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Glycine betaine-induced lead toxicity tolerance related to elevated photosynthesis, antioxidant enzymes suppressed lead uptake and oxidative stress in cotton

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Abstract: Anthropogenic activities contaminate soils with heavy metal concentrations including lead (Pb), cadmium (Cd), copper (Cu), and chromium (Cr). Pb has higher potential for ready accumulation, sedimentation, and poisoning of the soil than other heavy metals. The present study was conducted to induce Pb tolerance, in solution culture-grown cotton, by exogenous glycinebetaine (GB) application using 3 levels of Pb (0, 50, and 100 µm) and 2 GB levels (0 and 1 mM). The results revealed that Pb stress decreased gas exchange characteristics (net photosynthetic rate, stomatal conductance, transpiration rate, water use efficiency, chlorophyll, carotenoids, and SPAD value) and the performance of antioxidant enzymes. Toxic effects of Pb stress were mitigated by GB application, which in turn increased the plant growth and gas exchange characteristics by reducing the performance of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and electrolytic leakage. An increase in the activity of antioxidant enzymes helped in mediating the adverse effect of the Pb stress as compared to plants treated with Pb alone. The results revealed that exogenous application of GB significantly alleviated the Pb toxicity by improving the growth, biomass, and photosynthetic parameters in cotton (*Gossypium* L.) plants. GB increased Pb tolerance by enhancing the chlorophyll synthesis, photosynthetic activities, and antioxidant enzyme activities and by lowering the electrolytic leakage, MDA, and H_2O_2 levels.

Key words: Antioxidant enzymes, *Gossypium*, electrolyte leakage, growth, lead, glycinebetaine, photosynthesis

1. Introduction

Depending upon the oxidation state, heavy metals could be highly reactive and consequently toxic for most plant organisms. There is evidence that a plant's ability to mitigate the negative impacts of redox reactive heavy metal stress, by increased antioxidative protection, appears to be limited (Sekmen Esen et al., 2012; Ali et al., 2013a). Great attention has been focused on heavy metal pollution research with the progress in agriculture industry worldwide (Ali S et al., 2013a, 2013b). Lead (Pb) is found to be the most dangerous heavy metal, responsible for reduced soil fertility and elevated environmental pollution (Shahid et al., 2012). Lead toxicity causes the inhibition of seed germination and exerts adverse effects on growth and metabolic processes of plants, which retards plant and crop production.

The overproduction of reactive oxygen species (ROS) is the best indicator for secondary stress, which results in a number of toxic effects on biochemical processes in many plant cells (Ali S et al., 2013b). The overproduction of ROS due to Pb stress brings about changes in cellular membrane permeability, which in turn damages organelles such as nuclei, mitochondria, and chloroplasts in plant cells (Clemens, 2001; Ali et al., 2012).

Malondialdehyde (MDA) production is also a major indication of oxidative stress due to lipid peroxidation in plants exposed to stress conditions (Yamamoto et al., 2001). A significant increase in MDA content was reported in zinc-stressed duckweed seedlings (Radic et al., 2010), while Szőllősi et al. (2009) reported the same in cadmium-treated seeds of *Brassica juncea* L.

Glycinebetaine (GB) is synthesized abundantly in the chloroplast, where it plays a significant role in the defense and regulation of the thylakoid membrane by maintaining photosynthetic attributes (Allakhverdieva, 2001). In many crop plants the natural GB accumulates at levels that can counterbalance the adverse effects of various environmental stresses (Yancey, 1994; Subbarao et al., 2001). GB addition

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is equally beneficial for nonaccumulating and/or low-accumulating plants in ameliorating the adverse effect of environmental stresses (Agboma et al., 1997a, 1997b; Yang and Lu, 2005; Zhang 2013). The breakdown of naturally synthesized GB does not occur in plants (Bray et al., 2000) and it can be collected as an inexpensive by-product from plants (Rhodes and Hanson, 1993). The application of GB as well as its extraction is an economically feasible approach in reducing the negative effects of environmental stresses on crop productivity. There are many studies reporting significant effects of exogenous application of GB on plant yield and crop productivity under drought stress; examples include those in tobacco, barley, wheat, beans, soybean, and sorghum. Lutts (2000) reported that the negative effects of salt stress on plants can be mitigated by GB application and that, under salt stress, GB-treated plants had significantly higher K^+ and lower Na^+ concentrations in the shoots compared with untreated plants. In maize (*Zea mays* L.), exogenously applied GB improved growth, net photosynthesis, leaf water content, and the quantum yield of photosynthesis in salt-stressed plants (Yang and Lu, 2005). The defensive role of GB may either have a positive impact on enzymes and integrity of membranes or may act as an osmoprotectant that helps in protecting against environmental stress indirectly through the mechanism of signal transduction (Subbarao et al., 2000; Chen and Murata, 2011).

Therefore, researchers focus on reducing the toxic effect of Pb on plant growth. As a novel endogenous quaternary ammonium compound that is found in hemophilic archaebacteria, mammals, bacteria, invertebrates, and plants (Rhodes and Hanson, 1993; Chen and Murata, 2002, 2008), GB has beneficial physiological effects on plants. Glycinebetaine as a phytoprotectant against Pb stress in cotton (*Gossypium* L.) plants has not been reported yet. The present study investigated the potential role of GB in mediating harmful effects of Pb stress on cotton plants. Biochemical and physiological changes related to Pb-induced oxidative stress on plant growth and biomass, chlorophyll content, photosynthetic parameters, soluble protein, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content, and activities of antioxidant enzyme such as superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were assessed.

2. Materials and methods

2.1. Experimental sites

This study was carried out in the wire house of Ayub Agricultural Research Institute and in the labs of the Government College University Faisalabad and Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.

