ^a
Control		13.10	-270.14	1.11 \pm 0.02 ^f	2.88 \pm 0.01 ^f
Cypermethrin	10	15.79	-268.83	1.32 \pm 0.02 ^e	3.03 \pm 0.02 ^e
	50	18.09	-267.90	1.47 \pm 0.24 ^d	3.16 \pm 0.02 ^d
	100	22.21	-266.43	1.78 \pm 0.02 ^c	3.36 \pm 0.04 ^e
	150	27.45	-264.63	2.09 \pm 0.01 ^b	3.67 \pm 0.07 ^b
	200	36.42	-261.78	2.46 \pm 0.02 ^a	3.94 \pm 0.06 ^d
λ -cyhalothrin	10	14.64	-269.39	1.21 \pm 0.02 ^e	2.62 \pm 0.03 ^e
	50	17.33	-268.31	1.44 \pm 0.04 ^d	2.85 \pm 0.05 ^d
	100	20.20	-267.28	1.68 \pm 0.02 ^c	3.39 \pm 0.02 ^c
	150	22.47	-266.31	1.79 \pm 0.02 ^b	3.62 \pm 0.08 ^b
	200	30.04	-263.55	2.13 \pm 0.02 ^a	3.84 \pm 0.03 ^a

Notes: Redox potentials (E_h) of the glutathione redox couple were calculated from experimentally determined concentrations of GSH and GSSG using Nernst equation. E_o is the standard potential for the redox couple GSH/GSSG (-240 mV at pH 7). Oxidative stress index was calculated using glutathione redox couple equation (oxidative stress index = $((2 \times \text{GSSG})/\text{tGSH}) \times 100$). The results were presented as mean \pm SEM ($n = 3$) and analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test. The bivariate correlations analysis was performed, quoting Pearson correlation coefficient and test of significance using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at $P < 0.05$.


Figure 1. The level of lipid peroxidation (as quantified by malondialdehyde) in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ -Cyhalothrin). The values are mean \pm SD of triplicate determination of the results (nmol/mg proteins).

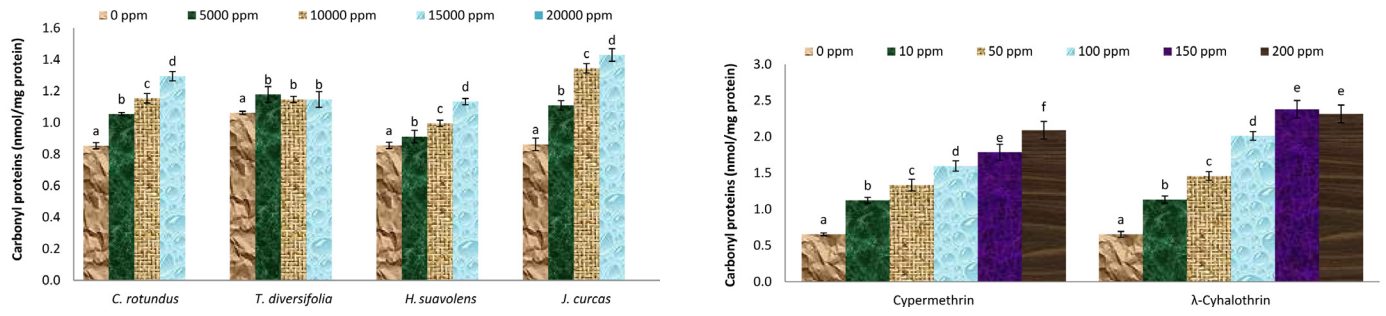


Figure 2. Level of carbonyl proteins in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ -cyhalothrin). The values are mean \pm SD of triplicate determination of the results (nmol/mg proteins).

