Exogenous glutathione attenuates lead-induced oxidative stress in wheat by improving antioxidant defense and physiological mechanisms

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Introduction

Lead (Pb) is highly persistent and naturally occurring toxic metal (Schreck et al. 2012). Mining, metal plating and finishing operations, batteries, pesticides, fertilizers, pigments and gasoline industries are major sources of Pb contamination (Ahmad et al. 2011). Lead contamination has been noticed in drinking and irrigation water, even in the air. Permissible quantity of Pb is not more than 5 mg L⁻¹ of irrigation water (Ayers and Westcot 1985) whereas in soil, the allowable range is in 10–30 μg g⁻¹. Lead contamination can reach 30–2,000 μg Pb g⁻¹. So, high water and soil Pb content have become acute environmental hazards (Silva et al. 2017). Lead is spread from the environment and enters into the human body through food ingestion, water and air (Piano et al. 2008; Cai et al. 2015).

Lead is a non-essential element but easily taken up by the plant. Lead-induced pro-oxidative effects disrupt biochemical/physiological processes and distort cellular structural components. Cell membrane structure, water and hormonal status, enzyme activity, uptake and distribution of mineral, chlorophyll (chl) biosynthesis, photosynthesis, transpiration, and DNA synthesis are negatively affected by Pb toxicity (Sengar et al. 2008). Lead stress adversely affects seed germination, growth, and development (Pourrut et al. 2011).

Many heavy metals including Pb causes excess generation of reactive oxygen species (ROS) such as superoxide anion (O₂⁻); hydrogen peroxide (H₂O₂), hydroxyl radical (·OH) and singlet oxygen (¹O₂) (Wahsha et al. 2012). These are produced due to aerobic metabolism and capable of oxidizing different molecules more strongly than molecular oxygen. Excessive ROS generation beyond plants scavenging capacity results in oxidative stress which affect protein, nucleic acids, lipids and activate programed cell death (PCD; Gill and Tuteja 2010; Hasanuzzaman et al. 2012). To combat oxidative stress plants have ROS scavenging or detoxification mechanisms. Antioxidant defense system comprises two major components such as non-enzymatic and enzymatic components. Along with ascorbic acid (AsA) and glutathione (GSH), non-enzymatic components may comprise phenolic compounds, non-protein amino acids, a-tocopherols, etc. The enzymatic component includes some antioxidant enzyme such as superoxide dismutase (SOD), catalase (CAT), peroxidases (AO and APX), the ascorbate-glutathione cycle (AsA-GSH cycle) and glutathione-S-transferase (GST).

This study aims to investigate how exogenous glutathione (GSH, 1.0 mM) affects the oxidative stress and antioxidant defense in wheat seedlings under lead (Pb) stress [0.5 and 1.0 mM Pb(NO₃)₂]. Lead treatment decreased growth, leaf relative water content, and chlorophyll (chl) content whereas raised proline (Pro) level. Lead stress increased H₂O₂ content, O₂⁻ generation rate, and membrane lipid peroxidation. Addition of Pb also disrupted antioxidant enzyme activities and status of endogenous ascorbate and GSH pool. The increase of methylglyoxal was evident under Pb stress. Glutathione supplementation under Pb stress increased antioxidant redox pool and augmented the activities of antioxidant enzymes, and decreased ROS production. Exogenous supplementation of GSH reverted the increase in the methylglyoxal level due to Pb stress due to increased activities of glyoxalase enzymes. Exogenous GSH also regulated Pro, well-maintained tissue water status and prevented chl degradation and increased plant growth and biomass.