2.2. Growth conditions

Healthy seeds of cotton genotype MNH 886 were immersed in a concentrated sulfuric acid solution for 15 min to remove short fibers from the surface of the seeds. Seeds were then thoroughly rinsed with distilled water and sown in 5-cm layers of sterilized quartz sand trays in a growth chamber with a photoperiod of 16 h of light and 8 h of dark with light intensity of 400 ± 25 μmol m^{-2} s^{-1}. The light/dark temperature was set at 30/25 °C with relative humidity of 85%. After 2 weeks of sowing, the uniform seedlings were wrapped with foam at a root shoot junction and transplanted in thermocol sheets having evenly spaced holes floating on iron tubs of 40 L in capacity, lined with polyethylene sheeting containing modified Hoagland's solution. The basic nutrient medium had the following composition: $Ca(NO_3)_2$ 2.5 mM, $MgSO_4$ 1 mM, KCL 0.5 mM, KH_2PO_4 0.5 mM, $FeCl_3$ 0.1 μM, $CuSO_4$ 0.2 μM, $ZnSO_4$ 1 μM, H_3BO_3 20 μM, H_2MoO_4 0.005 μM, $MnSO_4$ 2 μM. Continuous aeration was given by an air pump in the nutrient solution by making air bubbles. The solution was changed every week. A complete randomized design was applied. Two weeks after transplanting, 3 Pb levels (control and 50 and 100 μM) developed with $Pb(NO_3)_2$ and 2 levels of GB (control and 1 mM) with 3 replicates were applied. By adding 1 M H_2SO_4 and NaOH solution, the solution's pH was maintained at 6.0 ± 0.1.

2.3. Measurements of plant growth and biomass

Plants were harvested after 6 weeks of growth under Pb stress. Data regarding shoot and root length, and shoot and root fresh and dry weight, were determined.

2.4. Leaf area

Leaf area was determined with a leaf area meter (LI-2000, LI-COR, USA).

2.5. Gas exchange parameters

Six weeks after application of treatment, the photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), and water use efficiency (Pn/E) were determined using an Infra-Red Gas Analyzer (Analytical Development Company, Hoddesdon, UK).

2.6. SPAD value

The SPAD-502 (Soil–Plant Analyses Development) enables quick, easy measurement of chlorophyll. After 6 weeks of treatment, SPAD values were measured with the SPAD-502 meter (Zhejiang Top Instrument Co., Ltd., China).

2.7. Chlorophyll contents

Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were determined spectrophotometrically (Metzner et al., 1965). After 6 weeks of treatment, the topmost fully expanded fresh leaves were weighed and dipped in 85% (v/v) aqueous acetone for the extraction of the chlorophyll pigments. Supernatant taken was centrifuged at 4000 rpm for 10 min and diluted with

85% aqueous acetone to a suitable concentration for spectrophotometric measurements. The disappearance was calculated at absorbances of 452.5, 644, and 663 nm alongside a blank of untainted 85% liquid acetone. Chlorophyll a and b, total chlorophyll, and carotenoids were estimated using the following equations:

Chlorophyll a (μg/mL) = 10.3 × E663 – 0.98 × E644,

Chlorophyll b (μg/mL) = 19.7 × E644 – 3.87 × E663,

Total chlorophyll = chlorophyll a + chlorophyll b,

Total carotenoids (μg/mL) = 4.2 × E452.5 – [(0.0264 × chl a) + (0.426 × chl b)],

where E = absorbance.

Finally, these pigment fractions were calculated as mg/g fresh weight.

2.8. Estimation of electrolyte leakage

Electrolyte leakage was estimated by using the method of Dionisio-Sese and Tobita (1998). After treatment for 6 weeks, leaf samples were cut into small pieces of 5 mm in length and were placed in test tubes containing 8 mL of deionized distilled water. The tubes were placed in a water bath at 32 °C for 2 h. The initial electrical conductivity of the medium (EC_1) was assessed. The samples were then placed in an autoclave at 121 °C for 20 min to expel all electrolytes. Afterwards, the samples were cooled at 25 °C and a second electrical conductivity (EC_2) was measured. Total electrolyte leakage (EL) was calculated by using the following formula:

$EL = (EC_1 / EC_2) \times 100$.

2.9. Assay of antioxidant enzymes

Antioxidant enzymes such as SOD, POD, CAT, and APX in roots and leaves were determined spectrophotometrically.

After 6 weeks of treatment, fresh samples (0.5 g) of leaves and roots were ground with the help of a mortar and pestle and homogenized in 0.05 M phosphate buffer (pH 7.8) under chilled conditions. The homogenized mixture was filtered through 4 layers of muslin cloth and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was taken and Zhang's method (1992) was used for the measurement of SOD and POD activities.

CAT (EC 1.11.1.6) activity was determined by a standard method (Aebi, 1984). The assay mixture (3.0 mL) comprised 100 μL of enzyme extract, 100 μL of H_2O_2 (300 mM), and 2.8 mL of 50 mM phosphate buffer with 2 mM EDTA (pH 7.0). The CAT activity was assayed by monitoring the decrease in the absorbance at 240 nm as a consequence of H_2O_2 disappearance ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$).

APX (EC 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981). The reaction mixture consisted of 100 μL of enzyme extract, 100 μL of ascorbate (7.5 mM), 100 μL of H_2O_2 (300 mM), and 2.7 mL of 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). The oxidation of ascorbate was determined by the change in absorbance at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

2.10. Determination of soluble protein

The soluble protein content was analyzed according to the method of Bradford (1976) using Coomassie Brilliant Blue G-250 as dye and albumin as a standard.

2.11. MDA content

The level of lipid peroxidation in the leaf tissue was measured in terms of MDA content, a product of lipid peroxidation, determined by the thiobarbituric acid (TBA) reaction using the method described by Dhindsa et al. (1981) and Zhang and Kirham (1994). First, 0.25 g of leaf sample was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 5 min. To a 1-mL aliquot of the supernatant, 4 mL of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min, the absorbance of the supernatant at 532 nm was read and the value for the nonspecific absorption at 600 nm was subtracted. The MDA content was calculated by using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

2.12. Hydrogen peroxide contents

H_2O_2 was extracted by homogenizing 50 mg of leaf or root tissues with 3 mL of phosphate buffer (50 mM, pH 6.5) (Jana and Choudhuri, 1981). To measure H_2O_2 content, 3 mL of extracting solution was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and the mixture was centrifuged at 6000 × *g* for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm. H_2O_2 content was computed by using the extinction coefficient of 0.28 $\mu mol^{-1}\ cm^{-1}$.

