

Oxidative Stress Induced by Lead in Chloroplast of Spinach

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Abstract Seedlings of spinach were grown in Hoagland's medium containing 0, 20, 40, 60, 80, 100 μM PbCl_2 , respectively, for 4 weeks. Chloroplasts were assayed for overproduction of reactive oxygen species (ROS) such as superoxide radicals ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) and of lipid peroxide (malonyldialdehyde) and for activities of the antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase and glutathione content, oxygen-evolving rate, and chlorophyll content. Increase in both ROS and lipid peroxide content and reduction in photosynthesis and activities of the antioxidant defense system indicated that spinach chloroplast underwent a stress condition due to an oxidative attack. Seedling growth cultivated in containing Pb^{2+} media was significantly inhibited. The results imply that spinach chloroplast was not able to tolerate the oxidative stress induced by Pb^{2+} due to having no effective antioxidant defense mechanism.

Keywords Oxidative stress · Lead · Spinach chloroplast · Antioxidative enzymes · Lipid peroxides · Oxygen evolution

Introduction

Toxic metal pollution of surface and groundwater due to increased industrialization and geochemical activities is a major concern since it enters the food chain endangering life forms. Lead is a widespread nonessential heavy metal, which enters the ecosystems from natural (weathering of rocks) as well as anthropogenic sources (industrial effluents, agricultural runoffs). It is reported that heavy metals can induce oxidative stress by generating free radicals and toxic oxygen species [1, 2]. These species react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability. The deleterious effects resulting from

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the cellular oxidative state may be alleviated by enzymatic and nonenzymatic antioxidant machinery of the plant that vary at various cellular and subcellular levels in different plants. Plants use a diverse array of enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), as well as low molecular weight antioxidants like cysteine, nonprotein thiol, and ascorbic acid to scavenge different types of reactive oxygen species (ROS), thereby protecting potential cell injury against tissue dysfunction [3]. SOD is a key enzyme in protecting cells against oxidative stress and dismutates superoxide radical ($O_2^{\cdot-}$) to H_2O_2 and oxygen. However, hydrogen peroxide is also toxic to cells and has to be further detoxified by CAT and peroxidases to water and oxygen. Cysteine, thiols, and ascorbic acid can directly interact with and detoxify oxygen free radicals and thus contribute significantly to nonenzymatic ROS scavenging.

Spinach is a familiar vegetable and is widely planted in Suzhou suburb of China, and it is also a vegetable liable to heavy metals contaminated. Chloroplast is a site of photosynthesis which is capable of light absorption and transfer, light-induced charge separation, electron transfer between photosystem (PS) II to PSI, water photolysis, oxygen evolution, and CO_2 assimilation. Many reports are available on the accumulation and toxicity of lead in crops and animals [4–10], among these negative effects, reduction in chlorophyll content, PSII efficiency, photophosphorylation, and enzyme activity in the carbon reactions were reported [11–16]. We speculate that the inhibition of heavy metals toxicity to photosynthesis is related to oxidative stress and membrane lipid peroxidation caused by heavy metals, which lead to the destruction of the structure of chloroplasts and the inhibition of photosynthesis. However, no work has so far been carried out to study oxidative stress and antioxidant response of spinach chloroplast induced by lead and other heavy metals. In the present study, the involvement of various antioxidants (enzymatic and nonenzymatic) in the tolerance against lead-induced stress in spinach chloroplast is investigated, which is helpful in understanding the biochemical detoxification strategies that the plant adopts against oxidative stress induced by repeated metal exposure.

