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PHYTOTOXICITY OF LEAD AS ENVIRONMENTAL POLLUTANTS ON SOYBEAN (*GLYCINE MAX* L.) AND AMELIORATING EFFECT OF 5-AMINOLEVULINIC ACID

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Abstract

Lead is a toxic element distributed everywhere and is one of the most abundant minerals. The present work aimed to investigate whether exogenous pretreatment of soybean (*Glycine max* L.) with 5-aminolevulinic acid (ALA) could protect plants from Pb stress at 100µM. the activities of NADH-oxidase (EC, 1.6.3.1), superoxide dismutase (EC, 1.15.1.1), ascorbate peroxidase (EC, 1.11.1.11), glutathione reductase (EC, 1.6.4.2) and catalase (EC, 1.11.1.6) were increased under stress. Hydrogen peroxide (H_2O_2), malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), proline and protein carbonylation were increased. Soluble protein decreased gradually depending upon concentration. Aminolevulinic acid (ALA) treatment decreased H_2O_2, MDA and protein carbonylation. The results suggest that exogenous ALA may improve the tolerance against heavy metal stress.

Key words: Cadmium, Lead, *Glycine max*, ALA, Enzymes

Introduction

Heavy metals pollution is one of the major environmental problems in the world today. The unsuitable condition created by human activities, such as mining, smelting, energy transfer and intensive agriculture, has threatened the survival of man himself, as well as of other living organisms (Nedelkoska and Doran, 2000; Flora *et al*., 2008).Monitoring the pollution status of the environment using plants is one of the main topics of environmental biogeochemistry (Diatta *et al*., 2003; Ojekunle *et al*., 2018).

Heavy metals are known to be non-degradable, persist in nature for a longer period and toxic to living organisms as fairly low concentrations. Heavy metals are one of the major pollutants that are accumulated in environment. The stress by heavy metals causes various direct and indirect effects on practically all physiological processes in plants (Sethy and Ghosh, 2013).

In the environment the levels of heavy metals had never been a threat to health but in the recent years increased industrial activities leading to air borne emissions, autoexhausts, and sewage waters from industries as well as solid waste dumping have become the source of large quantities of heavy metals into the environmental (Saroware*t al*., 2012). Rising levels of heavy metal concentrations in the environmental has evolved increasing concern as can be realized from the levels of some metals in an urban environment. Plants are essential components of natural ecosystems and agroecosystems, and are the first component of the terrestrial food chain. The toxicity of heavy metals on plant physiology and metabolism are very complex, and they depend on plant sciences, nature of heavy metal and its concentration (Youssef and Azooz, 2013).

Soybean (*Glycine max*) is a species of Fabaceae family (bean family), which native to East Asia, widely grown for its edible bean. The plant is classed as an oilseed rather than a pulse by the UN Food and Agricultural Organization (FAO).It is favoured by wide variety of climates and soils and thus considered to be the most economical crop (Zaefarian and Rezvani, 2016). It contains about 20 % oil and 40 % high quality protein. Soybean protein is rich in valuable amino acids (5%) in which most of the cereals are deficient. In addition, it contains a good amount of minerals, unsaturated fats,

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salts and vitamins (thiamine and riboflavin) and its sprouting grains contain a considerable amount of Vitamin C (Imtiyaz *et al*., 2014; Miransari, 2016).

Lead continues to be used widely in many industrial processes and occurs as a contaminant in all environmental compartments (soils, water, the atmosphere, and living organisms). The prominence of environmental lead contamination results both from its persistence (Punamiya *et al*., 2010; Sethy and Ghosh, 2013) and from its present and past numerous sources.Lead is ubiquitously distributed toxic elements and one of the most abundant metals. It exerts adverse effects on growth and photosynthetic processes of plants and cause inhibition of enzyme activities, water imbalance, alteration in membrane permeability and disturbs mineral nutrition(Pingto *et al*., 2004).The aim of this study was to investigate the effect of lead (Pb) as environmental pollutants on the various metabolites and activities of some enzymes of *Glycine max* metabolism.

Materials and Methods

Growth of soybean

Soybean growth was carried out according to (Chakhchar *et al*., 2015). A variety of soybean (*G. max* L.) was provided by Ministry of Agriculture, Iraq. Seeds were surface-sterilized with 5% (v/v) sodium hypochlonite (NaOCl) for 10 min and rinsed several times with distilled water.

