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Combined Citric Acid and Glutathione Augments Lead (Pb) Stress Tolerance and Phytoremediation of Castorbean through Antioxidant Machinery and Pb Uptake

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Abstract: Lead (Pb) is one of the most toxic elements on earth. The main origins of Pb pollution are automobiles, paint and electroplating industries. Pb-induced stress has very toxic effects on plant growth and biomass. The concentration of reactive oxygen species (ROS) in plant cells significantly increases under Pb stress, which interrupts the biochemical cycles in cells and leads to cell death. Therefore, it is essential to clean up the Pb-polluted soils. Among all techniques that are used to clean soil that is metal-contaminated, the best technique is phytoremediation. The present study intends to determine the role of citric acid (CA) and glutathione (GSH) in the phytoremediation of Pb by using castor bean plants. Plant biomass was significantly reduced due to Pb stress. Lead toxicity was also harmful to the photosynthetic pigments and antioxidant enzymes activities. In reverse, the content of malondialdehyde (MDA), H_2O_2 concentration and electrolyte leakage (EL) were increased under Pb stress. The combined application of GSH and CA enhanced photosynthetic pigments, antioxidant enzyme activities and plant biomass and minimized MDA, H_2O_2 and EL under Pb stress. The amount of Pb in roots and leaves remarkably increased by the joint application of CA and GSH. The combined application of CA and GSH (5 mM + 25 mM, respectively) was proven to be beneficial compared to the control. From the present results, we can conclude that the combined application of CA and GSH promoted the phytoremediation of Pb and helped the host plant to combat Pb toxicity.

Keywords: oxidative stress; castor bean; chelator; heavy metal; phytoextraction

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1. Introduction

Lead (Pb) is a well-known recalcitrant pollutant that ranks second (after arsenic) among all hazardous materials known in the environment [1]. It originates from various sources such as weathering of rocks, volcanic eruption, mining, sewage sludge, storage batteries, explosives, vehicle exhausts and radioactive decay [2]. Naturally, Pb occurs in ranges of 15–40 mg/kg soil but it can exceed several thousand ppm as the result of environmental pollution. The higher levels of Pb in agricultural soils (due to both natural and anthropogenic sources) intensified its accumulation in plant tissues, which has ultimately decreased crop production. An excessive amount of Pb in plants indirectly activated the production of reactive oxygen species (ROS), which hampers the antioxidant defense machinery, degrades proteins, causes alterations in chloroplast structures, impairs nutrient uptake, causes a decline in photosynthetic efficiency, inhibits the division of cells and eventually reduces the growth of plants [3,4]. Pb-induced toxicity results in DNA damage, enhancement lipid peroxidation and lower production of ATPs. In addition, Pb strongly inhibits seed germination, root elongation, seedling development, plant growth, transpiration, chlorophyll production and water uptake by plants. The plants may enhance their immunity via enhanced enzymatic antioxidants in the form of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and ascorbate, intermediates of the ascorbate–glutathione cycle. Nonenzymatic antioxidants encircle glutathione, phenols, carotenoids anthocyanins and flavonoids to achieve considerable resistance against Pb-induced oxidative stress [5].

A lot of ex situ and in situ techniques have been employed by scientists for the remediation of soils polluted with trace elements such as Pb [6,7]. In situ phytoremediation (a method based on plants) has achieved enormous recognition and this in situ technique is cost-effective in comparison with ex situ techniques and deals with the problem of heavy metal contamination. Among different phytoremediation techniques, phytoextraction is an effective remediation technology dependent upon the use of species of plants for their ability to accumulate impurities in shoot parts and remove them from soil/water [8]. However, plants' ability to extract heavy metals (HMs) from roots to aerial parts relies on the target species of plant, soil types and the conditions of the environment [9]. Different plant species, for example, *Ricinus communis*, are used to remediate Cd, Cr, Cu and Pb contaminated soils [10].

In general, the low bioavailability of HMs in soil negatively affects the phytoextraction technique. However, the promising use of soil amendments (organic compounds) can enhance the solubilization of metals by forming complexes with target sites of metals [11]. Therefore, the use of organic chelating agents could be a potential approach for metal desorption in soil and facilitation in transport from soil to aerial plant parts. Previous studies have found that many organic chelators, for instance, glutathione (GHS; γ-glutamyl-cysteinyl-glycine) [12] and citric acid (CA) [13,14] can effectively interact with HMs, facilitating the immobilization or mobilization of HMs and diminishing or enhancing their bioavailability depending upon several conditions. GSH plays an important role in metal homeostasis and also works as a metal-binding peptide precursor [15]. CA is also known for its suitability as a metal chelation agent and alleviator of metals stress [16]. In a previous study, Mallhi et al. [12] reported that different levels of CA enhanced the Pb tolerance in castor bean (*Ricinus communis* L.) under Pb stress. However, the combined effects of GSH and CA have not been studied to remediate Pb-polluted soils by using castor bean.

The selection of the target plant was made based on its ability to yield higher biomass, cause rapid growth and withstand HMs, which is a prerequisite for the phytoextraction process. The purpose of the current study is to explore the effective use of castor bean plants assisted by the combined use of GSH plus CA for phytoextraction by castor bean from Pb-polluted soils. It was hypothesized that the combined application of GSH and CA would improve the growth and photosynthesis of castor bean plants, as well as assist the castor bean plants to enhance the phytoremediation potential of Pb by castor bean plants under Pb stress. In the current study, we attempted to (1) investigate the Pb-induced toxic effects on morpho-physiological attributes of castor bean plants, (2) assess the potential of combined use of GSH plus CA to ameliorate the phytoextraction of Pb-contaminated soils, and (3) to explore the possible impacts of GSH plus CA on the plants' growth and photosynthesis under Pb stress.