Abbreviations: AO- ascorbate oxidase; APX- ascorbate peroxidase; AsA- ascorbic acid (ascorbate); BSA- bovine serum albumin; CAT- catalase; CDNB-1-chloro-2, 4-dinitrobenzene; chl- chlorophyll; DHA- dehydroascorbate; DHAR- dehydroascorbate reductase; EDTA- ethylenediaminetetraacetic acid; Gly I- glyoxalase I; Gly II- glyoxalase II; GR- glutathione reductase; GSH- reduced glutathione; GSSG- oxidized glutathione; GPX- glutathione peroxidase; GST- glutathione S-transferase; MDA- malondialdehyde; MDHA- monodehydroascorbate; MDHAR- monodehydroascorbate reductase; MG- methylglyoxal; NADPH- nicotinamide adenine dinucleotide phosphate; NBT- nitroblue tetrazolium chloride; Pb- lead; PEG- polyethylene glycol; Pro- proline, ROS- reactive oxygen species; RWC- relative water content; SLG- S-D-lactoylglutathione; SOD- superoxide dismutase; TBA- thiobarbituric acid; TCA- trichloroacetic acid.
ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione S-transferase (GST) which dismutase or detoxify the ROS (Gill and Tuteja 2010; Hasanuzzaman et al. 2012).

Methylglyoxal (MG), the cytotoxic compound produced from triose-phosphate as reactive cytotoxic alpha-oxoaldehyde during glycolysis pathway. Methylglyoxal production is higher and harmful under environmental stress condition but remains balanced under suitable growth condition. Due to high reactive nature of MG, adverse effects of this component in biological processes have been reported in many studies. Some common adverse effects of MG are cell proliferation inhibition as well as degradation of protein and DNA which is correlated with the inactivation of antioxidant defense (Yadav et al. 2005a, 2005b). Glyoxalase system possesses two glyoxalase enzymes (glyoxalase I; Gly I and glyoxalase II; Gly II) and GSH converting toxic MG to d-lactic acid that is non-toxic to the cell. Glutathione is regenerated at the end of the detoxification process (Batth et al. 2017).

In a plant cell, GSH is major non-protein thiol component which is water-soluble antioxidant having multifarious biochemical roles in plant stress adaptation (Foyer and Noctor 2005a, 2005b). Among different cellular functions of GSH ROS scavenging is a major one. Glutathione directly scavenges some ROS and maintains some antioxidant components in active form, thus enhances oxidative stress tolerance (Hasanuzzaman et al. 2012). Thiol group has nucleophilic nature which some ROS and maintains some antioxidant components in active form, thus enhances oxidative stress tolerance (Hasanuzzaman et al. 2012). Thiol group has nucleophilic nature which forms mercaptide bonds with metals. Consequently, it protects plant cells from the toxic effects of metals by facilitating vacuolar sequestration of toxic metals (Foyer and Noctor 2005a, 2005b). Glutathione is an active component of glyoxalase system detoxifying MG. Under normal and abiotic stress condition, GSH helps in growth, development, gene expression, and protein activation by its diversified properties (Noctor et al. 1998; Shao et al. 2008; Hasanuzzaman et al. 2017a, 2017c). Glutathione can sequester the Pb which is immobilized on the thiol group of GSH. However, the underlying mechanisms of GSH to impart stress in plants are still under study. Among the damaging effects, oxidative stress is the most devastating. Therefore, the mechanisms how plants cope with metal-induced oxidative stress are important tasks for plant biologists. The present study reveals how exogenous GSH application alleviates Pb-induced damages, particularly, by coordinated actions on antioxidant metabolism and MG detoxification in improving tolerance of wheat plants subjected to Pb stress.

Materials and methods

Test plant, growth condition, and treatments

The seedlings were grown as per the methods described in Hasanuzzaman et al. (2017b). Uniform sized healthy seeds of wheat (Triticum aestivum L. cv. Pradip) were used for the experiment. The seeds were surface sterilized with ethanol (70% v/v). The seeds were then washed very well with hygienic water followed by soaking in distilled water for 10 min. After that, 10 ml of distilled water was added to each Petri dish (9 cm, containing 6 filter paper sheets) and sterilized seeds were sown on it. Finally, seeds were kept for germination up to 3 days. Petri dishes with germinated seedlings had been moved into growth chamber (CLE-303, Tomy Seiko, Tokyo) where the optimum growth conditions were maintained (light: 350 μmol photon m⁻² s⁻¹, temp: 25 ± 2°C, RH: 65–70%). As a plant nutrient, Hyponex (Japan) was used every day after 10,000-times dilution according to necessity. Lead [0.5 Pb(NO₃)₂ (mild stress) and 1.0 mM Pb(NO₃)₂ (severe stress)] and 1.0 mM glutathione (GSH, Wako, Japan); alone and in combination were applied on seven-day-old wheat seedlings. For growing control plants, the only Hyponex solution was used. Each Petri dish contained 20 mL of such solutions. After 48 h of Pb treatment, samples were collected, and various morphological and physiological parameters were measured.