Materials and Methods

Plant Cultivation and Treatment with Pb^{2+}

Seeds of *Spinacia oleracea* were scarified in 85% H_2SO_4 , rinsed in running water, and sterilized in 0.2% $HgCl_2$ for 10–15 min. Seeds were then planted in a perlite-containing pot and placed in porcelain dishes. Seven days after planting, seedlings were placed into 500 ml of modified Hoagland's medium containing 20, 40, 60, 80, and 100 μM $PbCl_2$ [7, 17], respectively, as described below. Plants were grown at 20°C using a 16/8 h light/dark cycle in a growth chamber under 400 $\mu mol\ m^{-2}\ s^{-1}$ of cool fluorescent light for 4 weeks. A control treatment contained only Hoagland's medium (no Pb^{2+}).

Chloroplast Preparation

The leaves of Pb^{2+} treated spinach and the control were homogenized in a prechilled mortar and pestle in ice-cold isolation buffer, which contained 400 mM sucrose, 10 mM NaCl, and 20 mM Tricine (pH 7.8). The slurry was filtered through five layers of cheesecloth, and the chloroplasts were sedimented at 3,000 $\times g$ for 5 min at 4°C. The supernatant was carefully discarded, and the pellet was retained. The pellet was washed and resuspended in a small volume of chilled suspension buffer that contained 100 mM sucrose, 10 mM NaCl,

2 mM $MgCl_2$, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5. Care was taken that the whole procedure was completely done under ice-cold conditions as quickly as possible to inactivate and prevent the degradation of chloroplast by proteolytic enzymes. Chlorophyll was extracted in chilled 80% acetone and estimated spectrophotometrically [18].

Pb^{2+} Content of Chloroplasts

The Pb^{2+} content of chloroplasts was measured by inductively coupled plasma mass spectrometry (POEMS, Thermo Jarrel Ash, USA).

ROS Assay of Chloroplast

Superoxide ion ($O_2^{\cdot-}$) was measured as described according to Able et al. [19], by monitoring the reduction of 3'-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) in the presence of $O_2^{\cdot-}$, with some modifications. The chloroplasts were homogenized with 2 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at $5,000\times g$ for 10 min. The reaction mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 7.5), 20 mg chloroplast supernatant proteins, and 0.5 mM sodium, XTTs. The reaction of XTT was determined at 470 nm for 5 min. Correction was made for the background absorbance in the presence of 50 U of SOD. The production rate of $O_2^{\cdot-}$ was calculated using an extinction coefficient of $2.16\times 10^4 M^{-1} cm^{-1}$.

H_2O_2 were extracted according to Wang and Luo [20]; the chloroplasts were homogenized in 3 ml of ice-cold acetone. The homogenate was centrifuged at $30,000\times g$ for 10 min, and the supernatant was used for assays of the contents of H_2O_2 . The reaction mixture contained 0.1 ml of 5% $Ti(SO_4)_2$, 0.2 ml of ammonia solution, and 1 ml of the H_2O_2 extract, which was centrifuged at $30,000\times g$ for 10 min. The precipitate was repeatedly washed with acetone until pigments were completely removed; it was then dissolved with 5 ml of 2 M H_2SO_4 , and the contents of H_2O_2 was measured at 415 nm [20].

Lipid Peroxide Content (MDA) Assay of Chloroplast

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive metabolites, mainly malonyldialdehyde (MDA), following the modified method of Heath and Packer [21]. Frozen chloroplasts were homogenized in a prechilled mortar and pestle with 2 vol of ice-cold 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 min at $15,000\times g$. Assay mixture containing 1-ml aliquot of supernatant and 2 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA was heated to $95^\circ C$ for 30 min and then rapidly cooled in an ice bath. After centrifugation ($10,000\times g$ for 10 min at $4^\circ C$), the supernatant absorbance (532 nm) was read, and values corresponding to nonspecific absorption (600 nm) were subtracted. MDA concentration was calculated using its extinction coefficient.

Antioxidant Enzyme Activity Assays of Chloroplast

The chloroplasts were homogenized in 1 ml of ice-cold 50 mM sodium phosphate (pH 7.0) that contained 1% polyvinyl pyrrolidone. The homogenate was centrifuged at $30,000\times g$ for 30 min, and the supernatant was used for assays of the actives of SOD, CAT, APX, and GPX.