2.13. Estimation of Pb concentration

After 6 weeks of treatment, the harvested cotton plants were washed thoroughly with tap water, distilled water, and deionized water in sequence. Plant samples were then separated into roots, stem, and leaves dried at 80 °C in an oven for 48 h and were ground into powder. Each sample (0.5 g) was dry-ashed, extracted with HCl, and centrifuged at 3600 rpm for 15 min. Concentrations of Pb in root, stem, and leaves were determined by flame atomic absorption spectrometry.

2.14. Statistical analysis

All values reported in this article are means of 3 replicates. Analysis of variance was done using SPSS 16.0 (SPSS, USA) followed by the Tukey test between the means of treatments to determine significant differences.

3. Results

3.1. Plant growth parameters

To investigate the impact of GB on Pb-induced inhibition of growth in cotton plants, plant height, root length, number of leaves per plant, and leaf area are presented in Figure 1. Addition of GB alone to culture solution caused

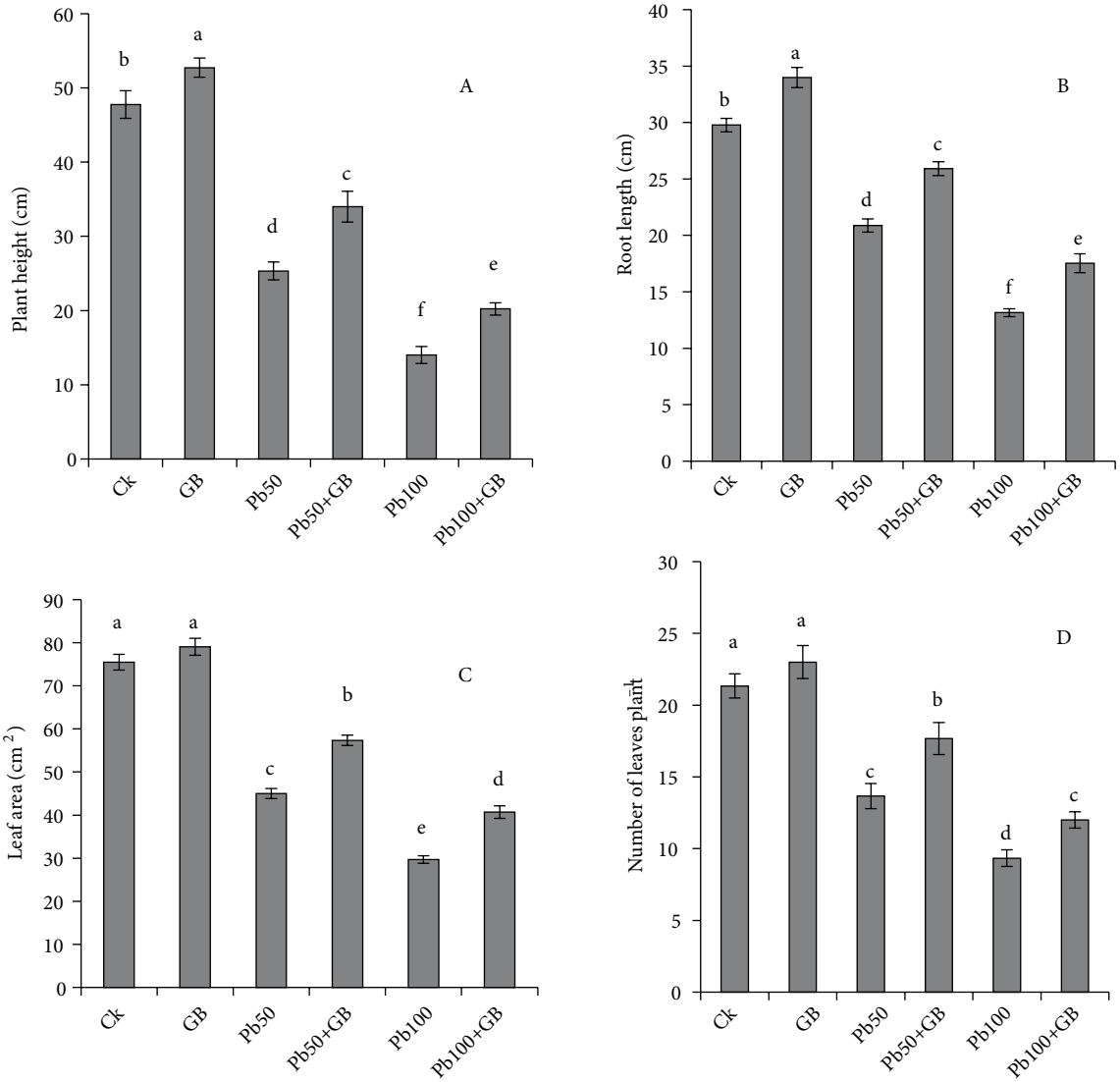

Figure 1. Effect of different concentrations of lead (Pb) (0, 50, and 100 µM) and GB (0 and 1 mM) on plant height (A), root length (B), leaf area (C), and number of leaves plant^{-1} (D) in cotton plants. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

a slight increase in growth compared to the control. Pb addition caused a significant reduction in all 4 cotton growth parameters at both Pb levels (50 and 100 µM) compared with the control and the decrease in growth was dose-dependent. GB alleviated the toxic effects of Pb on growth, reflecting increment in plant height, root length, number of leaves per plant, and leaf area. The appearance of the plants differed greatly between the treatments; control plants grown in the absence of GB (control) were shorter than those grown with GB (GB treatment). Similarly, the plants for the Pb treatment at both Pb levels (50 µM and 100 µM) were smaller than the plants grown with Pb + GB. The positive effect of GB was greater for plants treated with Pb as compared to control plants.

3.2. Plant biomass

The effect of exogenous GB on cotton biomass examined in terms of leaf fresh weight, stem fresh weight, root fresh weight, leaf dry weight, stem dry weight, and root dry weight under Pb stress is shown in Figure 2. Lead stress significantly decreased leaf fresh weight, stem fresh weight, root fresh weight, leaf dry weight, stem dry weight, and root dry weight at both stress levels (50 and 100 µM) and the inhibitory effect was more prominent at 100 µM. Biomass inhibition upon Pb stress in cotton plants was mitigated by GB addition and higher leaf fresh weight, stem fresh weight, root fresh weight, leaf dry weight, stem dry weight, and root dry weight were observed in GB-treated plants.