2. Material and Method

2.1. Pot Experiment

The experiment was carried out in the botanical garden of Government College University, Faisalabad at ambient conditions. Random soil samples were collected from an agricultural field of University of Agriculture Faisalabad and a homogenous composite sample was made. The soil was spiked with selected concentrations of Pb (0, 300 and 600 mg kg^{-1}) by using lead nitrate ($PbNO_3$) salt. The plastic pots were packed with sieved soil with a finalweight of 5 kg of soil per pot, and 5 seeds of castor bean were planted in every pot to perform pot experiments. Castor bean seeds were taken from Ayub Agriculture Research Institute, Faisalabad, Pakistan. The detailed characteristics of the soil used for the current experiment have been shown in our previous study [12] and also in Table 1.

Table 1. Soil physico-chemical properties used for the experiment.

Texture	Sandy Loam
Silt	15.0%
Sand	67.9%
Clay	17.1%
EC	1.96 dS m^{-1}
pH	7.61
Sodium adsorption ratio (SAR)	1.89 (mmol $L^{-1})^{1/2}$
Available P	2.11 mg kg^{-1}
Organic matter	0.59%
HCO_3	2.51 mmol L^{-1}
$SO_4{}^{-2}$	11.44 mmol L^{-1}
Cl^-	5.45 mmol L^{-1}
$Ca^{2+} + Mg^{2+}$	13.98 mmol L^{-1}
K^+	0.04 mmol L^{-1}
Na^2	5.23 mmol L^{-1}
Available Zn^2	0.77 mg kg^{-1}
Available Cu^{2+}	0.31 mg kg^{-1}
Available Cr^{+6}	0.16 mg kg^{-1}

2.2. Experimental Design

Plant thinning was performed after 1 week of germination, and two of the plants were placed out of five in every plastic pot. For experimental design, CRD design was applied for the placement of pots, including 3 replicates. The NPK fertilizers were used for plant fertilization at a concentration of 120:50:25 kg ha^{-1}, respectively [12]. Initially, a half dose of N was applied along with the full dose of P and K fertilizers, while the remaining half dose of N was applied later on. Used fertilizers (NPK) were manufactured by Engro Fertilizers in Karachi, Pakistan. Urea salt was used to obtain nitrogen, potassium sulfate for potassium and diammonium phosphate for phosphorus. The foliar spray of CA (5 mM) plus GSH (25 mM) was done on the experimental plants including control (without CA, GSH and Pb). The very first foliar spray of CA and GSH was given just after the thinning of the plants in respective pots and remaining foliar sprays were given after an interval of one week each. For all treatments, the total amount of 1.0 L of CA and GSH was used for each treatment and all replications of the treatment. The level of CA and GSH was selected based on separate studies related to CA [12] and GSH (unpublished data) in which different levels of these amendments were used under Pb stress with castor bean growth. Citric acid, GSH and Pb salts of analytical grades were purchased from Sigma-Aldrich, Germany.

2.3. Plants Harvesting

Castor bean plants were allowed to grow for 70 days after sowing and then different parts (leaves, stem and roots) of the bean plants were separated. The leaves and stem of castor bean plants were harvested with sharp stainless steel blade and roots were separated from soil and washing was done with distilled water. To wash the roots, one percent dilute

HCl was used followed by washing with purified water many times which helped in the elimination of acidic material completely. After separation and washing with distilled water, samples were separately dried in the oven at 70 °C for almost 72 h. The growth of plant and biomass such as length of root, length of shoot, dry weight of root, dry weight of shoot, number of leaves per plant, leaf areas were measured, individually and separately for each plant.

2.4. Chlorophyll Contents and Gas Exchange Parameters

The samples of fresh leaves (1.0 g) were extracted with 85% *v*/*v* acetone and centrifuged at 4000 rpm for chlorophyll content evaluation. Measurements were taken at appropriate wavelengths. The chlorophyll a, chlorophyll b and carotenoids wavelengths used were 644 nm, 663 nm and 452 nm with spectrophotometer (Halo DB-20/DB-20S, Dynamica Company, London, UK) and final measurements were calculated by using the following formulas [17–22].

$$\text{Chlorophyll a} = 10.3 \times \text{E664} - 0.98 \times \text{E644}$$

$$\text{Chlorophyll b} = 19.7 \times \text{E644} - 3.87 \times \text{E663}$$

$$\text{Carotenoids Contents} = 4.2 \times \text{E452} - [(0.0264 \times \text{Chl a}) + (0.426 \times \text{Chl b})]$$

In the maximum sunlight on the same day, transpiration rate, photosynthesis rate and stomatal conductivity were measured using infrared gas analyzers (IRGA, LCA-4, Analytical Development Company, Hoddesdon, UK). IRGA was run on three different leaves of each plant. While taking readings, it was ensured that the analyzer, as well as plant leaves, were set facing towards direct sunlight.

2.5. Estimation of MDA, EL, H_2O_2 and Antioxidants Enzymes

To calculate MDA contents, material used was 0.1 percent thiobarbituric acid [23,24]. For EL estimation, the Dionisio-Sese and Tobita [25] approaches were used. For this, measurement of initial as well as final EC of the solution was done by extraction of the sample at 32 °C for almost 2 h and same sample for 20 min at 121 °C, respectively. In order to calculate the contents of H_2O_2, the same method was adopted as used by Jana and Choudhuri [26]. In this method, extracts used were homogenized with a buffer of phosphorus (50 mM), at 6.5 pH. After this, the centrifugation of solution was done for 20 min then (20% *v*/*v*) H_2SO_4 was administered, and then centrifuged again for 15 min and adsorption was noted at 410 nm. In liquid nitrogen, the sample was ground for the analysis of POD and SOD activities and standardized at a pH of 7.8 in 0.5 M phosphate buffer [27]. The methods devised by Nakano and Asada [28] and Aebi [29] were used, respectively, for the measurement ofAPX and CAT activities.