The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 3-ml reaction mixture contained 50 mM

sodium phosphate (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 100 μM ethylenediamine tetraacetic acid (EDTA), and 200 μl of the enzyme extract. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan [22]. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a 1-ml reaction volume.

The activity of CAT was determined by measuring the rate of disappearance of H_2O_2 at 240 nm. Each 3-ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 12.5 mM H_2O_2 , and 200 μl of enzyme extract [23]. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 240 nm.

APX activity was assayed using the method described by Reuveni et al. [24]. A reaction mixture consisting of 100 μl supernatant, 17 mM H_2O_2 (450 μl), and 25 mM ascorbate (450 μl) was then assayed for 3 min at 290 nm. Activity was measured as disappearance of ascorbate. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 290 nm.

GPX activity was measured using the method described by Reuveni et al. [24]. A reaction mixture consisting of supernatant (100 μl), 17 mM H_2O_2 (450 μl), and 2% guaiacol (450 μl) was then assayed for 3 min at 510 nm. Activity was measured as appearance of tetra-guaiacol. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} at 510 nm.

Reduced Glutathione Assay

In order to perform the GSH assay, chloroplast was homogenized as described above. However, supernatants were not diluted fivefold as described in the case of the antioxidant enzyme assays. Reduced glutathione (GSH) content was estimated using the method of Hissin and Hilf [25]. The reaction mixture contained 100 μl of supernatant, 100 μl *o*-phthalaldehyde (1 mg ml^{-1}), and 1.8 ml phosphate buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8.0). Fluorometry was performed using F-4500 fluorometer (F-4500, Hitachi, Japan) with excitation at 350 nm and emission at 420 nm.

Activities of the Oxygen Evolution of Chloroplast

The oxygen evolution of chloroplasts isolated from spinach was measured with an Oxygraph oxygen electrode (Hansatech instruments, UK). The assay medium contained 0.5 M sorbitol, 10 mM KCl, 0.5 mM MgCl_2 , 0.05% (w/v) bovine serum albumin, 10 mM NaHCO_3 , and HEPES-KOH (pH 7.6). The concentration of the artificial electron acceptor was 2 mM for $\text{K}_3\text{Fe}(\text{CN})_6$. The concentration of the chloroplast was 20 $\mu\text{g ml}^{-1}$.

Growth Measurement

The fresh weight and dry weight of spinach were weighted at the 28th day. The chlorophyll contents were determined by Arnon's method [18].

All the experiments were independently performed at 25°C, and the presented data are the average of the recordings from five independent experiments.

Statistical Analysis

The analysis of variance appropriate for the design was carried out to detect the significance of differences ($P < 0.05$) among the treatment means, and the Scheffe test was performed to compare among experimental groups for significant differences.

Table 1 Pb²⁺ Content in Chloroplasts of Pb²⁺-treated Spinach

Pb ²⁺ treatment (μM)	0	20	40	60	80	100
Pb ²⁺ content (ng/g chloroplasts)	Not detected	131.55±6.58 ^a	185.77±9.29 ^a	251.69±12.58 ^a	289.66±14.48 ^a	325.77±16.29 ^a

The data presented were an average of the recordings from five independent experiments. Values represent means±SE, *n*=5

^a It is significantly different from 20 μmol/L Pb²⁺ treatment at the 5% confidence level

Results

Pb²⁺ Content in Chloroplasts

In order to prove whether Pb²⁺ had entered chloroplasts, we detected Pb²⁺ content of chloroplasts from five groups of spinach, respectively. Data in Table 1 prove that Pb²⁺ entered chloroplasts and Pb²⁺ contents of Pb²⁺-treated spinach chloroplasts were in direct proportion with Pb²⁺-treated concentrations, but the control was not detected.