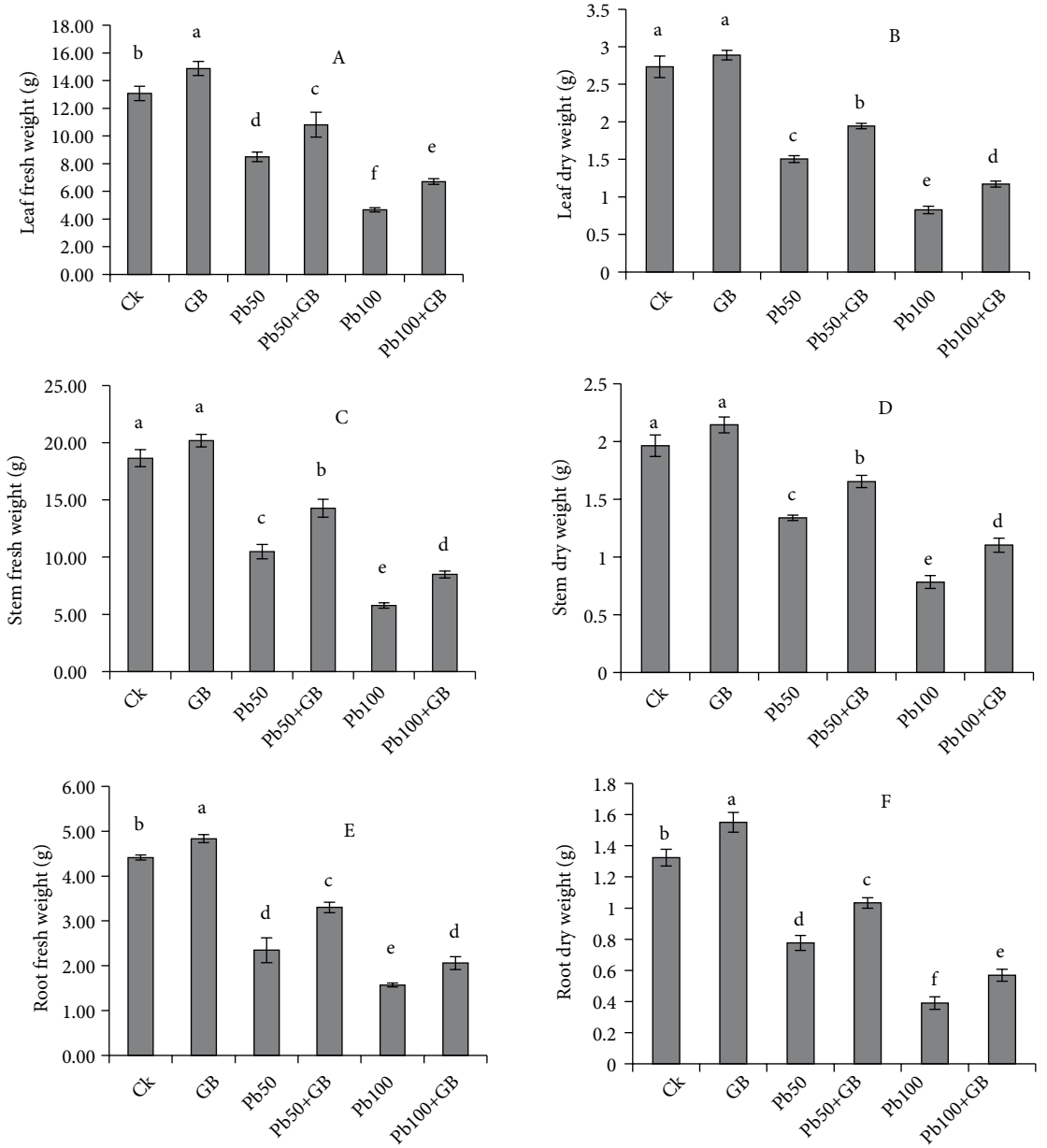

Figure 2. Effect of different concentrations of lead (Pb) (0, 50, and 100 μM) and GB (0 and 1 mM) on leaf fresh weight (A), stem fresh weight (B), root fresh weight (C), leaf dry weight (D), stem dry weight (E), and root dry weight (F) in cotton plant. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

3.3. Photosynthetic pigments and SPAD value

The Pb-induced cotton growth and biomass reduction was accompanied by a considerable decrease in chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, and SPAD value, as shown in Figure 3. The application of 2 Pb stress levels (50 and 100 μM) resulted in a significant decrease in photosynthetic pigments such as chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, and SPAD value in cotton plants relative to the control. GB application positively affected the photosynthetic pigments by enhancing the plant performance.

3.4. Gas exchange parameters and water use efficiency

Figure 4 depicts the gas exchange parameters expressed as net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), and water use efficiency (Pn/E) induced by Pb and GB alone or in combination. A gradual decrease was found in the gas exchange parameters with increasing Pb stress. However, the decrease at 100 μM Pb