2.6. Estimation of Pb Contents

The 1.0 g of each sample (n = 6) was digested by adding a 4:1 ratio of HNO_3:$HClO_4$ (*v*/*v*) on hot plate, and for the measurement of the concentration of Pb, an atomic absorption spectrophotometer (novAA ® 350–Analytik Jena, Germany) was used [30]. For the estimation of Pb concentration in the plants, we used the following formula:

$$\text{Pb concentration} = \text{Absorbance by Atomic Absorption Spectrophotometer} \times \text{Dilution Factor}$$

For the estimation of Pb accumulation in the plant we followed the following formula:

$$\text{Pb Total Accumulation} = \text{Pb concentration in plants} \times \text{Plant dry mass}$$

2.7. Statistical Analysis

One-way ANOVA was implemented for processing data at a probability level of 5% using the SPSS tool (Statistical software, Version 21.0) from the International Business

Machines Corporation (IBM) in New York, United States. For the multiple evaluations of means, the HSC post-hoc Tukey's test was used [31]. Statistical analysis was applied on the basis of means values and standard deviation.

3. Results

3.1. Plant Growth and Biomass

The growth of plant and biomass such as length of roots, length of shoots, dry weight of root, dry weight of shoots, the number of leaves per plant and leaf area were significantly decreased at both Pb levels (300, 600 mg kg^{-1}) in comparison with controls (Figure 1A–F). Maximum reduction in plant growth and biomass attributes was noticed at 600 mg kg^{-1} Pb as compared with control plants. The combined application of exogenous GSH and CA as a chelator, in Pb-treated plants significantly minimized the Pb toxicity by promoting the plant growth traits. Under non-stress conditions, GSH + CA notably ameliorated the plant growth and biomass attributes especially dry weight of root as well as shoots, followed by shoot and root lengths, the area of leaf and number of leaves per plant. Under 300 and 600 mg kg^{-1} Pb stress, the castor bean plants treated with CA (5 mM) and GSH (25 mM) significantly exhibited ameliorating effects on the biomass and growth of plant traits as compared with Pb-alone treatments.

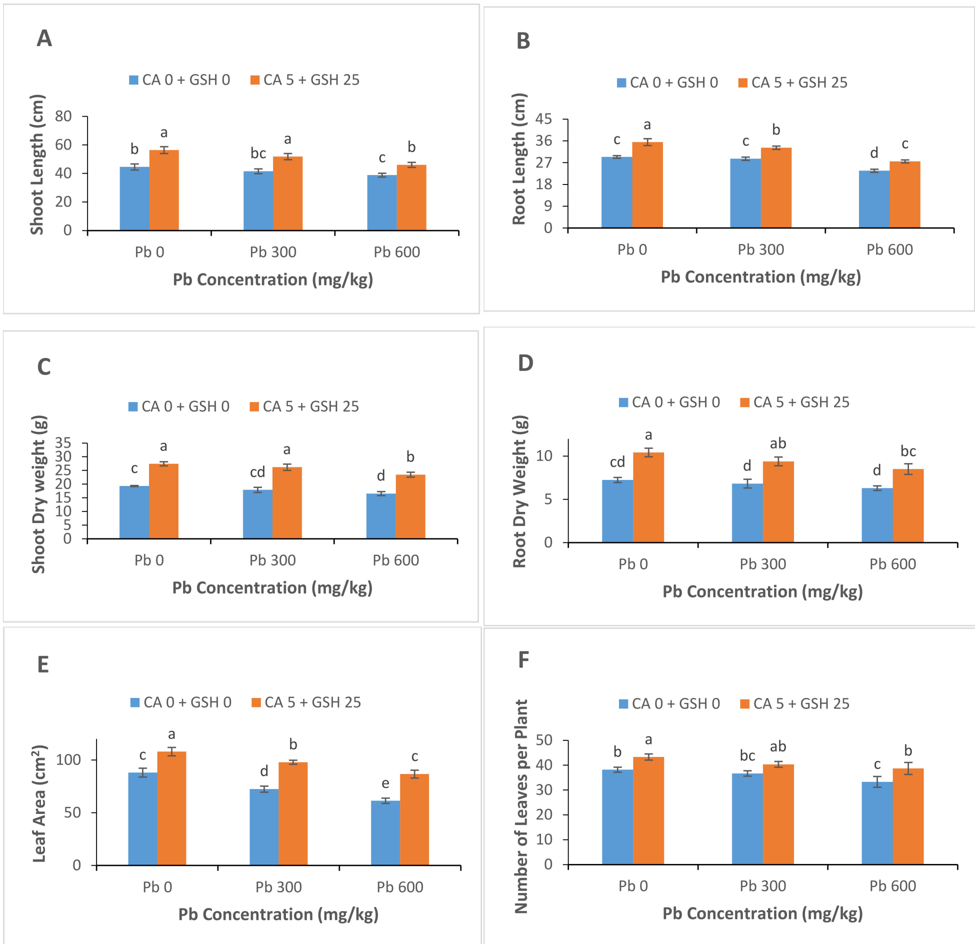

Figure 1. Different concentration effect of Pb (0, 300 and 600 mg kg^{-1}) and combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on length of the shoot (**A**), length of root (**B**), dry weight of shoot dry (**C**), dry weight of roots (**D**), area of the leaf (**E**) and number of leaves/plant (**F**) of castor bean plants. With the help of standard deviation, the values which had been reported are the mean of 3 replicate samples. The noteworthy difference between the treatments had been shown via lower case letters at $p \leq 0.05$.