Effect of Pb²⁺ on ROS Accumulation in Spinach Chloroplast

It is well known that ROS accumulation is one of the mechanisms of heavy metals on organism damage. Similarly, chloroplast damage cultivated in Pb²⁺-contained media is related to ROS production. The effects of treatments with various Pb²⁺ concentrations on the production rate of O₂^{•-} and H₂O₂ in spinach chloroplast are shown in Figs. 1 and 2. It can be seen that ROS was sharply enhance by Pb²⁺ treatments from 20 to 100 μM, i.e., O₂^{•-} generating rate increase of 4.57%, 13.27%, 22.17%, 30.27%, and 37.52%, respectively; H₂O₂ is 1.71, 2.12, 2.5, 2.83, and 3.98 times as the control, respectively, suggesting that exposure to Pb²⁺ causes a strong oxidative stress in spinach chloroplast.

Effect of Pb²⁺ on Lipid Peroxidation of Spinach Chloroplast

Analysis of lipid peroxide (MDA) content of spinach chloroplast, after 4 weeks of culture, was performed (Fig. 3). MDA content is 48.41%, 60.37%, 63.77%, 69.86%, and 72.60% higher in various Pb²⁺-treated spinach with respect to the control ones, indicating a lipid

Fig. 1 O₂^{•-}-generating rate (μmol mg⁻¹ chlorophyll min⁻¹) of chloroplast of spinach grown in the presence of Pb²⁺ for 4 weeks. The production rate of O₂^{•-} was calculated using an extinction coefficient of 2.16 × 10⁴ M⁻¹ cm⁻¹. Values represent means±SE, *n*=5

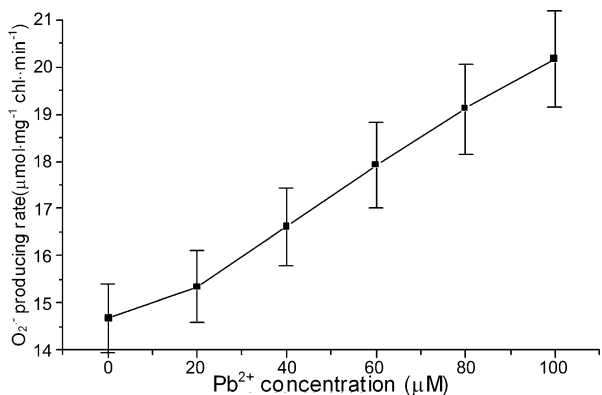
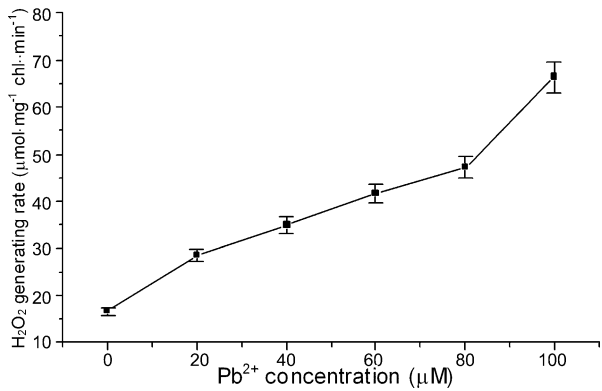


Fig. 2 H₂O₂-generating rate ($\mu\text{mol mg}^{-1}$ chlorophyll min^{-1}) of chloroplast of spinach grown in the presence of Pb²⁺ for 4 weeks. The content of H₂O₂ was measured at 415 nm. Values represent means \pm SE, $n=5$



peroxidation enhancement which is generally associated to an oxidative stress status in spinach chloroplast.

Antioxidant Defense

Figure 4 compares activities of SOD in spinach chloroplast exposed to various Pb²⁺ concentrations and the control. In 20 and 40 μM Pb²⁺-treated groups, chloroplast shows an elevated activity of SOD that increased by 7.73% and 10.50% followed by a significant decrease in 60, 80, and 100 μM Pb²⁺-treated groups, suggesting 2.61%, 27.54%, and 33.56% reduction, respectively.