Treatment

The hydroponics medium was Hoagland nutrient solution (Hoagland and Arnon, 1950), this being continuously aerated and replaced every day. After two weeks growth, plants were pretreated for 12h with 200µM ALA or nutrient solution (control). Afterwards, plants were subjected to heavy metal stress using 100µmol of $PbCl_2$ for 72h under the same light regime as indicated above.

Enzymes extraction

Collect approximately 100 grams of mixed leaf plant samples, wash material 3x in water andground to fine powder by liquid nitrogen and then homogenized in 5 ml of 100 mM Tris-HCl (pH 7.7) containing 100mM EDTA, 0.5 % (w/v) Triton x-100, 1 mM phenyl-methyl-sulfonyl fluoride (PMSF), and 5 mM dithiothreitol. For determination of APX activity, 2mM ascorbate was added into the homogenization buffer and polyvinyl pyrrolidone (2% w/v) was used instead of dithiothreitol. The homogenate was centrifuged at 10,000 g for 30 min. The resulting supernatant was used for determination of the enzymes activities and the various amylases (Bradford, 1976).

Enzymes assay

Total superoxide dismutase activity (SOD, EC: 1. 15. 1.1) activity was measured by the nitroblue tetrazolium (NBT) method of Becana *et al.* (1986). One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction under the assay condition. NADH-oxidase (EC, 1.6.3.1) activity was assayed by the method of Miramar *et al*. (2001). One unit of the enzyme was defined as one µmol NADH oxidized min^{-1} $protein^{-1}$ under the assay condition. Glutathione reductase (GR, EC: 1.6. 4. 2) activity was determined according to the method of Yan *et al*. (2012). One unit of GR activity was defined as the oxidation of 1 µmol NADPH per min at 25°C and pH 7.6. Catalase (CAT, EC: 1. 11. 1. 6) activity was estimated following the consumption of H_2O_2 at 240nm for 3min (Aebi, 1984). One unit of catalase was defined as the amount that decomposes 1µmol H_2O_2 per min at 25°C and pH 7.8. Ascorbate peroxidase (APX, EC: 1. 11. 1. 11) activity was assayed using the method of Nakano and Asada (1981). One unit of APX activity was defined as the amount oxidizing of 1Mmol ascorbate per min at 25°C and pH 7.0.

Determination of protein and protein carbonylation

The total soluble protein content of the enzyme extract was determined according to Bradford (1976) and employing BSA as the standard. On the other hand, in vitro carbonylation of proteinswas carried out according to the method of Pyngrope*et al*. (2013).

Determination of proline and lipid peroxidation

Proline content was measured according to the method described by Bates *et al*. (1973). Fresh leaves were ground in 1.5ml of 3% (w/v) and proline was estimated by ninhydrin reagent. The ninhydrin reaction mixture was partitioned against toluene and absorbance of the toluene phase was read at 520nm. Proline concentration was determined by plotting a standard curve, it was expressed in Mmol g-1 fresh weight. Lipid peroxidation was measured in terms of MDA content according to the method of Zhang *et al*. (2008).

Antioxidant capacity

Determination of H_2O_2 content

Fresh leaf tissue samples were ground in cold acetone (10% w/v) and centrifuged at 3000 g for 10min. Oneml of the supernatant was mixed with 0.1 ml titaniumreagent and 0.2ml of 17M ammonia solution and then centrifuged at 3000g for 10min. The precipitate was washed three times withacetone and dissolving in 3ml of 1 M H_2SO_4.

The absorbanceof the solution was measured at 410nm againstblanks, which had been prepared similarly but withoutplant tissue (Al-Aghabary *et al*., 2004).

Determination of glutathione

Total glutathione content was measured as described by Akerboom and Sies (1981). Samples were extracted with an equal volume of a solution containing 2m $HClO_4$ and 4mm EDTA. The preparation was mixed for 2min and centrifuged for 10min (7300g, Eppendorf 5415C microfuge) at 4°C. The pellet was retained for protein measurements and the supernatant was removed to a clean tube and neutralized with a solution containing 2 m KOH and 0.3 m 3^-](N-]morpholino) propane sulfonic acid. The samples were then mixed for 2min and left on ice for 10min. After centrifuging (7300g) for 10 min at 4°C, the supernatant was removed and placed on ice for measurements of glutathione.Reduced glutathione was determined as the difference between total glutathione and GSSG.