Lipid peroxidation, H₂O₂ level, and O₂⁻· generation rate

To determine the lipid peroxidation of the cell, malondialdehyde (MDA) content (derived from lipid peroxidation) was measured utilizing thiobarbituric acid (TBA) (Heath and Packer 1968). Hydrogen peroxide (H₂O₂) content was assayed following the procedure of Yu et al. (2003). The O₂⁻· generation of leaf sample was determined following the procedure according to Yang et al. (2011).

Histochemical detection of H₂O₂ and O₂⁻·

Histochemical localization of H₂O₂ and O₂⁻· of leaves were performed following the procedure described in Chen et al. (2010). For exposing the spots of H₂O₂ (brown spot) and O₂⁻· (dark blue) leaves were stained by 1% 3,3-diaminobenzidine (DAB) and 0.1% nitroblue tetrazolium chloride (NBT) solution, respectively.

Extraction and measurement of ascorbate and glutathione

To determine AsA and GSH, 5% meta-phosphoric acid containing 1.0 mM EDTA was used to homogenize leaf sample (0.5 g) followed by centrifugation (11,500 × g, 15 min) maintaining the temperature of 4°C. Ascorbate content was determined by following the procedure explained in Nahar et al. (2016b). Adding dithiothreitol (0.1 M) for 1 h at 25°C, an oxidized fraction of AsA was reduced. Then 1.0 unit ascorbate oxidase (AO) was mixed with the incubated mixture and measured spectrophotometrically at 265 nm. To get oxidized ascorbate (DHA), reduced AsA was subtracted from the total AsA. Glutathione and GSSG were estimated following the technique of Yu et al. (2003) where a slight modification was done (Nahar et al. 2016b). Oxidized GSH was subtracted from the total GSH for getting GSH. Standard curves of GSH and GSSG were prepared with various concentrations and GSH pool of plant sample determined.

Protein determination and enzyme extraction and assays

Leaf sample (0.5 g) was homogenized in pre-cold mortar pestle using extraction buffer which contained 1 ml of 50 mM K-P buffer (pH 7.0) including 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10% (w/v) glycerol, and subjected for centrifugation (11,500 × g, 10 min). Supernatants were used for determining enzyme activity at 0–4°C. The protein
concentration was determined following the procedure described in Bradford (1976).

APX (EC: 1.11.1.11) activity was determined according to Nakano and Asada (1981) and calculated using extinction co-efficient of 2.8 mM$^{-1}$ cm$^{-1}$.

MDHAR (EC: 1.6.5.4) activity was determined according to Hossain et al. (1984) using an extinction coefficient of 6.2 mM$^{-1}$ cm$^{-1}$ had utilized to calculate the activity.

DHAR (EC: 1.8.5.1) activity was measured according to Nakano and Asada (1981) using extinction coefficient of 14 mM$^{-1}$ cm$^{-1}$.

GR (EC: 1.6.4.2) activity was measured as per the methods of Cakmak et al. (1993) using extinction coefficient of 6.2 mM$^{-1}$ cm$^{-1}$.

SOD (EC 1.15.1.1) activity was measured according to El-Shabrawi et al. (2010) following a xanthine–xanthine oxidase system. The reaction mixture contained K-P buffer (50 mM), catalase (0.1 units), NBT (2.24 mM), xanthine (2.36 mM), xanthine oxidase (0.1 units). Then the change in absorbance of the solution was recorded for 1 min at 560 nm. SOD activity and expressed as min$^{-1}$ mg$^{-1}$ protein.

CAT (EC: 1.11.1.6) activity was measured as described in Nahar et al. (2016b) using extinction coefficient of 39.4 mM$^{-1}$ cm$^{-1}$.