Figure 5 presents CAT activities of chloroplast of spinach grown in Pb²⁺-contained media. During the growth period, control chloroplast exhibits high CAT activities (33.87 U mg^{-1} protein min^{-1}), while CAT activities in Pb²⁺-treated groups decrease sharply, showing 20.60%, 22.11%, 24.06%, 33.86%, and 35.34% reduction, respectively.

Figure 6 shows APX activities of spinach chloroplast exposed to various Pb²⁺ concentrations and the control (no Pb²⁺). In Pb²⁺-treated groups, chloroplast exhibits a significantly reduced activity of APX, which is 85%, 75%, 27.86%, 14.29%, and 5.71% compared to the control, respectively.

The GPX activities of the chloroplast of spinach grown in the presence of various Pb²⁺ concentrations and the control (no Pb²⁺) are shown in Fig. 7. GPX activities of chloroplast of spinach exposed to various Pb²⁺ concentrations are significantly lower than the control, having 7.60%, 9.11%, 20.64%, 26.80%, and 34.54% reduction, respectively.

Fig. 3 MDA content ($\mu\text{mol mg}^{-1}$ chlorophyll) of chloroplast of spinach grown in the presence of Pb²⁺ for 4 weeks. MDA concentration was calculated using its extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$). Values represent means \pm SE, $n=5$

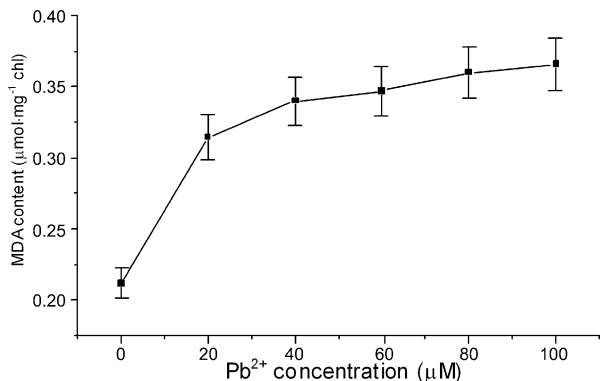
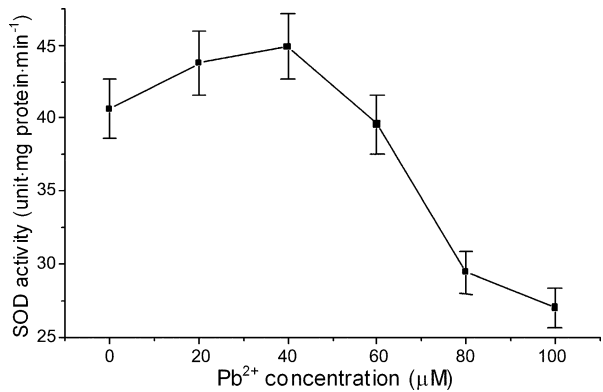


Fig. 4 SOD activities ($\text{U mg}^{-1} \text{ protein min}^{-1}$) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of NBT. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a 1-ml reaction volume. Values represent means \pm SE, $n=5$



The effects of Pb^{2+} on GSH content of spinach chloroplast are presented in Fig. 8. Growth in Pb^{2+} concentrations leads to a sharp decrease in GSH content relative to the control ($P < 0.05$).

Effect of Pb^{2+} on Oxygen Evolution Rate of Chloroplast

Figure 9 compares the oxygen evolution rate of chloroplast of spinach grown in Pb^{2+} -contained media. During the growth period, the control chloroplast exhibits a high oxygen evolution rate ($282.6 \mu\text{mol mg}^{-1} \text{ chlorophyll h}^{-1}$), while oxygen evolution rate in Pb^{2+} -treated groups decreased sharply, showing 83.86%, 43.88%, 34.43%, 26.05%, and 17.99% compared to the control, respectively.