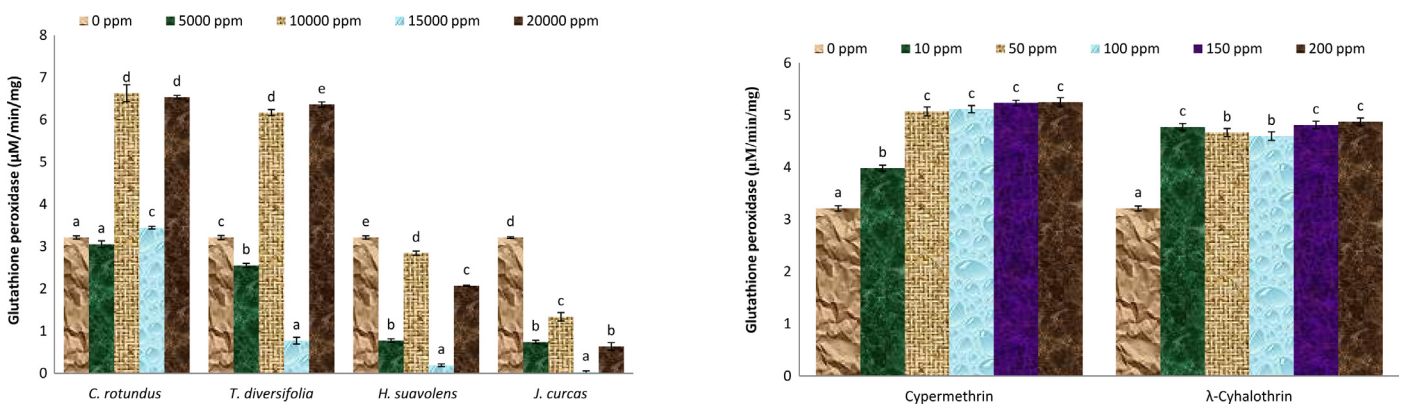


Figure 3. The Glutathione peroxidase level evaluated from the cowpea storage bruchid exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ -cyhalothrin). Each column represents the mean \pm SEM of triplicate determinations. The value is μ M/min/mg proteins.

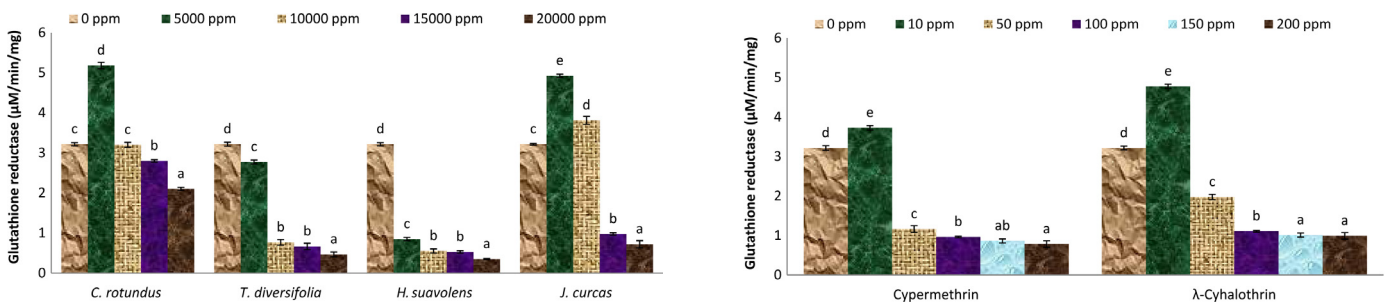


Figure 4. The glutathione reductase levels evaluated from the cowpea storage bruchid exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ -cyhalothrin). Each column represents the mean \pm SEM of triplicate determinations. The value is μ M/min/mg proteins.

Discussion

The result showed that oxidative and nitrosative stresses were detected in the whole-body homogenate of the bruchid exposed to different concentrations of bio-insecticides and insecticides (Table 1). This indicated generation and a significant positive correlation between the oxidative stress and nitrosative stress. It showed intoxication. These results are in agreement with

earlier reports^{34,35} that insecticides are responsible for causing oxidative stress and nitrosative stresses. In this study, the complex ratio of GSH–GSSG, an intracellular redox condition, was used as index of oxidative stress.^{36–38} The nitrosative stress was the measure of nitrates and nitrites contents, a major end product of NO metabolism. The presence of GSSG and GSH allows dynamic control of the redox state. Altered glutathione