3.2. Light-Harvesting Pigments

Light-harvesting pigments including carotenoid, total chlorophyll, chlorophyll a (chl a) and chlorophyll b (chl b) were remarkably decreased in the leaves of castor bean plants grown in Pb-spiked soil in comparison with non-spiked ones (Figure 2A–D). Maximum reduction (even non-significant) in the values of these photosynthetic pigments was noticed at 600 mg kg^{-1} Pb in comparison with other Pb-treatment and control. Combined application of GSH (25 mM) + CA (5 mM) along with both Pb levels (300 and 600 mg kg^{-1}) remarkably increased chlorophyll a, chlorophyll b and total chlorophyll, as well as carotenoids contents as compared to all Pb-alone treatments. Under the same (GSH + CA) treatment, a more pronounced escalation in the values of photosynthetic pigments was noticed under 300 mg kg^{-1} Pb stress in comparison with 600 mg kg^{-1} Pb treatment.

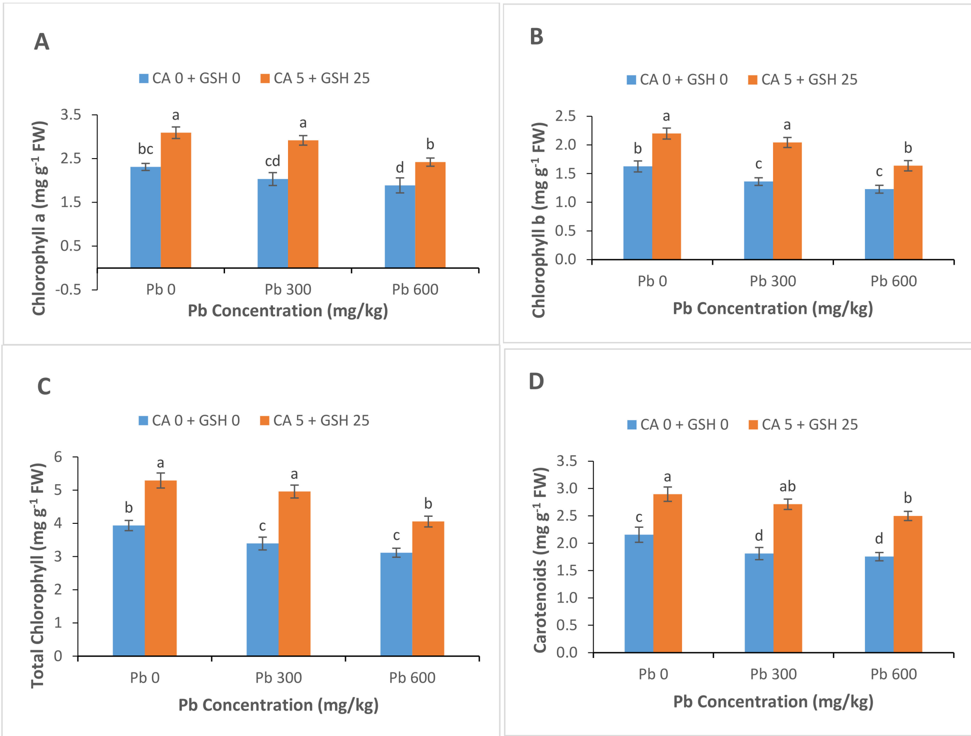

Figure 2. Different concentration effect of Pb (0, 300 and 600 mg kg^{-1}) and combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on the content of chlorophyll (**A**), the content of chlorophyll b (**B**), total chlorophyll (**C**) and the content of carotenoids content (**D**) of castor bean plants. With the help of standard deviation, the values which had been reported are the mean of 3 replicate samples. The noteworthy difference between the treatments had been shown via lower case letters at $p \leq 0.05$.

3.3. Gas Exchange Attributes

A non-significant reduction in gas exchange parameters (transpiration rate, photosynthetic rate, stomatal conductance and the efficiency of use of water) was noted in plant leaves under increasing Pb treatments (300 and 600 mg kg^{-1}). Maximum reduction in these attributes was observed at 600 mg kg^{-1} Pb when compared to other Pb-treatment and relative controls. The addition of GSH (25 mM) + CA (5 mM) along with Pb-stressed plants gradually enhanced the stomata condensation, transpiration rate, water use efficiency and photosynthesis rate when compared with all Pb-alone treatments. Under non-stress conditions, the combined effect of GSH and CA noticeably promoted the gas exchange parameters with the maximum escalation in the efficiency of water use, followed by a rate of transpiration and stomatal conductance, as well as a photosynthetic rate when compared to no-chelator applications (GSH+CA) (Figure 3A–D).