Effect of Pb^{2+} on Chlorophyll Content of Spinach

During spinach growth period, the leaves damaged by various Pb^{2+} treatments were observed, exhibiting yellow white or curly. The chlorophyll content of spinach is presented in Fig. 10. It can be seen that the total chlorophyll content in Pb^{2+} -treated groups is lower than the control, which is decreased by 28.21%, 30.91%, 34.32%, 49.78%, and 62.35%, respectively; the ratio of chlorophyll *a*/chlorophyll *b* shows 0.08, 0.14, 0.27, 0.47, and 0.49 reductions, respectively. The results suggest that Pb^{2+} can inhibit chlorophyll synthesis or

Fig. 5 CAT activities ($\text{U mg}^{-1} \text{ protein min}^{-1}$) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. The activity of CAT was determined by measuring the rate of disappearance of H_2O_2 at 240 nm. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 240 nm. Values represent means \pm SE, $n=5$

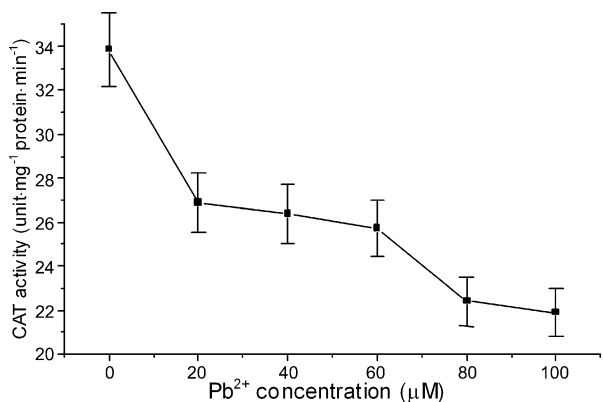
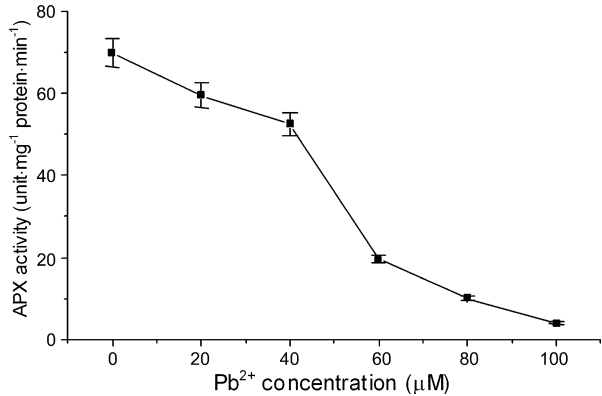


Fig. 6 APX activities ($\text{U mg}^{-1} \text{ protein min}^{-1}$) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. APX activity was assayed for 3 min at 290 nm and measured as disappearance of ascorbate. One unit of enzyme activity was defined as a decrease in absorbance of 0.001 min^{-1} at 290 nm. Values represent means \pm SE, $n=5$



ROS accumulated by Pb^{2+} oxidize chlorophyll, particularly chlorophyll *a*, thus resulting in photosynthesis decline.

Effect of Pb^{2+} on Spinach Growth

Figure 11 shows that exposure to Pb^{2+} has negative effects on spinach growth, particularly the dry weight of spinach is decreased significantly; that is, the fresh weight of a single plant in Pb^{2+} -treated groups is 61.73%, 31.65%, 29.61%, 22.05%, and 15.59% and the dry weight is 43.13%, 24.50%, 23.50%, 14.75%, and 8.43% compared to the control, respectively. It demonstrates that Pb^{2+} impairs accumulation of the organic substances in spinach, which is closely related to photosynthesis damaged by oxidative stress in chloroplast.