GST (EC: 2.5.1.18) activity was determined following the method of Hossain et al. (2006) using extinction coefficient of 9.6 mM$^{-1}$ cm$^{-1}$.

GPX (EC: 1.11.1.9) activity was measured according to Elia et al. (2003) using extinction coefficient of 6.62 mM$^{-1}$ cm$^{-1}$.

Glyoxalase I (EC: 4.4.1.5) activity was determined as per the method of Hossain and Fujita (2009) using extinction coefficient of 3.37 mM$^{-1}$ cm$^{-1}$.

Glyoxalase II (EC: 3.1.2.6): The activity of Gly II was measured according to Principato et al. (1987) using extinction coefficient of 13.6 mM$^{-1}$ cm$^{-1}$.

Quantification of methylglyoxal content

Methylglyoxal (MG) had been determined according to Nahar et al. (2016b) as modified from Wild et al. (2012). Perchloric acid (5%) was used for homogenizing leaf sample. Then for resulting solution was then mixed with sodium dihydrogen phosphate and N-acetyl-L-cysteine. After 10 min, N-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine was formed. Then it had been recorded spectrophotometrically at 288 nm wavelength. The content of MG was calculated based on the standard curves from the absorbance of known samples.

Measurement leaf relative water content

Relative water content (RWC) was estimated following the procedure described by Barrs and Weatherly (1962). After taking the fresh weight (FW), leaves were floated on distilled water for 12 h for determination of the turgid weight (TW). Drying the turgid leaves at 80°C for 48 h dry weight (DW) had been recorded. For calculating leaf RWC with a formula: RWC(%) = [(FW− DW)/(TW− DW)] × 100. Proline (Pro) content was measured following the well-established method of Bates et al. (1973).

Determination of chlorophyll content

To estimate chl content, leaf (0.5 g) was extracted with by acetone (80% v/v), and then the homogenates were centrifuged for 10 min at 5000 × g. Finally, the absorbance of the supernatant was read spectrophotometrically at 663 and 645 nm for chl a and chl b content, respectively and computed according to Arnon (1949).

Growth measurement

For plant height measurement, the length from the base up to the top leaves of ten plants was measured, and average values were taken. Average fresh weight was taken from ten randomly selected seedlings. For dry weight, the seedlings were dried at 80°C for 48 h.

Statistical analysis

The experiment was arranged following completely randomized design. In this study analysis of variance (ANOVA) was used and mean differences were compared using Tukey’s honest significant difference (HSD) test trough XLSTAT v. 18.07 software (Addinsoft 2017) from three replicates. The significance of differences was determined at P ≤ 0.05.

Results

ROS generation and induction of oxidative stress

Compared to control, MDA content (an indicator of lipid peroxidation) was augmented by 58 and 179% under mild and severe stress, respectively (Figure 1(A)). Similarly, Pb stress aggravated H$_2$O$_2$ generation and induced oxidative stress. Mild and severe stress increased H$_2$O$_2$ levels by 41 and 95%, compared to control (Figure 1(B)). A similar increase of O$_2^•$ generation rate was noticed at either level of Pb when compared to the control (Figure 1(C)). However, exogenous GSH application in Pb-treated seedlings showed a lower amount of MDA, H$_2$O$_2$ and O$_2^•$ in the tissue and on the leaf surface and compared to Pb alone (Figure 1(A–C)).

Histochemical recognition of H$_2$O$_2$ and O$_2^•$ in leaf

Hydrogen peroxide and O$_2^•$ were detected in wheat leaves by histochemical staining of DAB and NBT. Lead stress increased spots of H$_2$O$_2$ and O$_2^•$ in wheat leaves, but exogenous GSH addition with Pb decreased those spots indicating the reduction of oxidative stress (Figure 2).