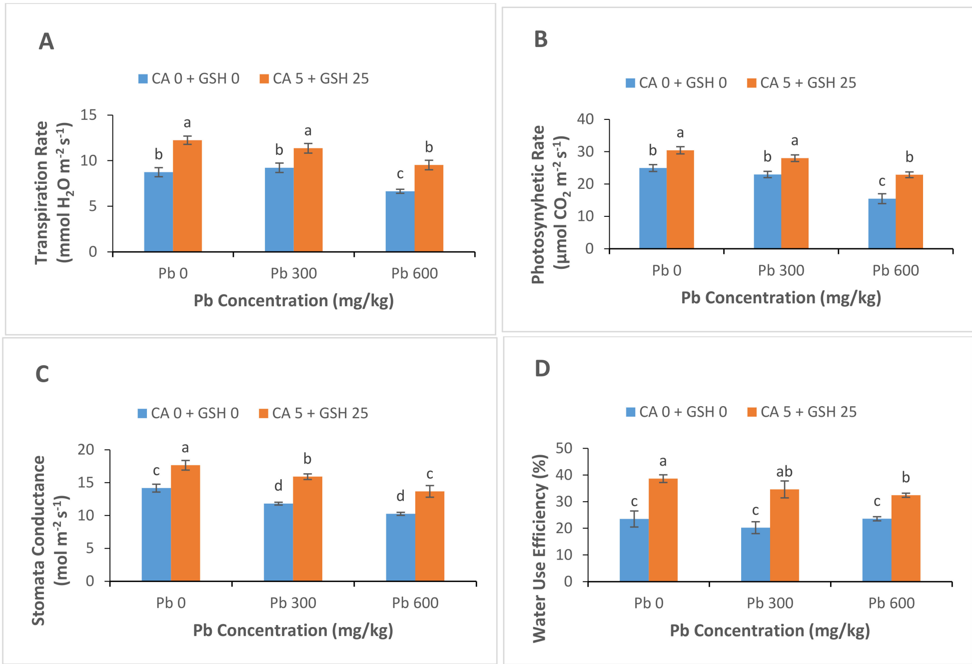

Figure 3. Different concentration effect of Pb (0, 300 and 600 mgkg^{-1}) and combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on the rate of transpiration (**A**), rate of photosynthetic (**B**), the conductance of stomata (**C**) and water use efficiency (**D**) of castor bean plants. With the help of standard deviation, the values which had been reported are means of 3 replicate samples. The noteworthy difference between the treatments had been shown via lower case letters at $p \leq 0.05$.

3.4. Electrolyte Leakage, Hydrogen Peroxide and Malondialdehyde Contents

The increasing levels of Pb (300 and 600 mg kg^{-1}) induced the contents of malondialdehyde (MDA), electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) in the leaves and roots of castor bean plants in comparison with non-stress Pb-alone treatments (Figure 4A–F). The accumulation of the above-mentioned oxidative markers was more pronounced in roots than leaves irrespective of treatments. Interestingly, the addition of combined chelators (GSH + CA) showed a slight reduction in EL, MDA and H_2O_2 concentration in both roots and leaves. Furthermore, the addition of GSH (25 mM) + CA (5 mM) along with Pb-stress considerably declined EL, MDA, and H_2O_2 concentration as compared to Pb-alone treatments.

3.5. Antioxidant Enzyme Activities

The potential effects of CA + GSH were analyzed on the activities of antioxidant enzymes viz. POD, CAT, APX and SOD of Pb-treated castor bean plants (Figure 5A–F). At both levels of Pb (i.e., 300 and 600 mg kg^{-1}), the increasing trends in the antioxidant enzyme activities were noticed in tissues of plants especially at 600 mg kg^{-1} Pb in comparison to respective controls. Overall, high activities of antioxidant enzymes were noted in roots than leaves at all treatments. Interestingly, non-stressed plants exhibited a slight increase in the activities of antioxidant enzymes under GSH + CA applications. The exogenous addition of both GSH and CA (combine) into Pb-treated plants further ameliorated the antioxidant enzyme activities and demonstrated synergistic effects on castor bean plants under Pb stress. At GSH (25 mM) + CA (5 mM) under Pb stress, maximum escalation in antioxidant enzyme activities was observed in comparison with Pb-alone treatments.