Statistical analysis

The data were subjected to ANOVA and the mean values were separated based on Least Significant Difference (LSD) at 0.05 probability level using COSTAT 6.3 program.

Results and Discussion

Effect of Pb on the activities of antioxidant enzymes

The results in the present work reveal that treatment of *G. max* seedlings with Pb enhanced the activities of the tested five enzymes NADH-oxidase, superoxide dismutase (fig. 1), ascorbate peroxidase, catalase (fig. 2), and glutathione reductase (fig. 3). The elevated activities of all enzymes were dependent on the concentrations of the tested metal. Many studies have shown that increased plasma membrane NADH-oxidase activity was associated with increased $O2^{-}$ and H_2O_2 production following biotic and abiotic stresses (Lara-Nunez *et al*., 2006). It seems likely that NADH-oxidase is one of the generation sites for ROS in *G. max*.

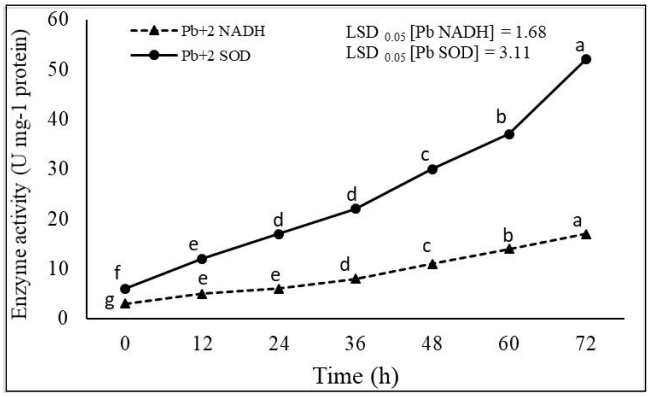

Fig. 1: The activities of NADH-oxidase and SOD in 5-day old seedlings treated with $PbCl_2$.

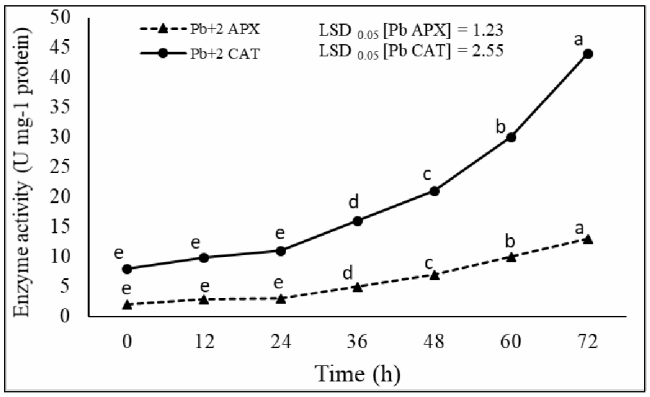

Fig. 2: The activities of APX and Catalase in 5-day old seedlings treated with $PbCl_2$

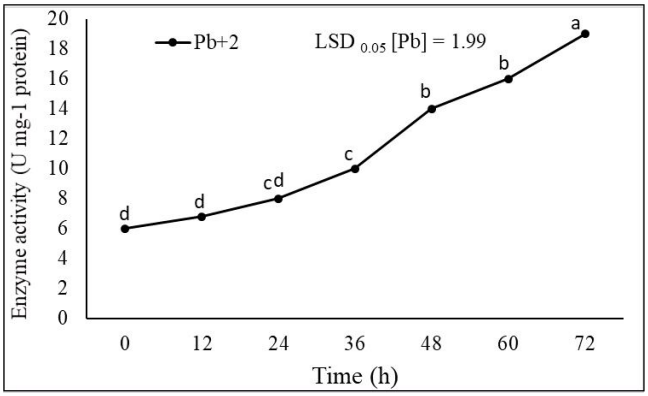

Fig. 3: The activities of glutathione reductase in 5-day old seedlings treated with $PbCl_2$.