Ascorbate and glutathione levels

Ascorbate level decreased with the increase of Pb concentration in the growing media. Exogenous GSH supplementation with Pb increased the AsA levels by 11 and 28%, compared to Pb treated seedlings without GSH (Figure 3 (A)). Endogenous GSH increased under mild stress whereas it decreased under severe stress condition, compared to control. But endogenous GSH increased by 18, and 51% in GSH supplemented mild and severe stress, compared to Pb stress.
alone (Figure 3(B)). Lead affected seedlings showed high GSSG level (Figure 3(B–D)). Exogenous GSH addition reduced GSSG level and increased GSH level which increased the ratio of GSH/GSSG markedly in contrast to Pb stress alone.

**Activities of antioxidant enzymes**

Lead stress affected the enzymes of the antioxidant defense system differentially. Wheat seedlings showed higher APX activity under Pb stress, compared to control. Its activity was not increased further when Pb affected seedlings were supplemented with GSH (Figure 4(A)). The activities of MDHAR and DHAR were decreased in a dose-dependent manner under Pb stress, compared to control. Exogenous GSH application under non-stress condition did not change the activities of MDHAR and DHAR, compared to control. In contrary, Pb affected plants when added-on with exogenous GSH, the activity of MDHAR was increased by 45 and 53%, and DHAR activity was increased by 55 and 35% under mild and severe stress, compared to the Pb treated plants.
The activity of GR was increased under mild stress but diminished due to severe stress, compared to control. Exogenous GSH application augmented GR activity by 29% due to mild and 126% due to severe stress exposure, compared to Pb treatment devoid of GSH (Figure 4(D)).

The activity of SOD was increased by 10 and 35% upon exposure to 0.5 and 1.0 mM Pb(NO₃)₂ and it increased further when supplemented with exogenous GSH (Figure 5(A)). Lead stress decreased CAT activities by 16 and 31% (Figure 5(B)), decreased GPX activities by 15 and 28%, whereas increased GST activities by 30 and 44% under 0.5 and 1.0 mM Pb.
Exogenous GSH application with both levels of Pb stresses increased CAT and GPX activities (Figure 5(B and D)), in contrast to Pb stress alone. The activity of GST remained similar in GSH supplemented wheat seedlings when compared to Pb affected seedlings alone (Figure 5(C)).

**Glyoxalase system enzymes and MG**

Both the activities of Gly I and Gly II were diminished under Pb stress (except Gly II activity under mild stress) with an associated increase of MG content. Exogenous GSH supplementation amplified Gly I activity by 19 and 39% and amplified Gly II activity by 21 and 48% under mild and severe Pb treatment, compared to Pb stress alone (Figure 6(A and B)). The decrease of MG contents in GSH supplemented leaves treated with 0.5 and 1.0 mM Pb were 22 and 27%, compared to Pb treatment alone (Figure 6(C)).

**Leaf relative water content**

Leaf relative water content was decreased in wheat plants due to Pb stress, compared to control (Figure 7(A)). After application of exogenous GSH, the leaf RWC of wheat seedlings increased by 13 and 8% due to 0.5 and 1.0 mM Pb stresses, compared to Pb affected plants without GSH addition (Figure 7(A)).

**Proline content**

Lead stressed wheat seedlings were characterized by high Pro level in the present study. Proline level increased markedly at both levels of Pb stresses. Exogenous GSH supplementation was able to reduce the Pro level in Pb affected seedlings (Figure 7(B)). Proline content decreased by 24, and 22% in GSH supplemented Pb affected seedlings under 0.5 and 1.0 mM Pb stress, compared to Pb treatment only (Figure 7(B)).

**Chlorophyll contents**

Lead treatment reduced photosynthetic pigments contents in the present study. Chlorophyll a and chl b contents were reduced which resulted in a reduction of chl (a + b) under 0.5 and 1.0 mM Pb stress, compared to control. Glutathione addition with Pb stress increased chl contents, compared to Pb treatment alone (Figure 8).

**Growth parameters**

Lead stress hampered the growth of wheat seedlings. Plant height, fresh weight and dry weight of seedlings were reduced with the increase of Pb concentration (Table 1). Mild and severe Pb stress decreased plant height by 19% and 40%, fresh weight by 23 and 43%, dry weight by 21% and 50%, compared to control plants. Exogenous application of GSH in Pb stressed plants increased plant height, fresh weight, and dry weight, compared to the Pb treated plants without GSH (Table 1).