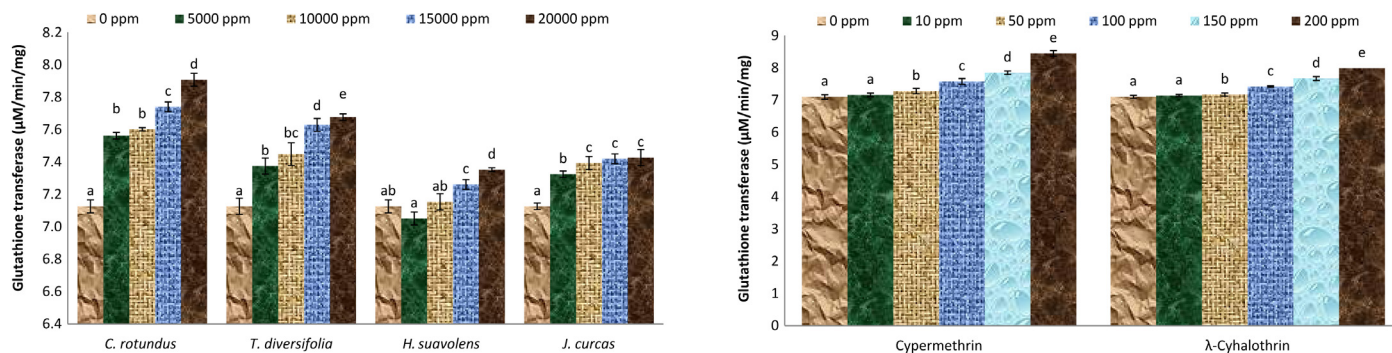


Figure 5. The glutathione transferase level evaluated from the cowpea storage bruchid exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ-cyhalothrin). Each column represents the mean ± SEM of triplicate determinations. The value is µM/min/mg proteins.

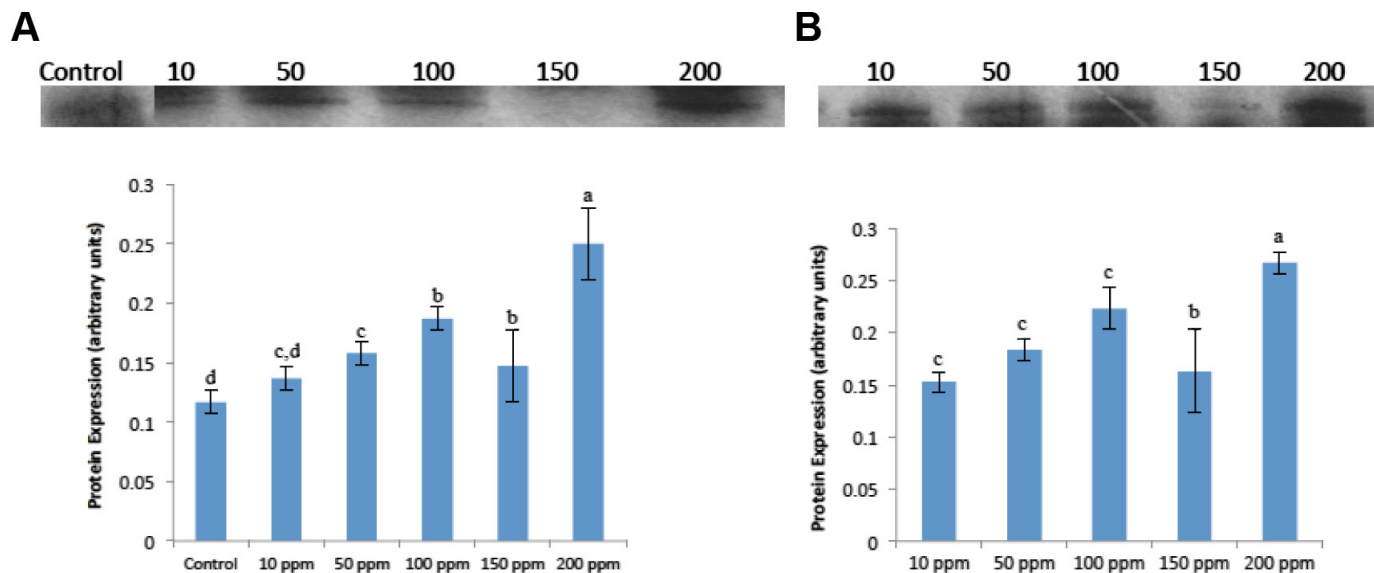


Figure 6. Western blot showing protein level and expression of glutathione transferase in cowpea storage bruchid exposed to different concentrations of Cypermethrin and λ-cyhalothrin. Bar graphs of expressed *C. maculatus* glutathione transferase show band intensity. Each column represents the mean ± SEM of triplicate determinations.