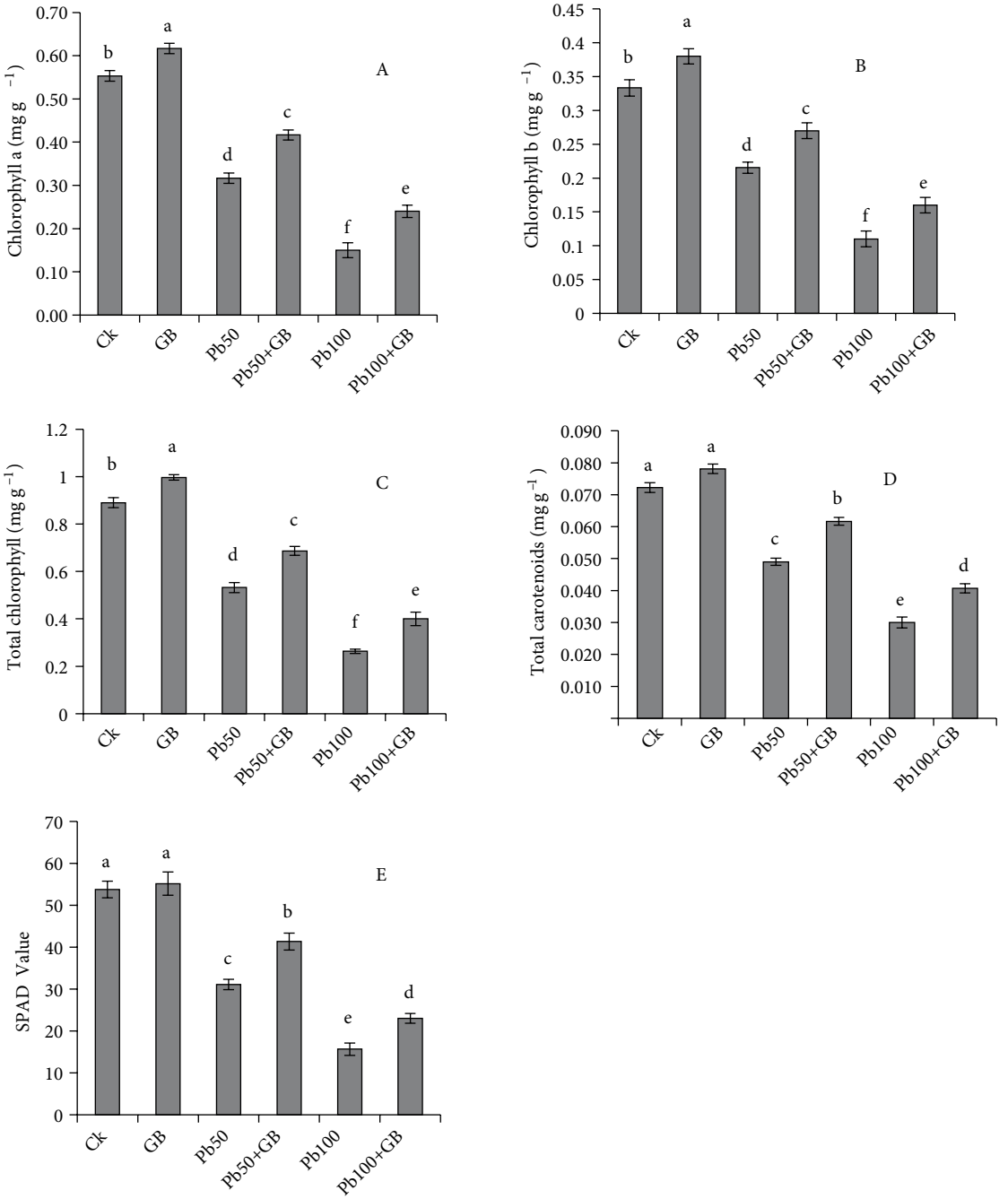

Figure 3. Effect of different concentrations of lead (Pb) (0, 50, and 100 μM) and GB (0 and 1 mM) on chlorophyll a (A), chlorophyll b (B), total chlorophyll (c), total carotenoids (d), and SPAD value (E) in cotton plants. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different using the Tukey test at $P \leq 0.05$.

stress level was significant and much more pronounced as compared to the control plants. Exogenous application of GB helped the cotton plants to overcome Pb stress and recover gas exchange parameters significantly as compared to control plants. Moreover, GB markedly and significantly enhanced all gas exchange parameters of cotton plants under both Pb levels (50 and 100 μM).

3.5. Activities of antioxidative enzymes

Lead stress induced numerous changes in the antioxidative system of cotton plants. Plants under Pb stress conditions