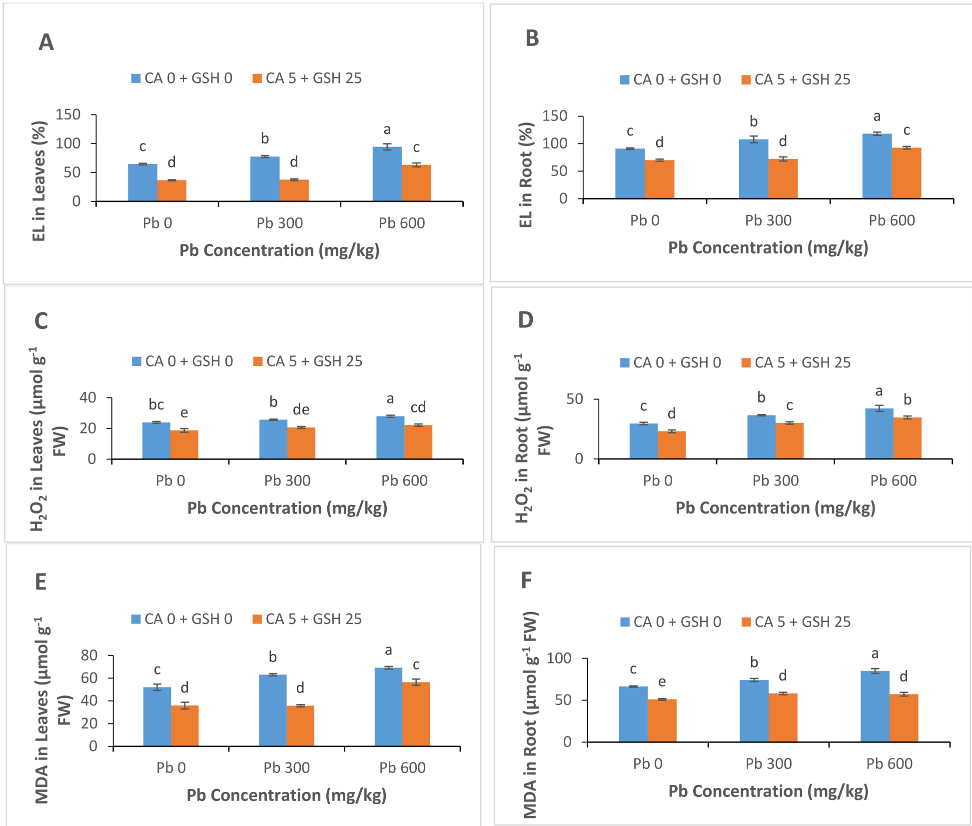

Figure 4. Different concentration effect of Pb (0, 300 and 600 mg kg^{-1}) combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on leaf's EL (**A**), root's EL (**B**), content of leaf H_2O_2 (**C**), content of roots H_2O_2 (**D**), content of leaf MDA (**E**) and content of roots MDA (**F**) of castor bean plants. With the help of standard deviation, the values which had been reported are means of 3 replicate samples. The noteworthy difference between the treatments has been shown via lower case letters at $p \leq 0.05$.

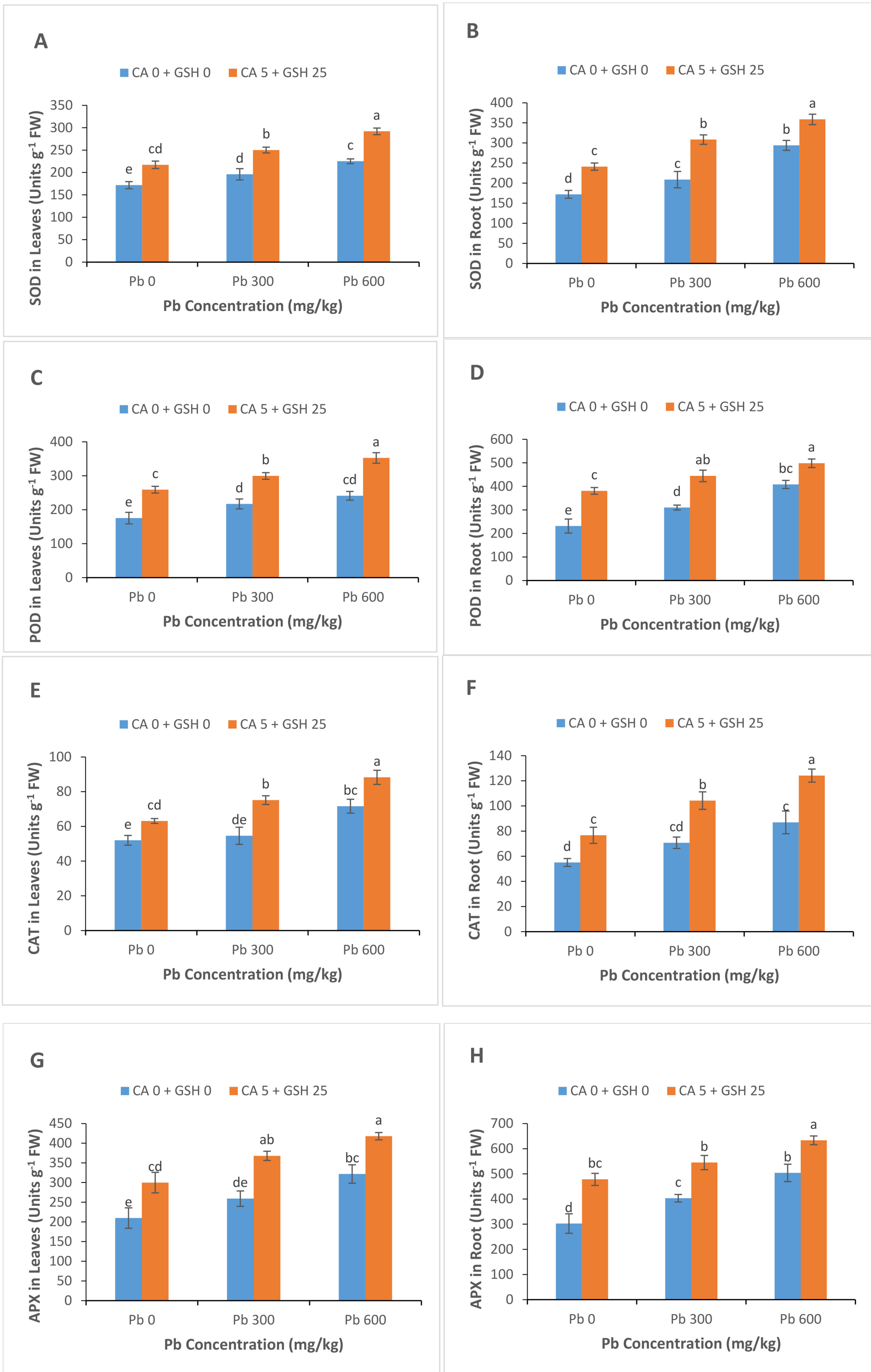

Figure 5. Different concentration effect of Pb (0, 300 and 600 mg kg^{-1}) and combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on the content of leaf SOD (**A**), the content of roots SOD (**B**), the content of leaf POD (**C**), the content of roots POD (**D**), the content of leaf CAT content (**E**), the content of roots CAT (**F**), the content of leaf APX (**G**) and the content of roots APX (**H**) of castor bean plants. With the help of standard deviation, the values which had been reported are the mean of 3 replicate samples. The noteworthy difference between the treatments had been shown via lower case letters at $p \leq 0.05$.