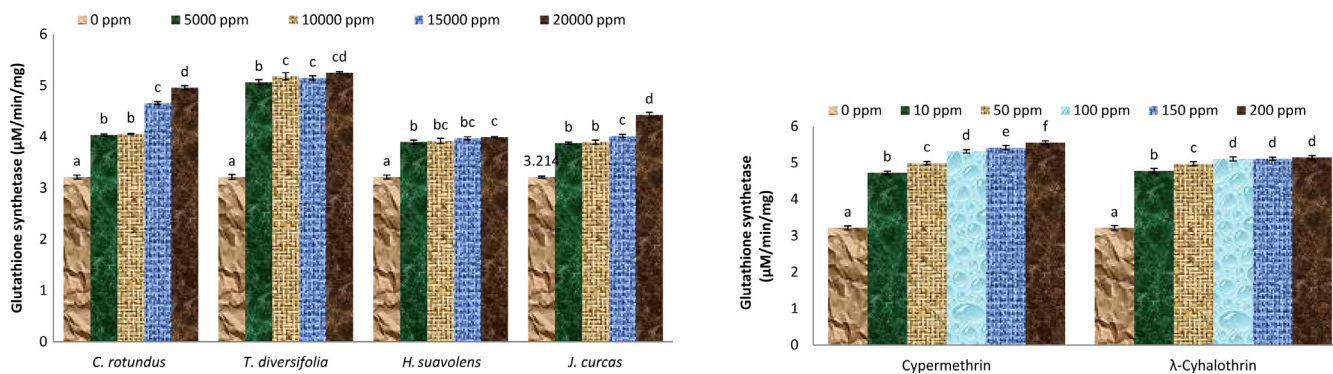


Figure 7. The glutathione synthetase level evaluated from the cowpea storage bruchid exposed to different concentrations of bio-pesticides (*T. diversifolia*, *C. rotundus*, *H. suavolens* leaves, and *J. curcas* seed) and pesticides (Cypermethrin and λ-cyhalothrin). Each column represents the mean ± SEM of triplicate determinations. The value is µM/min/mg proteins.



homeostasis, in association with increased oxidative stress, has been implicated in the pathogenesis of many diseases.³⁹ The increase in the oxidative stress, as shown in our results, is consequent upon formation of GSSG. GSSG formation from GSH brings about acceleration of oxidative process and consequently altered the redox status homeostasis without a significant change in total glutathione content. It seems that the bruchid cannot preserve its redox homeostasis and this impaired glutathione redox status could be responsible for the efficacy of the insecticides and bio-insecticides used. It has been earlier reported that cells resistant to apoptosis preserved high intracellular pools of GSH by enhancing pathways for establishment and maintenance of high intracellular redox potential.⁴⁰ With these, however, a change in GSSG has a potential utility in providing biomarker that could be used in environmental monitoring system.

The result also showed that NO was produced during the oxidative burst triggered by the insecticides and bio-insecticides. NO has been reported to be involved in protecting membrane integrity and protecting lipid derived radicals, and thereby, antagonizing oxidative stress.⁴¹ However, the simultaneous increase in both NO (Table 1) and LPO (Fig. 1) values upon exposure could indicate that NO might not be involved in signaling or in protection against LPO or the NO might appear to be inadequate to cope with oxidative stress. All these might suggest that the bio-insecticides and chemical stress affect the metabolism, growth, and development⁴² of the bruchid and its mortality. The signs of oxidative stress could be extensive as shown by the result of CP and LPO (Figs. 1 and 2). CPs formation is a result of protein oxidation. ROS directly attack proteins and lead to the formation of carbonyl.⁴³ These biotic stressed cowpea bruchids showed a higher concentration of protein carbonyl, a measure of ROS and reactive nitrogen species (RNS) mediated damage, and a symptom of oxidative stress.⁴⁴ This could be a reflection of steady oxidative stress. ROS directly attack the protein and lead to the formation of protein carbonyl. It indicates that the insecticides and bio-insecticides have elicited a toxic effect at critical targets. The formation of protein carbonyl is irreversible, causing conformational changes, compromised catalytic activity of enzymes, and increased susceptibility to protease action.⁴⁵ At various dosages of both insecticides and bio-insecticides, detoxification processes might have been activated early to avoid oxidative damage. However, cowpea storage bruchids suffered membrane damage as shown by the increase LPO because of inadequate protective mechanism.