**Discussion**

Considering dynamic physiological properties of GSH including antioxidative capacity, protection of biomembrane and cellular ultrastructural components, metal chelation, redox regulation and stress signal transduction this study revealed the ameliorative role of GSH supplementation in...
improving wheat seedlings tolerance to Pb stress. In the present study, exogenous GSH application rendered a slight increase of endogenous GSH in control plant. It might be due to the maintenance of GSH homeostasis in the plant through cellular regulation. But in Pb treated plant, exogenous GSH addition increased endogenous GSH level (compared to the Pb treated seedlings without GSH). This increased GSH level has a role in metal chelation, GSH also acts as a potent antioxidant, and it interacts with other signaling molecules during biotic and abiotic stress. In our experiment, Pb-induced oxidative stress decreased noticeably after GSH supplementation mainly because of the enhancement of antioxidant enzymes and glyoxalase system. In the present study, exogenous GSH improved Pb tolerance which is evidenced by the different physiological and biochemical parameters studied in Pb affected wheat seedlings. There were various possible dimensions of confirmation of Pb tolerance induced by GSH. Glutathione itself a vital non-enzymatic antioxidant, it keeps other antioxidant components active and improves their activities (Hasanuzzaman et al. 2017a).

Lead toxicity resulted in a burst of \( \text{H}_2\text{O}_2 \), \( \text{O}_2^{\cdot -} \) and lipid peroxidation in the seedlings which is also designated by histochemical localization of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^{\cdot -} \). Lead has a high affinity for \(-\text{SH}\) groups of enzymes. It can replace the cations from binding sites of enzymes and thus inactivate the enzymes of the antioxidant system (Mishra et al. 2006; Pouriut et al. 2011). Lead has been reported to overproduce ROS including \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^{\cdot -} \) (Verma and Dubey 2003; Bai et al. 2015). Lead-induced generation of ROS interrupted antioxidant balance, and distorted lipid metabolism has been reported by Ahamed and Siddiqui (2007). In the present study, after supplementation of exogenous GSH, the oxidative stress was reduced markedly as indicated by the reduction of \( \text{H}_2\text{O}_2 \), \( \text{O}_2^{\cdot -} \) and lipid peroxidation. Glutathione reacts with \( \text{H}_2\text{O}_2 \), \( \text{OH}^{\cdot} \), and exogenous GSH addition in the present

Figure 6. Activities of glyoxalase system enzymes [Gly I (A) and Gly II (B)] and MG content (C) in wheat leaves induced by exogenous glutathione under Pb stress. Pb0.5, Pb1.0, GSH, GSH + Pb0.5 and GSH + Pb1.0 indicate 0.5 mM Pb(NO\(_3\))\(_2\), 1.0 mM Pb(NO\(_3\))\(_2\), GSH (1.0 mM), GSH + 0.5 mM Pb(NO\(_3\))\(_2\), GSH + 1.0 mM Pb(NO\(_3\))\(_2\) treatment, respectively. Mean (±SD) was calculated from three replicates for each treatments. Bars with different letters are significantly different at \( P \leq 0.05 \) applying Tukey’s HSD test.

Figure 7. Leaf relative water content (A) and proline content (B) in wheat leaves induced by exogenous glutathione under Pb stress. Pb0.5, Pb1.0, GSH, GSH + Pb0.5 and GSH + Pb1.0 indicate 0.5 mM Pb(NO\(_3\))\(_2\), 1.0 mM Pb(NO\(_3\))\(_2\), GSH (1.0 mM), GSH + 0.5 mM Pb(NO\(_3\))\(_2\), GSH + 1.0 mM Pb(NO\(_3\))\(_2\) treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at \( P \leq 0.05 \) applying Tukey’s HSD test.
study might accelerate ROS detoxification and oxidative stress reduction.