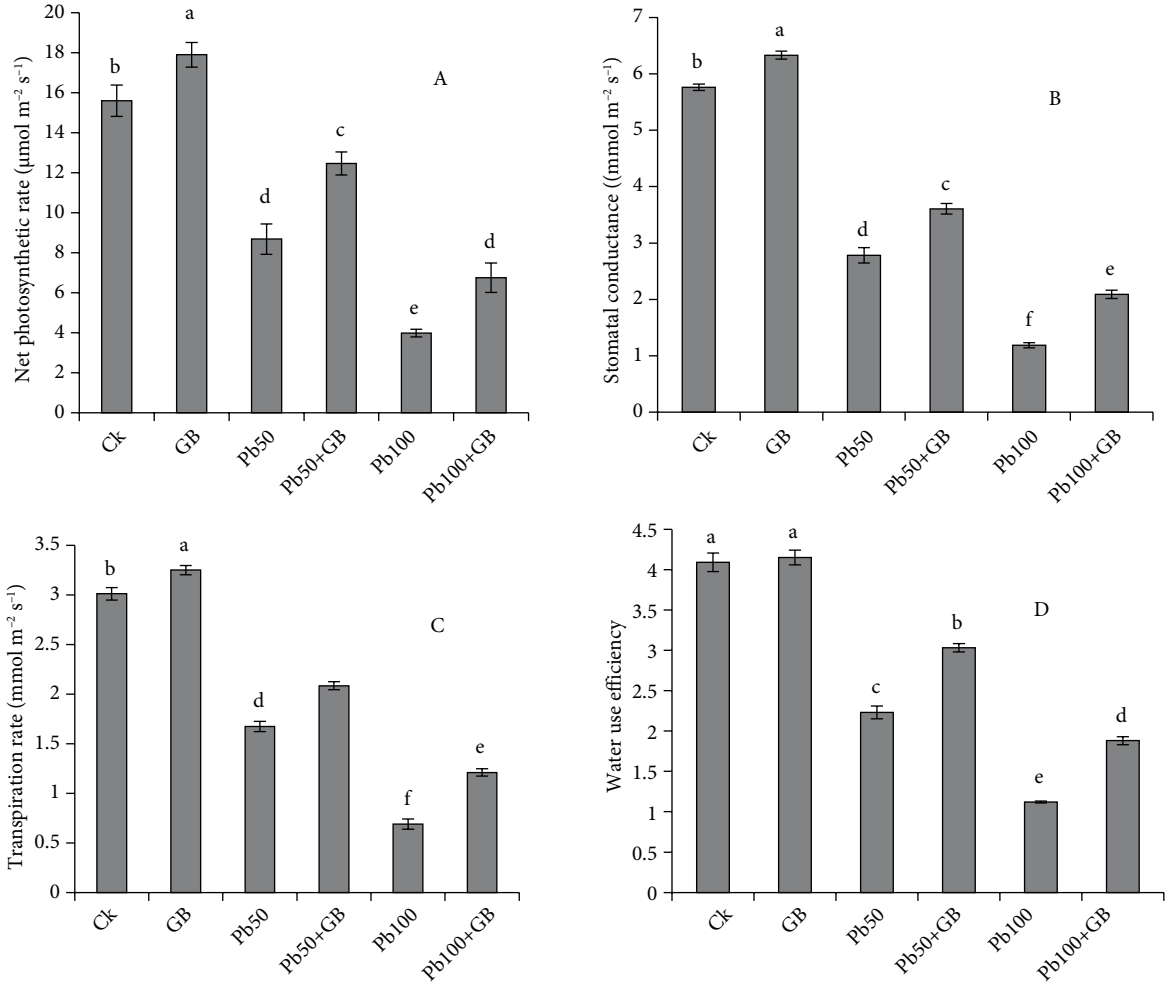

Figure 4. Effect of different concentrations of lead (Pb) (0, 50, and 100 μM) and GB (0 and 1 mM) on net photosynthetic rate (A), stomatal conductance (B), transpiration rate (C), and water use efficiency (D) in cotton plants. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

showed higher SOD, POD, CAT, and APX in both leaves and roots at the lower Pb level of 50 μM as compared to control plants (Table 1). Higher antioxidative enzymatic activities indicated that it could clean out more ROS and decrease the degree of membrane lipid peroxidation and hydrogen peroxide. However, a decrease was again observed in all antioxidant enzymes tested at the higher Pb stress level of 100 μM. Meanwhile, GB improved the antioxidative defense of cotton plants under both Pb levels (50 and 100 μM) by increasing the activities of antioxidant enzymes such as SOD, POD, CAT, and APX in both roots and leaves of cotton plants.

3.6. Electrolyte leakage, MDA, and H_2O_2

The present results revealed significant Pb effect on electrolyte leakage, MDA, and H_2O_2 in cotton leaves and roots level (Table 2). Under Pb stress, the levels of electrolyte leakage, MDA, and H_2O_2 contents in cotton plants increased significantly with the increasing Pb stress, indicating a greater degree of membrane damage and lipid peroxidation with the increasing level of Pb stress. However, GB application significantly lowered electrolyte leakage, MDA, and H_2O_2 contents in cotton plants.

3.7. Soluble protein

The effects of Pb and GB treatments on soluble protein contents in leaves and roots of cotton plants are shown in Figure 5. Soluble protein contents in both roots and leaves of the cotton plants were significantly reduced by the addition of Pb at both levels (50 and 100 μM) in nutrient solution. However, the reduction was significantly alleviated by the exogenous application of GB at both Pb levels. Moreover, in the absence of Pb, exogenous GB application also increased the soluble protein contents in both leaves and roots compared with the control.

Table 1. Effect of different concentrations of lead (Pb) (0, 50, and 100 µM) and GB (0 and 1 mM) on antioxidant enzymes in cotton plants.

Treatments	SOD (units g^{-1} FW)		POD (units g^{-1} FW)		CAT (units g^{-1} FW)		APX (units g^{-1} FW)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Ck	47 ± 1.15e	62.65 ± 4.91e	334 ± 8.74f	1081 ± 76.94e	889.52 ± 6.06e	1037 ± 158.5318	133 ± 2.081666e	118 ± 3.46e
GB	54.33 ± 5.17e	71 ± 5.20e	449 ± 30.12e	1432.39 ± 90.81d	989.73 ± 6.06e	1314.71 ± 130.32	166 ± 3.61e	133 ± 2.65e
Pb50	109.27 ± 4.33b	143.33 ± 3.38b	932.12 ± 33.82b	2793 ± 51.54b	2353 ± 94.64b	2886.12 ± 231.06	424 ± 14.47b	320.62 ± 10.59b
Pb50+GB	137 ± 1.52a	176.30 ± 4.29a	1112 ± 15.63a	3576.81 ± 70.56a	2763.60 ± 67.33a	3700.21 ± 109.12	550.41 ± 11.26a	398.67 ± 9.84a
Pb100	67.12 ± 2.33d	98 ± 4.73d	640.68 ± 20.54d	1932 ± 61.16c	1497.33 ± 85.58d	2058.09 ± 290.16	250.05 ± 10.01d	200 ± 6.08d
Pb100+GB	93.61 ± 3.39c	122.21 ± 2.02c	800.59 ± 12.71c	2498.66 ± 16.04b	1987.49 ± 89.76c	2555.92 ± 269.28	350.19 ± 12.98c	270 ± 8.74c

Abbreviations: Ck, Control; GB glycinebetaine. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

Table 2. Effect of different concentrations of lead (Pb) (0, 50, and 100 µM) and GB (0 and 1 mM) on MDA, H_2O_2, and electrolyte leakage of leaf and root in cotton plants.

Treatments	MDA (µM g^{-1} FW)		H_2O_2 (µM g^{-1} FW)		Electrolyte leakage (%)	
	Leaf	Root	Leaf	Root	Leaf	Root
Ck	4.73 ± 0.16f	3.19 ± 0.03e	80.81 ± 0.81e	70 ± 4.93e	17.10 ± 1.05e	23.10 ± 0.93e
GB	8.97 ± 0.44e	4.13 ± 0.07e	96.99 ± 1.79e	67.33 ± 2.73e	18.99 ± 1.08e	23.23 ± 0.92e
Pb50	24.44 ± 0.98b	13.15 ± 0.36b	281.67 ± 6.34b	208 ± 6.24b	42.85 ± 0.53b	48.63 ± 1.45b
Pb50+GB	14.99 ± 0.94d	8.05 ± 0.16d	180.52 ± 6.48d	146 ± 3.51d	27.11 ± 1.06d	33.67 ± 1.45d
Pb100	31.34 ± 0.98a	18.08 ± 0.58a	333.31 ± 8.41a	247.69 ± 4.67a	52.94 ± 1.60a	59 ± 1.52a
Pb100+GB	19.81 ± 1.17c	10.57 ± 0.76c	221.39 ± 2.60c	67.30 ± 5.36c	35.02 ± 0.73c	42.33 ± 2.03c