In summary, the level of modified lipids, compromised membrane integrity, and protein carbonylation are joint consequent nitrosative and oxidative stress in cowpea storage bruchid. All these reflect a severe disorder in metabolism. These are accompanied by the modulation of the glutathione system of antioxidant defense. Glutathione and glutathione-dependent enzymes have been known to play a central role in protection and detoxification of peroxides, hydroperoxides,

and xenobiotic and maintaining homeostatic dynamics of GSH.^{19,46} These systems play a crucial role in protecting the structure and functioning of the membrane system and maintaining cellular redox state.⁴⁷ The possible linkage between cowpea storage bruchid (*C. maculatus*) glutathione antioxidant defense system and insecticidal efficacy of some reported bio-insecticides and commercially available synthetic insecticides was sought. The relevance of the glutathione system in the defense against chemical stress is of importance to give a thorough perspective of glutathione metabolism in the stressed state, where the process is of fundamental importance for the mechanism of toxicity, and also, to provide possible path of adaptation evolution and eventual compensatory mechanism for the stressed condition. These alterations of the insect glutathione-dependent and detoxification system activities with insecticides and bio-insecticides reflect the presence of a functional defense mechanism against oxidative and nitrosative stresses related firmly to glutathione metabolism. The stimulation is important and could sustain the defense mechanism.

GSH is a co-factor for glutathione peroxidase. Glutathione peroxidase is known to detoxify a variety of organic hydroperoxides produced in LPO to the corresponding hydroxyl compounds, utilizing GSH and/or other reducing equivalents.⁴⁸ Glutathione peroxidase protects the cell against a low level of oxidative stress.⁴⁹ The increase in glutathione peroxidase activity (Fig. 3) could be from the increase in the concentration of H₂O₂ and organic hydroperoxides (ROOH) as shown by the resulting oxidative stress. It appears that oxidative stress was initiated within a short space of time of exposure to the insecticides and bio-insecticides. This clearly shows that there is a relationship between glutathione peroxidase, redox homeostasis and lipid peroxidation. The glutathione antioxidant defense induction appears to be inadequate to avoid oxidative damage. The role of GR is to maintain the cytosolic concentration of GSH. The inhibition of glutathione peroxidase activity by the insecticides and bio-insecticides used in this study may lead to GSH depletion, if the depletion cannot be corrected by the synthesis of new glutathione.⁵⁰ The detoxification of possible ROS and hydroperoxides implies the oxidation of GSH to GSSG by glutathione peroxidase. GSSG is then reduced to GSH by GR at the expenses of NADPH.⁵¹ The GR activity is compromised (Fig. 4). This statistically significant negative correlation of Glutathione peroxidase (GPx) and GR activities in the stressed bruchid followed the same pattern in the insecticides and bio-insecticides and could be interpreted as insufficient to meet ROS demands in these chemical stress conditions. It might be an inadequate adaptive response to oxidative stress and the accumulation of ROS. The low GR activity as shown in our result is unusual. Other workers have reported elevated GR activity under a stress condition.^{29,52} However, despite the relevance of GR in the tolerance to oxidative stress, its activity strictly depends on NADPH availability.⁵²

GST catalyzes the conjugation of GSH with a variety of electrophilic metabolites. The enzyme participates in defense against oxidative stress by detoxifying endogenous harmful compounds like hydroxylalkenal and base propenal or DNA hydroperoxides and electrophilic xenobiotic, and is known to provide protection against oxidative/nitrosative stress by GSH mediated process of reactive products of LPO.³⁰ Similar to GPx, GST activity was induced and might indicate an adaptive response to the oxidative stress. There was an increase in GST activity. This suggests involvement and activation of GST-dependent xenobiotic metabolism. Our earlier results^{53,54} have demonstrated the involvement of bruchid glutathione transferases to insecticides and bio-insecticides toxicity. The induction of GST is considered beneficial to handle environmental stress.⁵⁵ The induction of the insect GST was supported by the result from western blotting (Fig. 6). Overexpression of GST could be an important means of cell protection during physiological stress as such posed by the bio-insecticides and insecticides. The expression appears to be significant. It is probable that the bruchid is overstressed by the exposure and this can be the possible reason for GST expression. However, the mortality might not be compromised by the induction of the GST. With this, GST activity may not be among the best explanatory variables of insecticides and bio-insecticides mortality, although this enzyme is directly linked to its detoxification. This could indicate that the role of glutathione in extending survival would be more related to the removal of free radicals than to the detoxification of insecticides and bio-insecticides stresses. Alternatively, the response of GST activity might be inadequate. The reduction in the activity might have shown that the disruption of the compensatory mechanism will help the insect to detoxify the abiotic stresses.