3.6. Lead Uptake and Accumulation

A significant induction (increasing trend) in the amount and accumulation of Pb in castor bean plants was found with increasing Pb concentrations from 300 to 600 mg kg^{-1} in the soil as compared to control (Figure 6A–D). At 600 mg kg^{-1} Pb, the maximum amount and accumulation of Pb in plant tissues was found. Irrespective of the applied doses, relatively higher Pb concentration (at all doses) and accumulation was found in roots than leaves. The combined applications of GSH (25 mM) + CA (5 mM) resulted in a significant accumulation of Pb in plant tissues as compared to respective Pb treatments alone. These findings demonstrated that the combined application of GSH + CA exhibited as an efficient chelating agent to enhance the translocation and uptake of Pb in almost all the parts of castor bean plants under a stressful environment.

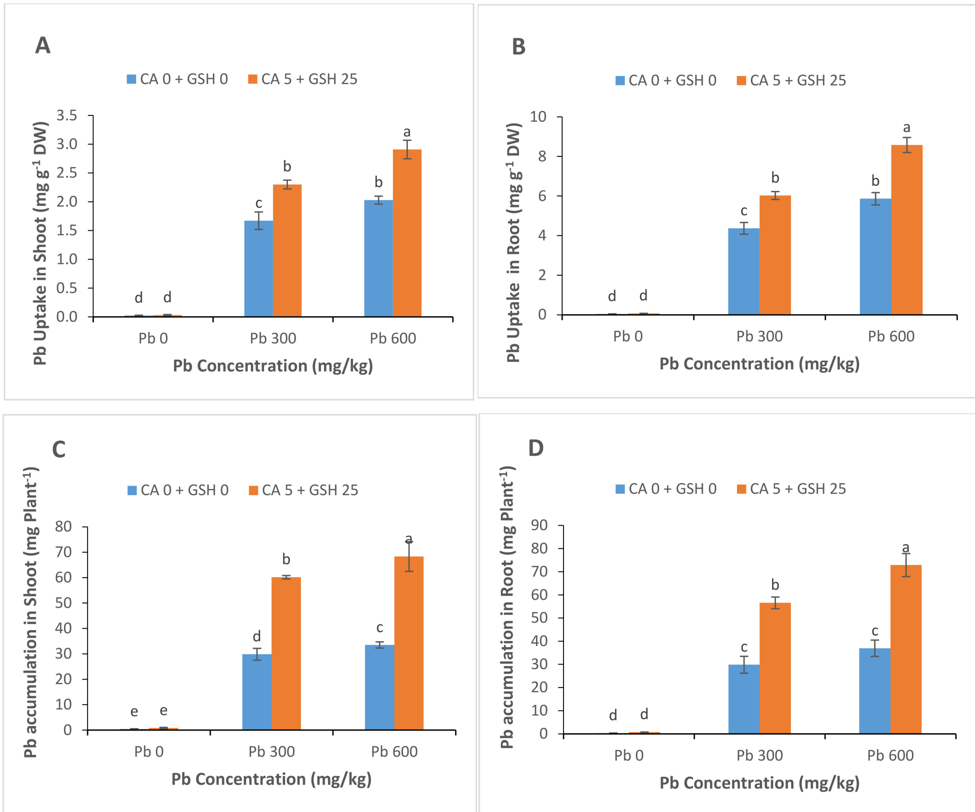

Figure 6. Different concentration effect of Pb (0, 300 and 600 mg kg^{-1}) and combined use of citric acid and glutathione (5 mM + 25 mM, respectively) on Pb concentration to the plant shoot (**A**), Pb concentration to the plant root (**B**), Pb accumulation in the shoot (**C**) and Pb accumulation in the root (**D**) of castor bean plants. With the help of standard deviation, the values which had been reported are means of 3 replicate samples. The noteworthy difference between the treatments had been shown via lower case letters at $p \leq 0.05$.

4. Discussion

The increasing concentration of Pb displayed detrimental effects on plants and ultimately reduced biomass production and plant growth (Figure 1A–D). Many HMs (e.g., Pb, copper, cobalt, nickel and cadmium) can affect plant agronomic parameters in a very toxic way [17,18]. Similarly, many researchers have reported that there is a great reduction in biomass of plants, as well as the growth of plants due to metal stress in wheat, rice and *Brassica napus* L. [19–21]. In the present study, the reduction in plant agronomic characteristics with increasing concentrations of Pb was in accordance with the work done by previous researchers [20,22,23]. The inhibited agronomic traits were a prominent indication of Pb stress in plants [3,24].

The combined effect of GHS and CA resulted in amplified resistance to Pb toxicity as compared to the one grown only in soil contaminated with Pb. In previous studies, the supportive role of CA is reported, which showed the increase in biomass production and growth of plants in *B. napus* L. under Pb and Cu stress after treatment with CA [14,25,26]. The supportive effect of CA on plants under metal stress can also increase the availability of nutrients to plants, such as iron, magnesium and zinc [27,28]. The GSH facilitates different cellular defense mechanisms in plants against toxic metals [29]. These mechanisms include the formation of the complex with metals to reduce the entry of the metals that are toxic from the medium of growth to the roots of plants and facilitate the synthesis of phytochelatins that bind and sequester metals in complexes that are stable in vacuolar regions [30–32]. Our results revealed that during Pb stress, the supportive role of both acids in a combined form is much higher than Pb treatments alone (Figure 6).