SOD is considered as a first defense against ROS as it acts on superoxide radicals, which are produced in different compartments of the cell and precursors of the other ROS (Alscher *et al*., 2002). SOD activity was increased with Pb treatments. The enhanced SOD activity in the presence of the metal ions is possibly circumstantial evidence for the production of reactive oxygen species (ROS). The increase in SOD activity in *G. max* is attributed to the increase in superoxide radical concentrations, this is likely due to de novo synthesis of enzyme protein (Lamhamdi *et al*., 2011), which is possibly attributed to transcription of SOD genes by a superoxide-mediated transduction signal (Asada and Takahashi, 1987).

The results show that the increase in SOD activity is correlated with the increased H_2O_2 production in plant tissues. H_2O_2 produced is a potent oxidizing agent. H_2O_2 like other ROS can be expected to be responsible for lipid peroxidation, so plants need to destroy it by CAT and APX. The increase in SOD activity in response to heavy metals is comparable to the reported results for

rape leaves and seedlings of tomato cultivated under heavy metal stress (Cho and Park, 2000).

APX reduces H_2O_2 into H_2O using ascorbate (as the electron donor, resulting in the formation of dehydroascorbate. The latter is recycled back to ascorbate using reduced GSH as an electron donor and the GSSG is converted back to GSH by NADPH. The increased APX activity under Pb stress suggests its role in the detoxification of H_2O_2 in *G. max* seedlings. The stimulation of APX activity has also been reported in several plants subjected to Pb (Sarowar *et al*., 2012). Also, enhancement of APX activity has also been reported in various plants treated with Pb (Rucinska *et al*., 1999; Shaw, 1995).

CAT activity was higher than APX activity in *G. max* under Pb stress. It can be suggested that Pb induced H_2O_2 production which, was successfully detoxified by both CAT and APX, but it appears that CAT is more important than APX in detoxification of H_2O_2. Catalase activity was found to increase under Pb phytotoxicity.

GR catalyzes the reduction of GSSG to GSH at the expense of NADPH. This is an important determination in the maintenance of glutathione pool. GR activity was enhanced in soybean seedlings in presence of Pb. GR activity was enhanced in soybean seedlings in presence of Pb (Chaoui *et al*., 1997; Prasad *et al*., 1999).

Enzymes of ascorbate-glutathione cycle are localized mainly in chloroplasts and also in other cellular organelles and cytoplasm, where they play important role in combating oxidative stress. SOD, APX, GR and CAT show simultaneous induction in soybean, which may be due to their coregulation (Sharma and Dubey, 2004).

Effect of Pb on MDA and H_2O_2 contents

Exposure of *G. max* seedlings to Pb induced lipid peroxidation (fig. 4). MDA is the product of membrane lipid peroxidation and its content reflects the degree of cell membrane damage when exposed to ROS. The lipid peroxidation was dependent on the concentration of Pb. Also, H_2O_2 content increased gradually in response to the concentration of Pb (fig. 5).

Lipid peroxidation was observed in Pb toxicity. Malondialdehyde (MDA) is a well-known lipid peroxidation indicator (Mittler, 2002) and its content increased with Pbtreatment of *G. max* seedlings. Reasons for lipid peroxidation after heavy metal exposure are not completely known, but it is believed that exposure to heavy metals may allow free radicals which can attack double bonds in membrane lipids and result in an increase in lipid peroxidation. Thus, the enhanced activities of antioxidant enzymes soybean under Pb treatment may be considered as circumstantial evidence for tolerance mechanism developed by soybean. It is well known that lead toxicity resulted in enhanced ROS generation (Lamhamdi *et al*., 2011).

Lipid peroxidation requires active O_2 uptake and involves the production of superoxide radical ($O_2^{\cdot-}$) (Fridovic, 1986). The other highly reactive chemical species are singlet oxygen (O_2), hydroxyl free radical ($OH^{\cdot}$) and H_2O_2. Which, initiate lipid peroxidation (Dhindsa *et al*., 1981). Hence, constitution and/or induced activity of SOD and other antioxidants enzymes such as APX, CAT and GR are essential.