Abbreviations: Ck, Control; GB, glycinebetaine. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

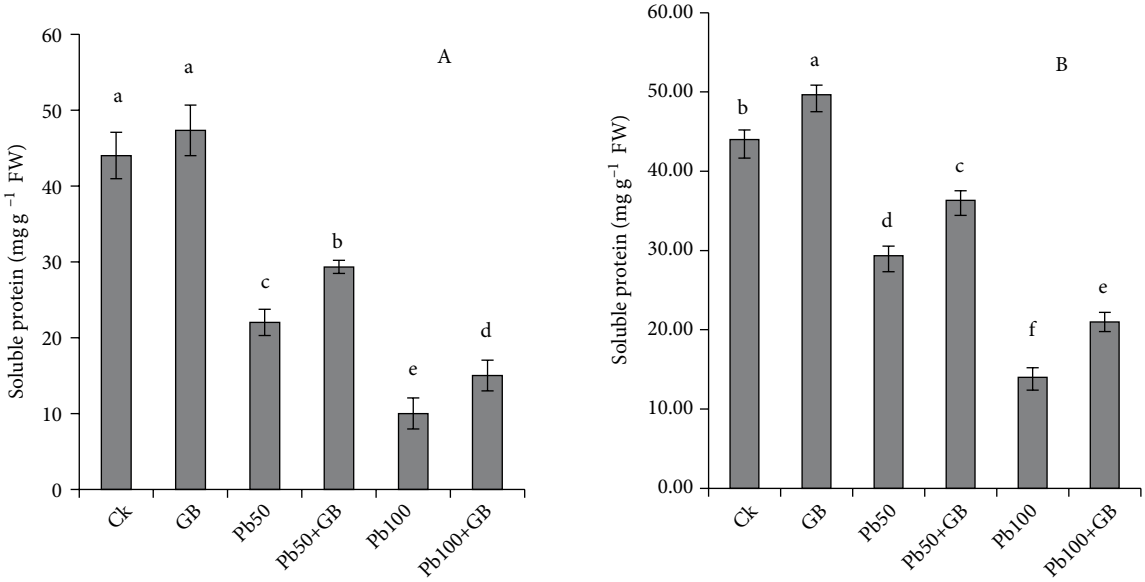

Figure 5. Effect of different concentrations of lead (Pb) (0, 50, and 100 μM) and GB (0 and 1 mM) on soluble protein contents of leaf (A) and root (B) in cotton plants. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

3.8. Lead contents

Pb contents in roots, stems, and leaves are shown in Figure 6. Pb addition caused a significant increase in Pb concentration in all 3 observed plant parts, with the roots having significantly higher levels than the stems and leaves. However, exogenous addition of GB to culture solution reduced Pb concentrations in all 3 parts of the cotton plants. These results indicated that GB might ameliorate the toxic effects of Pb by preventing Pb uptake in cotton plants.

4. Discussions

A number of studies have been conducted on the alarming problem of heavy metal pollution, which causes a number of negative and adverse effects on plant growth and development and leads to aggravated environmental deterioration (Atici et al., 2005). Moreover, heavy metal pollution results in extremely negative and unfavorable impacts on food production and human health. With heavy metal toxicity, their ions can exert certain limits of inhibitory effects on plant growth and development by repressing or destroying plant tolerance and resistances (Ali B et al., 2013a, 2013b). It was found that heavy metals at higher concentrations inhibit germination of seed and plant growth and disturb many biochemical and physiological processes (Clemens, 2001; Farooq et al., 2013). Heavy metal ions at higher concentrations can also inhibit photosynthetic characteristics and the breakdown of protein synthesis, and they can disturb the antioxidative defense of plants by affecting antioxidative enzymes such as SOD, CAT, APX, and POD, thus causing decreased plant tolerance and/or resistance to both abiotic and biotic stresses (Atici et al., 2005; Shao et al., 2008). Many studies also reported that uptake and hyperaccumulation of metals in the food chain by plants is extremely harmful to animal and human health (Sanita and Gabbrielli, 1999).

In the recent past, a number of researchers found beneficial effects of GB on the growth and development of numerous plants. To date, no study has been reported that investigates the impacts of exogenous application of GB on heavy metal tolerance to metals such as Pb. To the best of our knowledge, this was the first study of the interaction of GB and Pb tolerance in plants. Our present results show that plant growth and biomass decreased significantly under Pb stress. It was already observed in a number of studies that Pb inhibits germination, plant growth and development, and biomass by disturbing plant metabolism (Atici et al., 2005; Strubinskai and Hanaka, 2011; Yang et al., 2012; Szalai et al., 2013). Moreover, our present results show that Pb stress decreased gas exchange characteristics such as the net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), water use efficiency (Pn/E), chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, and SPAD value. Pb-induced reduction in chlorophyll synthesis and photosynthetic activities was reported by many researchers (Gajewska et al., 2006; Hamid et al., 2010).

In our present study, it was found that GB markedly alleviated Pb-induced reduction in growth, biomass, and photosynthetic parameters in cotton plants. It was