Lead stress induced toxicity in plants reduced carotenoids, chlorophyll contents and gas exchange parameters, whereas the application of CA and GSH reduced the Pb-induced toxicity and improved the photosynthesis in stressed plants (Figures 3 and 4). The reduced contents of chlorophyll under metal stress were due to the deformation of chloroplast [33,34]. It is well reported that Pb stress altered the ultrastructure of chloroplast in plants, which ultimately decreased the photosynthetic activity [35,36]. Under metals stress, the overproduction of H_2O_2 and enzymatic degradation of chlorophyll by chlorophyllase may also cause reduction of photosynthetic pigments [37,38]. Further, the replacement of Mg with HMs from chlorophyll molecules also contributed to photosynthetic pigments reduction [39]. The treatment of plants with CA and GHS enabled the plants to tolerate the Pb induced toxicity and sustained the chloroplast activity. The CA has been well reported to support plant defensive systems against metals toxicity and supports the normal functioning of chloroplast and stomata under metals stress [26]. The prominent CA role in facilitating photosynthesis process under metals stress has been reported by various researchers [10,40,41]. The application of GSH on cotton plants under Cd stress increased the content of chlorophyll [38]. It is obvious from results that collective action of CA and GHS sustained the plants to alleviate Pb stress and supported the plants to carry on its photosynthetic process efficiently under metal stress. The combined application of CA and GHS displayed the highest concentrations of chlorophyll and carotenoids in control plants and also supported the plants even under the highest concentration of Pb.

Plant natural defense systems consist of various enzymes with antioxidant properties such as CAT, POD and SOD, which may activate in response to enormous different abiotic and biotic stresses [42]. These enzymatic activities have a directly proportional relationship with ROS scavenging [43]. The stress of oxidation triggered by MDA and H_2O_2 due to metal toxicity tends to boost these enzymatic activities [42,44]. Various growth regulators and stress alleviating chemicals alleviate the metal stress in plants. The high concentration of Pb declined the enzymatic activities in castor bean plants. This decrease in enzymatic activities proved to be directly proportional to the increasing amount of Pb which indicated the severity of stress at higher Pb concentration [45].

In Pb exposed plants, the effect of CA and GHS assisted different plants to alleviate the metal toxicity by the initiation of defense system of plants. When CA is combined with GHS it restores the enzymatic activities in castor bean plants even at a high concentration of Pb. Both acids showed a synergistic relationship and protected the plants from the harmful effects of metal toxicity. The role of CA in strengthening the plant defensive system and reducing oxidative stress under metal stress was previously reported [46,47]. The combined role of CA and other organic acids in reducing metal toxicity and protecting plants from different metals has been reported earlier [38,48,49].

4.1. H_2O_2, MDA and Electrolyte Leakage (EL) Contents

The plant affected with heavy meals stress produces more ROS in comparison with non-stressed ones [50]. As Pb concentration increased in soil, MDA production and EL in roots and leaves of castor bean plants increased. The EL and MDA are the stress indicators of any plant under various types of biotic and abiotic stresses. Pb has been found to increase oxidative stress and lipid production in different plants [51]. The high level of EL imposed severe stress on the plants [52]. The plant failed to sustain the normal functioning of electron transportation and to absorb sufficient amounts of micro- and macronutrients under stressful conditions [53,54]. The rising oxidative stress in castor bean plants further decreased plant growth and inhibited the production and functioning of photosynthetic pigments [55,56]. The acid-amended plants exhibited adequate tolerance to Pb toxicity and the reduced oxidative stress as compared to non-amended plants. The CA and GHS combined presented a prominent role to reduce oxidative stress in treated plants, which indicated a promising role of CA and GHS in metal stress reduction [30,48]. The CA also showed its unique role in strengthening the plant defense system, reducing oxidative stress and supporting the normal functioning of plants under severe metal stress [13]. The application of GHS reduced ROS production under metals stress by promoting the activities of heme-based molecules as well as antioxidant enzymes in plants [30,38].

4.2. Deposition of Pb in Roots and Leaves

The Pb stressed plant treated with GHS and CA shows variations in concentration, as well as an accumulation of Pb in different parts of the plants. Lead uptake and accumulation by plants have been already well explained by many researchers [57,58]. The increasing accumulation of Pb in plants causes negative effects on the accumulation and uptake of essential nutrients by plants, plant functioning and, ultimately, altered normal growth pattern of the plants [59,60]. The failure of plants to absorb essential nutrients also severely affects the photosynthesis, transpiration process, promotes leaf chlorosis and damages the extracellular structure [27,56,61]. Pb uptake by plants is very much affected by soil types such as the Pb uptake by plants is normally higher in acidic soil medium than that of the basic soil medium. Citric acid with other organic acids increases the availability or metal solubility of organic chelates due to their chemical nature [14,26]. For enhancing phytoextraction of metals, the application of CA is a practical approach [27]. The results indicated that Pb stressed plants treated with CA exhibited a high concentration and deposition of Pb in tissues of the plant without compromising plant health and is grown without CA treated plants. Similarly, GHS also contributes in maintaining the growth of plants and their normal functioning under metal stress [32,38]. The castor bean plant grows in Pb enriched soil medium and is treated with CA plus GHS, which showed higher Pb accumulation and better growth as compared to non-treated plants. The collective role of GHS- and CA-assisted Pb stressed plants alleviated metal toxicity and achieved high deposition of Pb in plant leaves and roots.

5. Conclusions

The current study determined that combined application of CA and GSH remarkably alleviated Pb-induced toxicity at biochemical and morpho-physiological levels in castor bean plants. Our results revealed that Pb toxicity reduced the castor bean growth by stimulating the ROS. However, the exogenous application of CA plus GSH alleviated the Pb-induced toxicity. Both CA and GSH improved castor bean growth, as well as fresh and dry biomass, by enhancing the antioxidant enzyme activities and by overcoming ROS production under Pb stress. The joint application of GSH and CA remarkably improved the amount of Pb uptake by castor bean by enhancing plant growth and antioxidant defense system of plants that support plant metabolism and normal functioning under metal stress.

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