Glutathione synthetase was specifically assayed to provide insight into possible compensatory of possible depletion of GSH during oxidative and nitrosative stresses. Our results showed that glutathione synthetase was recruited when the bruchid is exposed to either bio-insecticides or insecticides (Fig. 7). Its involvement indicates the need to regulate glutathione steady-state levels and demonstrate distinct compensatory responses in the glutathione pathway following the stressed conditions to maintain the redox homeostasis. The increase in activity might indicate induction of de novo glutathione biosynthesis by glutathione synthetase; and, is likely to play a crucial role. Our result is not unusual. Nitrosative and oxidative stresses induced the glutathione synthetase gene expression in *Saccharomyces cerevisiae* and *Saccharomyces pombe*.³⁰ The variable induction by the insecticides and the bio-insecticides is a function of the nature and extent of the oxidative stress.

Altogether, these present data add to the growing position of how and to what extent enzymatic antioxidant defense of cowpea storage bruchid was altered in response to insecticides and bio-insecticides application. The synergy in the modulation of GPx, GST, and GS has supported

collaboration among these enzymes in the detoxification mechanism of the insecticides and bio-insecticides and maintaining GSH concentration for redox homeostasis. The accumulation of ROS does not make the GSH-related antioxidant systems lose their function but appears inadequate in terms of the inhibition of GR activity. The exogenous application of GSH to assuage *in vivo* GSH demand could not assist.

Author Contributions

Conceived and designed the experiments: AOK. Analyzed the data: AOK, ANK. Wrote the first draft of the manuscript: AOK. Contributed to the writing of the manuscript: AOK, ANK. Agree with manuscript results and conclusions: AOK, ANK. Jointly developed the structure and arguments for the paper: AOK, ANK. Made critical revisions and approved final version: AOK, ANK. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Labeyrie V. Vaincre la carence proteique par le developpement des legumineuses alimentaires et la protection de leurs recoltes contre les bruches. *Food Nutr Bull.* 1981;3:24–38.
2. Baributsa D, Lowenberg-DeBoer J, Mudrock B, Moussa L. Profitable chemical free cowpea storage technology for smallholder farmers in Africa: opportunities and challenges. In: *10th International Working Conference on Stored Product Protection*. Edited by: Carvalho MO, Fields PG, Adler CS, Arthur FH, Athanassiou CG, Campbell JF, et al. Julius-Khun Archives, Berlin, Germany; 2010;425:1046–1052.
3. Murdock LL, Shade RE, Kitch LW, et al. Post harvest storage of cowpea in sub-Saharan Africa. In: Singh BB, Mohan-Raj DR, Dashiell KE, Jackai LEN eds. *Advances in Cowpea Research*. Ibadan, Nigeria: International Institute of Tropical Agriculture; 1997:302–312.
4. Zhu-Salzman K, Murdock LL. Cowpea: insects, ecology and control. In: *The Encyclopedia of Pest Management*. Editor: David Pimentel. CRC Press, Boca Raton, USA; 2006:1–3.
5. Guo F, Lei J, Sun Y, et al. Antagonistic regulation, yet synergistic defense: effect of bergapten and protease inhibitor on the development of cowpea bruchid *Callosobruchus maculatus*. *Plos One.* 2012;7(8):e41877.
6. Banks HJ, Annis PC, Rigby GR. Controlled atmosphere storage of grain: The known and the future. In: Fleurat-Lassard F, Ducom P eds. *Proceedings of the 5th working conference on stored products protection*. Imprimerie du Médoc. Bordeaux, France; 1990:695–706.
7. Govindan K, Jeyarajan Nelson S, David PMM. Fly ash—excellent filler for black pepper, *Piper nigrum* dust formulation against *Callosobruchus maculatus* (F.). *J Biopesticides.* 2010;3(1):320–324.
8. Park C, Kim S, Ahn YJ. Insecticidal activity of asarones identified in *Acorus gramineus* rhizome against three coleopteran stored product insects. *J Stored Prod Res.* 2003;39:333–342.
9. Jackai LEN, Adalla CB. Pest management practices in cowpea: a review. In: Singh BB, Mohan Raj DR, Dashiell KE, Jackai LEN eds. *Advances in Cowpea Research*. Ibadan, Nigeria: IITA/JIRCAS; 1997:240–258.
10. Singh SR, Jackai LEN. Insect pests of cowpeas in Africa: their life cycle, economic importance and potential for control. In: Singh SR, Rachie KO eds. *Cowpea Research, Production and Utilization*. Chichester, UK: John Wiley & Sons, Ltd; 1985:217–232.
11. Wells C, Mongin A, Bertsch W. A study of photosensitive insecticidal volatile compounds in marigold (*Tagetes minuta*). *J High Resolution Chromatogr.* 1993;16: 53–55.
12. Després L, David J-P, Gallet C. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol Evol.* 2007;22(6):298–306.
13. Ahn JE, Zhou X, Dowd SE, Chapkin RS, Zhu-Salzman K. Insight into hypoxia tolerance in cowpea bruchid: metabolic repression and heat shock protein regulation via hypoxia-inducible factor 1. *Plos One.* 2013;8(4):e57267.
14. Caregnato FF, Koller CE, MacFarlane GR, Moreira JCF. The glutathione antioxidant system as a biomarker suite for the assessment of heavy metal exposure and effect in the grey mangrove, *Avicennia marina* (Forsk.). *Vierb Mar Pollut Bull.* 2008;56:1119–1127.
15. Guengerich FP. Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem Res Toxicol.* 1991;4:391–407.