Lead exposure decreased AsA content which was resulted from reduced MDHAR and DHAR activities those are liable for AsA regeneration. The decreased MDHAR and DHAR activities together with decreased AsA level increased the H$_2$O$_2$ production of Pb-stressed wheat seedlings. These results were in the same line with the findings of previous studies (Islam et al. 2008; Nahar et al. 2016a, 2016b). Exogenous GSH addition with Pb enhanced MDHAR and DHAR activities but maintained the AsA at the same level in contrast to the Pb treatment alone. Endogenous GSH was increased under 0.5 mM Pb stress but decreased under 1.0 mM Pb stress, whereas the GSSG content and GSH/GSSG ratio decreased irrespective of Pb doses, compared to Pb stress alone. The differential modulation of GSH recycling enzyme GR and biosynthesis of GSH are responsible for altered lead and GSSG pool. Regulation of GSH or GSSG levels and their ratio by Pb and other heavy metal stress was described previously (Verma and Dubey 2003; Nahar et al. 2016a, 2016b). However, exogenous GSH addition efficiently increased GR activity and GSH content; decreased GSSG level and restored GSH/GSSG ratio in exogenous GSH supplemented Pb treatment (compared to Pb stress alone). These are supported by the previous study where exogenous GSH improved GSH redox and the activities of GR and other antioxidant enzymes in Vigna radiata seedlings subjected to cadmium (Cd) and aluminum stress (Nahar et al. 2016a, 2016b).

Superoxide dismutase is the first line of enzyme that dismutates O$_2^{-}$ by converting it to H$_2$O$_2$ (Hasanuzzaman et al. 2012). Superoxide dismutase activity increased under severe stress in wheat seedlings, compared to control. Higher CAT activity is vital to reduce H$_2$O$_2$ (Tao et al. 2012). Reduction of CAT activity under Pb stress was responsible for increasing H$_2$O$_2$ production in Pb affected wheat seedlings in the present study. Lead-induced modulations of APX, SOD, and CAT activities have been reported in previous studies (Verma and Dubey 2003; Islam et al. 2008; Bai et al. 2015). Role of GPX is vital in reacting with H$_2$O$_2$ and lipid hydroperoxides with the help of GSH which prevents ROS overproduction and defends oxidative damage (Gill and Tuteja 2010; Hasanuzzaman et al. 2012). Increased GST activity is also important in ROS and metal detoxification and abiotic stress tolerance (Hasanuzzaman et al. 2012; Nahar et al. 2016a). In this study, GPX activity decreased, and GST activity increased in Pb treated seedlings, compared to control. Supplementation of exogenous GSH with Pb did not increase SOD activity further but the increased CAT, GPX and GST activity which together scavenged ROS and reduce oxidative injury in wheat seedlings, compared to Pb stress alone. Our results are corroborated by Nahar et al. (2016a) who observed that exogenous GSH addition increased SOD, CAT, GPX and GST activities in mung bean plants subjected to cadmium (Cd) stress.

Modulation of the components of MG detoxification system was found as an effective way to minimize the overproduction of MG under heavy metal stress (Nahar et al. 2016a). Wheat seedlings showed higher MG content under different levels of Pb stresses in our study which is corroborating with decreased activities of Gly I and Gly II which play a pivotal role in detoxifying MG. But when exogenous GSH was added with Pb, the wheat seedlings showed higher Gly I and Gly II activities and higher GSH level (compared to Pb treatment alone) which reduced MG content. The role of exogenous GSH in alleviating MG levels in tobacco was reported by Yadav et al. (2005b). Glyoxalase overexpressing transgenic tobacco plants exhibited higher GSH/GSSG, higher Gly I activity, reduced MG level and improved salt tolerance (Yadav et al. 2005a, 2005b). Improved Gly I and Gly II activities with elevated GSH content efficiently reduced MG toxicity in mung bean plants subjected to Cd treatment (Nahar et al. 2016a).