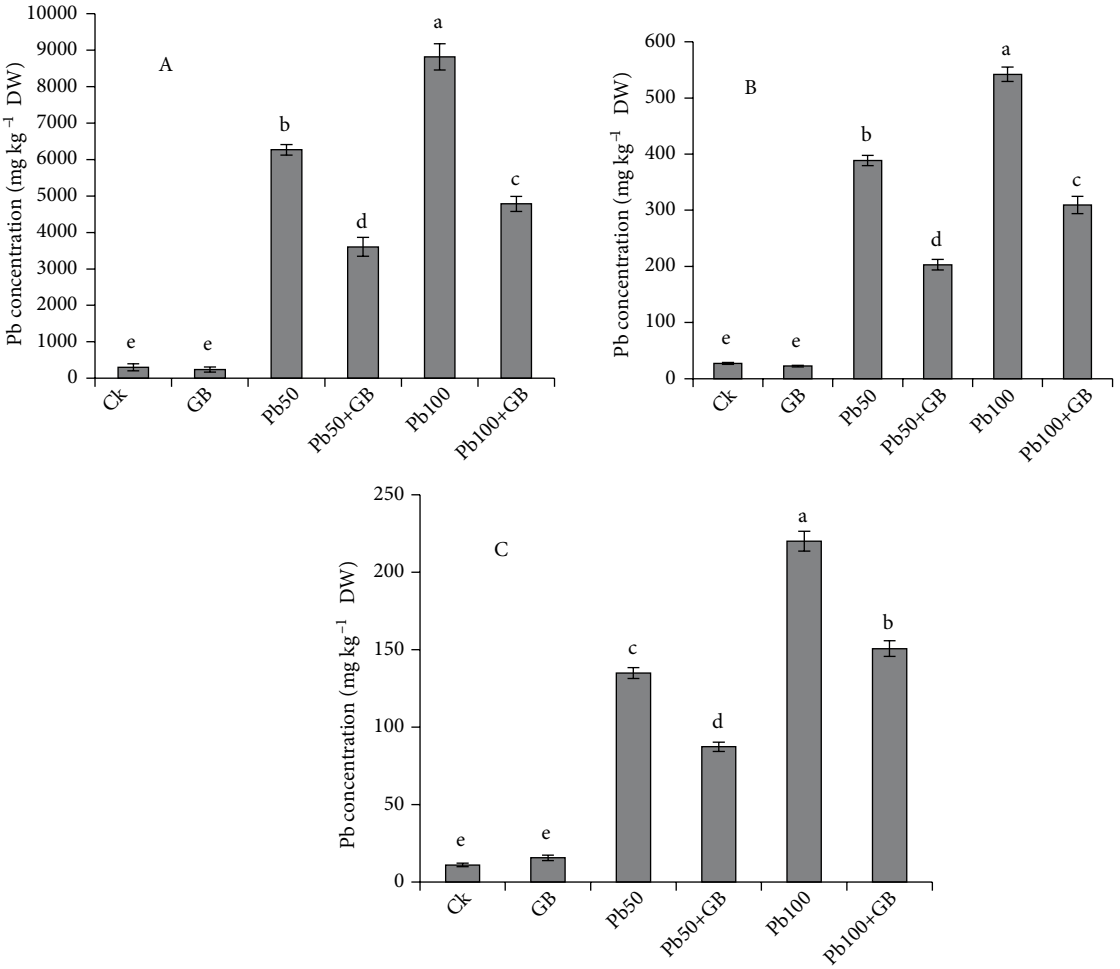

Figure 6. Effect of different concentrations of lead (Pb) (0, 50, and 100 μM) and GB (0 and 1 mM) on concentrations of Pb in roots (A), stems (B), and leaves (C) in cotton plants. Values show the means of 3 replicates ± SE. Means followed by the same small letters are not significantly different at $P \leq 0.05$ using the Tukey test.

demonstrated by many other researchers that exogenous application of GB enhanced tolerance to heavy metals and other stresses such as drought, salinity, heat, and cold (Allakhverdiev et al., 2007; Yang et al., 2008; Islam et al., 2009). GB may maintain photosynthetic capacity by increasing stomatal conductance and maintaining RuBisCo activity and chloroplast ultrastructure under drought stress (Nomura et al., 1998).

This study reports Pb-inhibited soluble protein contents in cotton plants in both roots and stems. This might be due to more oxidative damage occurring under Pb stress conditions that decreased the protein contents (Gupta et al., 2009). It was also observed in the present investigation that Pb stress enhanced ROS, as indicated by electrolyte leakage and MDA and H_2O_2 contents. It was already demonstrated by many researchers that Pb stress and other metals caused oxidative damage (MacFarlane, 2003). To overcome oxidative damage, plants developed complex antioxidant systems. Alleviation of toxic effects of metals and other stresses by GB was related to protection against oxidative damage due to oxidative stress (Park et al., 2004). GB alleviated Cd stress in tobacco by preventing oxidative damage by enhancing antioxidant enzyme activity (Islam et al., 2009). Similarly, it was found by many researchers that GB alleviated oxidative stress under different stresses in different crops (Meloni et al., 2003; Farooq et al., 2010). The GB-induced inhibition of electrolyte leakage and MDA and H_2O_2 contents indicates that GB could significantly alleviate the harmful effects of Pb stress in cotton plants.

In the present investigation, exogenous application of GB in solution culture showed significant beneficial effects on MDA, H_2O_2, and electrolyte leakage and antioxidant activities of SOD, APX, POD, and CAT under Pb stress. Similarly, it was found that GB enhanced the antioxidant enzymes and decreased oxidative stress in different stress

conditions (Meloni et al., 2003; Islam et al., 2009; Farooq et al., 2010). From our present results, it might be suggested that GB addition could markedly enhance the capacity of defense against oxidative damage induced by Pb toxicity in cotton seedlings.

Plants have developed numerous ways and mechanisms to maintain and regularize cellular metal homeostasis by preventing uptake and accumulation of high concentrations of free heavy metal ions (Clemens, 2001; Hall, 2002). The Pb contents in all 3 parts of cotton plants in the present study increased as we increased the Pb concentrations in solution culture. In this investigation, Pb was less translocated from roots to shoots in cotton plants. Mostly, the plants with the highest resistance take up a lower proportion of the total solution metal and have the lowest upper ground metal contents (Liu et al., 2004). However, exogenous application of GB inhibited the uptake of Pb in all 3 parts of plants such (roots, stems, and leaves) and translocation of Pb compared to Pb alone. Similarly, it was found that exogenous application of GB decreased the uptake and accumulation of Cd in tobacco plants by sequestering Cd (Islam et al., 2009).

In conclusion, GB is very important and beneficial for growth and development of many plant species exposed to different types of abiotic and biotic stresses. The present results revealed that Pb caused significant decrease in cotton growth, biomass, pigments, photosynthetic characteristics, and protein. GB addition in nutrient solution protects cotton seedlings against Pb stress by lowering MDA, H_2O_2, and electrolyte leakage and by enhancing antioxidant enzymes. It can be expected that GB would be a useful tool in lowering and reducing Pb contamination.

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