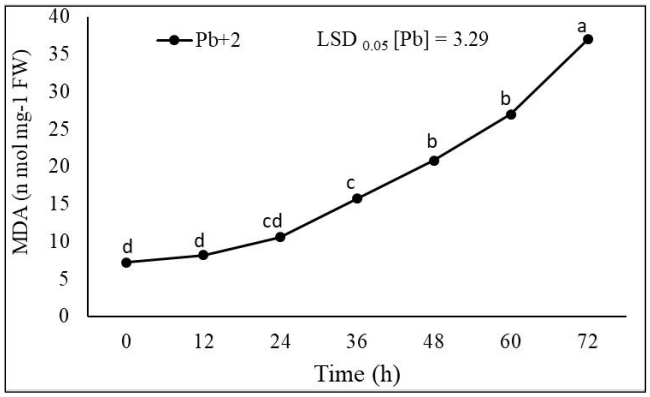

Fig. 4: Malondialdehyde (MDA) content in 5-day old seedlings treated with $PbCl_2$.

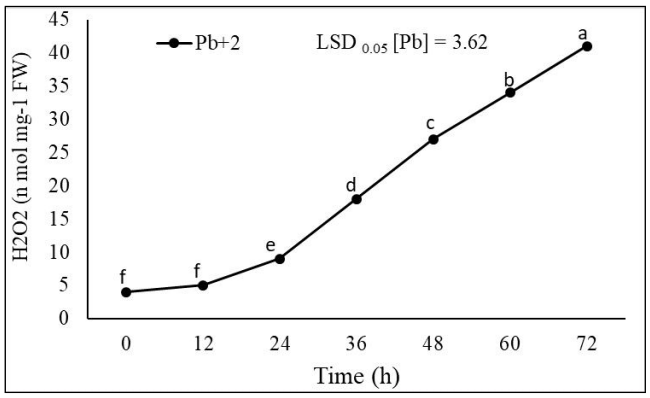

Fig. 5: Hydrogen peroxide content in 5-day old seedling treated with $PbCl_2$.

Effect of Pb on reduced glutathione and oxidized glutathione contents

The results in the present investigation indicate that both GSH (fig. 6) and GSSG (fig. 7) contents were increased by treatment of seedlings with Pb. However, the content of GSH was reduced gradually after 48h in case of Pb-treatment. In contrast, GSSG content reached its highest content at 36h after which it declined continuously in presence of the Pb. It was observed that the content of GSH was higher than that recorded for GSSG throughout the experimental period. Also, the ratio

of GSH/GSSG (fig. 8) increased with increasing the concentrations of metal. The results indicate increased level of GSH and GSSG at the lower concentrations in Pb-treated seedlings of *G. max*. This suggests active participation of GSH in detoxification of oxygen species and free radicals (Asada and Takahashi, 1987).

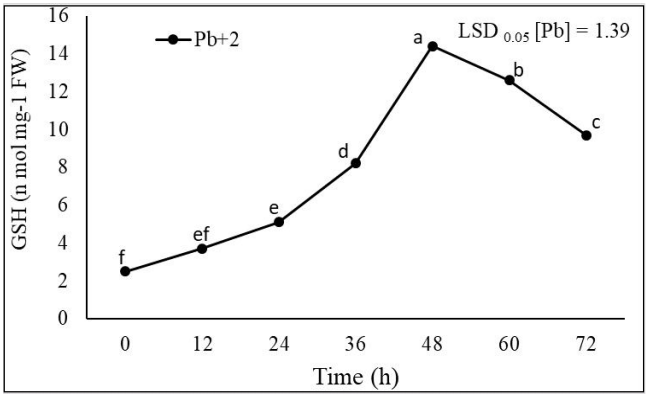

Fig. 6: GSH content (n mol mg^{-1} protein) in 5-day old seedlings treated with $PbCl_2$.

The GSSG content in the present investigation was lower than that of GSH. However, it has been reported that the concentration of GSSG increased at the expense of GSH. In severe stress conditions a high GSH/GSSG ration is necessary to sustain the role of glutathione as an antioxidant and a reductant (Foyer *et al*., 1997). Pb treatments for soybean enhanced the GSH/GSSG ratio. Studies carried out on several plants species subjected to various abiotic stresses indicated that a high GSH /GSSG ratio maintained by increased GSH synthesis and /or GSSG (Mendoza-Cozati *et al*., 2005). Reduction this may be necessary for efficient tolerance of plant against heavy metal stress. In addition, it is essential to keep glutathione in its reduced from for its incorporation into phytochelatins, which chelate metals (Cobbett, 2000). Consumption of GSH by phytochelatins synthesis induces an increase in the rate of GSH synthesis to restore basal levels(Mendoza-Cozati *et al*., 2005).