**Table 1.** Plant height, fresh weight and dry weight of seedling induced by exogenous glutathione (GSH) under Pb stress.

| Treatments  | Seedling height (cm) | Fresh weight of seedlings (g) | Dry weight of seedlings (g) |
|-------------|----------------------|-------------------------------|----------------------------|
| Control     | 14.12 ± 1.20 ab      | 1.35 ± 0.11 ab                | 0.14 ± 0.006 a             |
| Pb0.5       | 11.46 ± 0.97 b       | 1.04 ± 0.19 bc                | 0.11 ± 0.012 bc            |
| Pb1.0       | 8.47 ± 0.25 c        | 0.76 ± 0.03 c                 | 0.07 ± 0.003 d             |
| GSH         | 14.33 ± 1.22 a       | 1.38 ± 0.11 a                 | 0.14 ± 0.006 a             |
| GSH + Pb0.5 | 12.69 ± 1.08 ab      | 1.26 ± 0.10 ab                | 0.13 ± 0.006 ab            |
| GSH + Pb1.0 | 12.46 ± 0.75 ab      | 1.19 ± 0.08 ab                | 0.09 ± 0.004 c             |
| Pb0.5, Pb1.0, GSH, GSH + Pb0.5 and GSH + Pb1.0 indicate 0.5 mM Pb(NO$_3$)$_2$, 1.0 mM Pb(NO$_3$)$_2$, GSH (1.0 mM), GSH + 0.5 mM Pb(NO$_3$)$_2$, GSH + 1.0 mM Pb(NO$_3$)$_2$, treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Tukey’s HSD test.
Plants exposed to heavy metal reduce water uptake. This reduction of water uptake is associated with high Pro level which is an indicator of stress, and the increased Pro level is suggested to act as an osmoprotectant. In the present study, wheat seedlings exposed to Pb also showed high Pro level. In the previous study, rapeseed plants showed increased Pro level with rising concentrations of Pb (Gohari et al. 2012). John et al. (2009) observed elevated Pro accumulation while studying with Brassica juncea under Pb and Cd stresses. However, the present study showed decreased Pro levels but increased leaf RWC after exogenous GSH addition which is an indication of a reduction of water stress within the plants.

Lead-induced ROS production might also be involved in the breakdown of chl pigments which was evident in the present study. We noticed a reduction of chl content under Pb stress (compared to control) which was reversed by exogenous GSH addition with Pb stress. In some previous reports, a GSH-induced increase of photosynthetic pigment was demonstrated. Glutathione-induced oxidative stress reduction was supposed to prevent damage to chl biosynthesis enzymes (Kattab 2007). Addition of exogenous GSH (20 mg L⁻¹) reduced chlorosis and necrosis in leaves in Cd affected barley genotypes (Chen et al. 2010). In the present study, physiological parameters were improved by exogenous GSH application and the integrative effect was noticed in case of the growth of wheat seedlings. Pb treatment decreased the height, fresh weight, and dry weight of seedlings. The positive effect of exogenous GSH was noticed in Iris lactea under Pb stress. Exogenous GSH application increased root and shoot GSH synthesis at endogenous cellular level, regulated phytochelatin synthesis, decreased Pb accumulation and improved growth (Yuan et al. 2015). Similarly, GSH-induced Pb tolerance was reported in Sedum alfredii (Gupta et al. 2010).

**Conclusion**

From this study, it is observed that exogenous application of GSH elevated Pb-induced oxidative damages. Enhancement of antioxidant enzyme activities and an increase of the non-enzymatic antioxidant level contributed to alleviate oxidative stress. Specifically, GSH supplementation in Pb affected seedlings improved the AsA and GSH pool and increased the activities of MDHAR, DHAR, GR, CAT and GPX where the seedlings exposed to Pb (without GSH supplementation) failed to do so. Increased GSH level and the higher activities of Gly I and Gly II helped in MG detoxification. Exogenous GSH supplementation also increased chl content, regulated Pro content and improved the seedling growth. Although we propose a possible mechanism of GSH-mediated Pb tolerance in plants the molecular mechanisms and genetic bases of such effects would be a subject of further studies. Moreover, the signaling roles of GSH in modulating biosynthesis of biomolecules and interaction between or among GSH and other biomolecules in imparting stress tolerances require more investigation.

**Acknowledgments**

We acknowledge Khursheeda Parvin, Mazhar Ul Alam, Taufika Islam Anee and Farah Tasmin, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for the critical reading and editing of the manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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