16. Vranova E, Inze D, Van Breenegem F. Signal transduction during oxidative stress. *J Exp Bot.* 2002;53:1227–1236.
17. Srivalli B, Chinnusamy V, Chopra RK. Antioxidant defense in response to abiotic stresses in plants. *J Plant Biol.* 2003;30:121–139.
18. Prochazkova D, Sairam RK, Srivastava GC, Singh DV. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Sci.* 2001;161:765–771.
19. Hidalgo MC, Exposito A, Palma JM, de la Higuera M. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). *Int J Biochem Cell Biol.* 2002;34:183–193.
20. Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 1983;52:711–760.
21. Adedire CO, Akinneye JO. Biological activity of tree marigold, *Tithonia diversifolia*, on cowpea seed bruchids, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Ann Appl Biol.* 2003;144:185–189.
22. Hwang SY, Lin HW, Chern RH, Lo HF, Li L. Reduced susceptibility to water logging together with highlight stress is related to increases in superoxide dismutase and catalase activity in sweet potato. *Plant Growth Regul.* 1999;27: 167–172.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein–dye binding. *Anal Biochem.* 1976;72:248–254.
24. Boehme DS, Hotchkiss JA, Henderson RF. Glutathione and GSH dependent enzymes in broncho-alveolar larvae fluid cells in response to ozone. *Exp Mol Pathol.* 1992;56:37–48.
25. Díaz-Flores M, Angeles-Mejia S, Baiza-Gutman LA, et al. Effect of an aqueous extract of *Cucurbita ficifolia* Bouché on the glutathione redox cycle in mice with STZ-induced diabetes. *J Ethnopharmacol.* 2012;144(1):101–108.
26. Yokoi I, Habu H, Kabuto H, Mori A. Analysis of nitrite, nitrate, nitric oxide synthase activity in brain tissue by automated flow injection technique. *Methods Enzymol.* 1996;268:152–159.
27. Lushchak VI. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol.* 2011;101:13–30.
28. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun.* 1976;71:952–958.
29. Saydam N, Kirb A, Demirb O, et al. Determination of glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferase levels in human lung cancer tissues. *Cancer Lett.* 1997;119:13–19.
30. Kim SJ, Shin YH, Kim K, Park EH, Sa JH, Lim CJ. Regulation of the gene encoding glutathione synthetase from the fission yeast. *J Biochem Mol Biol.* 2003;36(3):326–331.
31. Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* 1981;77:398–405.
32. De Luca A, Mangiacasale R, Severino A, Malquori L, Baldi A. A E1A deregulates the centrosome cycle in a Ran GTPase-dependent manner. *Cancer Res.* 2003;63:1430–1437.
33. Brooks SP. A simple computer program with statistical tests for the analysis of enzyme kinetics. *Biotechniques.* 1992;13(6):906–911.
34. Uner N, Oruc EO, Sevgiler Y, Sahin N, Durmaz H, Usta D. Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of *Oreochromis niloticus*. *Environ Toxicol Pharmacol.* 2006;21:241–245.
35. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. *J Amino Acids.* 2012;26. Article ID 736837.
36. Ritola O, Lyytikainen T, Pylkko P, Molsa H, Lindstrom-Seppa P. Glutathione dependent system and monoxygenase enzyme activities in Arctic charr *Salvelinus alpinus* (L) exposed to ozone. *Aquaculture.* 2000;185:219–233.