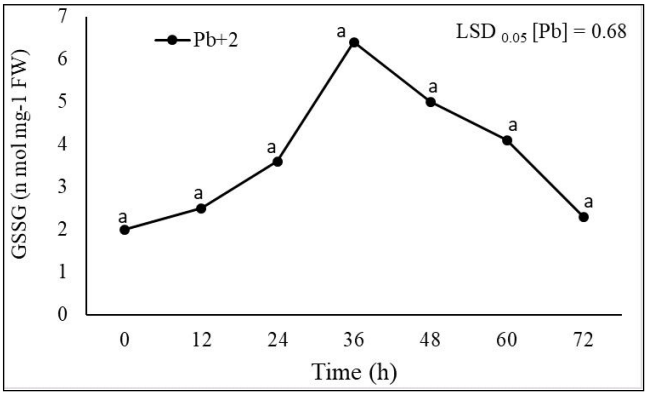

Fig. 7: GSSG content (n mol mg^{-1} protein) in 5-day old seedlings treated with $PbCl_2$.

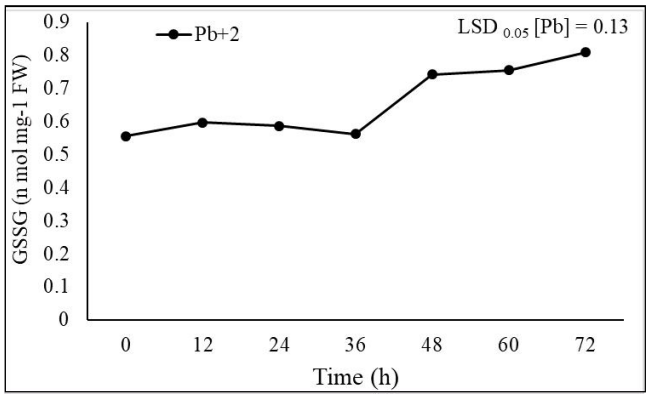

Fig. 8: Glutathione nedox state [GSH/(GSH+GSSG) ratio].

Effect of protein carbonylation

The results in the present investigation show that there was a corresponding increase in protein carbonylation in response to the increase in the concentration of Pb (fig. 9). The present results show occurrence of protein carbonylation in *G. max* and this process is increased with increasing metal concentration. Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. It is usually referred to a process that forms reactive ketones or aldehydes that can be reacted by 2, 4-dinitrophenylhydrazoine to form hydrazones. Direct oxidation of side chains of lysine, arginine, proline and threonine residues, among other amino acids in the "Primary protein carbonylation" reaction produces DNPH detectable protein products (Suzuki *et al*., 2010). It is a good index of oxidative stress (Debska *et al*., 2012; Moller *et al*., 2007).

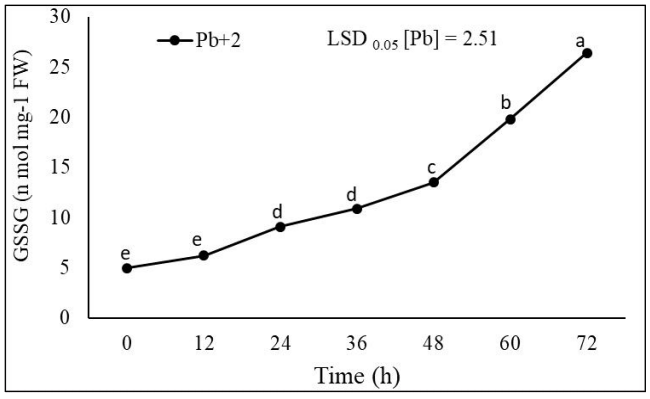

Fig. 9: Carbonyl content (protein oxidation) in 5-day old seedlings treated with $PbCl_2$.