37. Birk J, Meyer M, Aller I, et al. Endoplasmic reticulum: reduced and oxidized glutathione revisited. *J Cell Sci.* 2013;126:1604–1617.
38. Serru V, Baudin M, Ziegler F, et al. Quantification of reduced and oxidized glutathione in whole blood samples by capillary electrophoresis. *Clin Chem.* 2001;47:1321–1324.
39. Reid M, Jahoor F. Glutathione in disease. *Curr Opin Clin Nutr Metab Care.* 2001; 4:65–71.
40. Voehringer DW, Hirschberg DL, Xiao J, et al. Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. *Proc Natl Acad Sci U S A.* 2000;97:2680–2685.
41. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol.* 2008;183:795–803.
42. Caceres L, Necakov AS, Schwartz C, Kimber S, Roberts IJH, Krause HM. Nitric oxide coordinate metabolism, growth and development via the nuclear receptor E75. *Genes Dev.* 2011;25(14):1476–1485.
43. Bairy ACD, Saito E, Carvalho PSM, Junqueira VBC. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquat Toxicol.* 1996;34:151–162.
44. Lushchak VI. Free radical oxidation of proteins and its relationship with functional state of organisms. *Biochemistry (Moscow).* 2007;72(8):809–827.
45. Zhang X, Yang F, Zhang X, et al. Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD). *Aquat Toxicol.* 2008;86:4–11.
46. Li Z, Zlabek V, Grabic R, Li P, Randak T. Modulation of glutathione-related antioxidant defense system of fish chronically treated by the fungicide propiconazole. *Comp Biochem Physiol C.* 2010;152:392–398.
47. Baisak RD, Rana PBB, Acharge MK. Alterations in the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. *Plant Cell Physiol.* 1994;35:489–495.
48. Uner N, Oruc E, Sevgiler Y. Oxidative stress-related and ATPase effects of cotaxazole in different tissues of *Oreochromis niloticus*. *Environ Toxicol Pharmacol.* 2005;20:99–106.
49. Yan H, Harding JJ. Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. *Biochem J.* 1997;328:599–605.
50. Elia AC, Galarini R, Taticchi MI, Dorr AJM, Mantilacci L. Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. *Ecotoxicol Environ Saf.* 2003;55:162–167.
51. Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med.* 2009;30:1–12.
52. Peña-Llopis S, Ferrando MD, Peña JB. Fish tolerance to organophosphate induced oxidative stress is dependent on the glutathione metabolism and enhanced by *N*-acetylcysteine. *Aquat Toxicol.* 2003;65:337–360.
53. Kolawole AO, Okonji RE, Ajele JO. *Tithonia diversifolia*, *Cyperus rotundus* and *Hypis suaveolens* ethanol extracts combinatorially and competitively inhibit affinity purified cowpea storage bruchid (*Callosobruchus maculatus*) glutathione S-transferase. *Arthropod-Plant Interact.* 2011;5:175–184.
54. Kolawole AO, Ajele JO, Sirdeshmuhk R. Studies on glutathione transferase of cowpea storage bruchid, *Callosobruchus maculatus* F. *Pesticide Biochem Physiol.* 2011;100:212–220.
55. Van der Oost R, Beyer J, Vermeulen NPE. Fish bioaccumulation and biomarkers in environmental risk. *Environ Toxicol Pharmacol.* 2003;13:57–149.