Effect of Pb on soluble protein and proline contents

The effect of lead on soluble protein content in *G. max* was examined at two different concentrations (fig.10). Soluble protein content showed a remarkable decrease trend with the increase in the concentration of the tested metal ion. The proline content (fig. 10) of *G. max* leaves was greatly enhanced by Pb treatment and

the increase of proline concentration was dose-dependent. The increase in proline is in harmony with the results of Lamhamdi *et al*. (2011) and Singh *et al*. (2012). The increased level of proline in seedlings under heavy metal stress might be due to protein degradation. Proline has been shown to alleviate metal-induced oxidative stress by scavenging harmful ROS (Tripathi and Gam, 2004). Proline is known to accumulate under heavy metal exposure and considered to be involved in stress resistance. Free proline has been found to chelate metal ion in plants by forming non-toxic metal –proline complex and protect enzymes and cellular structure (Spiripoornadulsil *et al*., 2000).

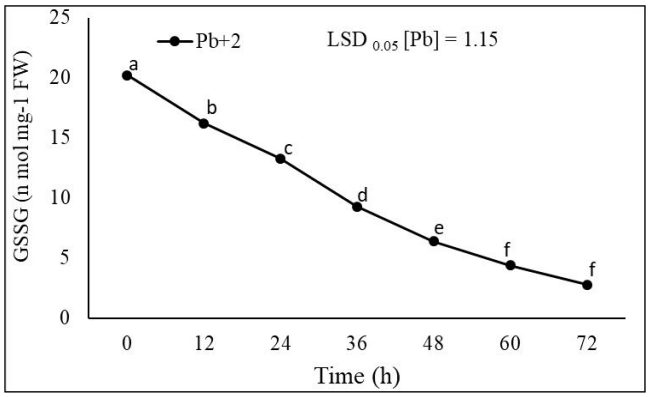

Fig.10: Protein content in 5-day old seedlings treated with $PbCl_2$.

enhancement of protein synthesizing machinery (El-khallal *et al*., 2009). Pretreatment with ALA ameliorated Cd-induced biochemical changes in seedlings of oilseed rape (Ali *et al*., 2013). Also, ALA was found to improve the plant growth by ameliorating stress. ALA may reduce the content of MDA, H_2O_2 and protein carbonylation through induction of antioxidant enzymes which can scavenge free radicals such as $O_2^{\cdot-}$ and H_2O_2 which are responsible for the damaging of the cell (Garcia *et al*., 2012; Xu *et al*., 2012). In conclusion, the results show that pretreatment of seedlings with ALA reduced MDA content and H_2O_2 production as well as protein carbonylation induced by Pb treatment.

Table 1: Alleviation the toxic effect of $CdCl_2$ and $PbCl_2$ on MDA, H_2O_2 and protein carbonylation.

Treatment	MDA (nmol mg^{-1} FW)	H_2O_2 (nmol mg^{-1} FW)	Carbonyl content (nmol mg^{-1} protein)
Control	8.0 ± 0.05	5.0 ± 0.001	5.0 ± 0.01
100 µM $CdCl_2$	55.4 ± 0.7	53.7 ± 0.6	34.7 ± 0.7
100 µM $PbCl_2$	38.3 ± 0.6	42.0 ± 0.7	27.4 ± 0.5
200 µM ALA + 100 µM $CdCl_2$	18.2 ± 0.2	21.4 ± 0.4	13.4 ± 0.2
200 µM ALA + 100 µM $PbCl_2$	11.0 ± 0.09	16.0 ± 0.3	10.7 ± 0.1

Effect of ALA pretreatment on H_2O_2, MDA and protein carbonylation

Pretreatment of *G. max* with exogenous ALA at 100 µM resulted in decreasing of H_2O_2 content, MDA content and protein carbonylation compared with non-treated seedlings (table 1). The reduction in protein content may be due to alteration of the transitional process or the transcriptional of some enzymes responsible for

Conclusion

Findings of the present study revealed that lead induced considerable changes in all the biochemical parameters studied in *Glycine max*. Lead toxicity has proved to be more consistent in its effect on the various plant parameters studied. The effect of lead was comparably more at higher concentrations (100µM). The activities of studied enzymes were increased under stress. While, soluble protein decreased gradually depending upon concentration. Aminolevulinic acid treatment decreased H_2O_2, MDA and protein carbonylation. The results suggest that exogenous ALA may improve the tolerance against heavy metal stress.

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