Discussion

The effect of lead on oxidative stress of spinach chloroplast and growth of the plant grown in lead-containing media was investigated. The question was raised whether oxidative stress of Pb^{2+} can occur in chloroplast by varying Pb^{2+} concentration during growth of spinach plants. The observed oxidative stress grown plants to Pb^{2+} ions appear to not indicate the presence of a protective mechanism against lead toxicity.

Fig. 7 GPX activities ($\text{U mg}^{-1} \text{ protein min}^{-1}$) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. GPX activity was assayed for 3 min at 510 nm and measured as appearance of *tetra*-guaiacol. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} at 510 nm. Values represent means \pm SE, $n=5$

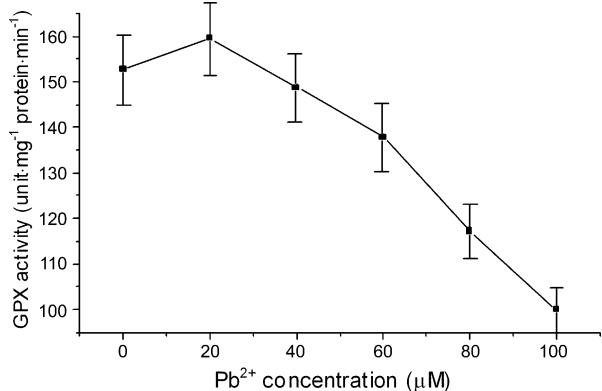
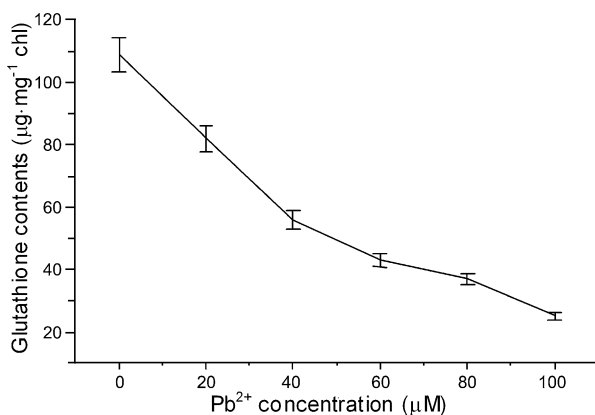


Fig. 8 GSH content ($\mu\text{g}/\text{mg}$ chlorophyll) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. GSH was performed using a fluorometer with excitation at 350 nm and emission at 420 nm. Values represent means \pm SE, $n=5$



One of heavy metal toxic mechanisms on chloroplast is an oxidative stress, probably because an imbalance between ROS and their removal makes macromolecules and membranes damaged, thus leads to photosynthesis inhibition. In plants, O_2 can be reduced to H_2O , when not reduced completely, and ROS that has strong oxidative ability emerges [26]. In the chlorenchyma, ROS is mainly from the photosynthetic electron transfer system [27]. Many studies proved that superabundant ROS could destroy the PS significantly. Miyao also proved that ROS such as $^1\text{O}_2$ has destructive effects on D1 protein and central complexes of PSII [28]. Our data showed that the production rate of $\text{O}_2^{\bullet-}$ and H_2O_2 in chloroplast of Pb^{2+} -treated spinach was significantly ascending (Figs. 1, 2), indicating that $\text{O}_2^{\bullet-}$ and H_2O_2 were constantly accumulated in spinach chloroplast. Interaction between H_2O_2 and $\text{O}_2^{\bullet-}$ can create $\cdot\text{OH}$ and $^1\text{O}_2$, which are far more destructive and can peroxidate the unsaturated lipid of the cell membrane [29]. As one kind of peroxide, MDA can intensively react with various cellular components; hence, enzymes and membranes are seriously damaged and membranous electric resistance and fluidity fall, and this eventually leads to the destruction of the membrane structure and physiological integrity [30]. Luo et al. pointed out that the formation of MDA under H_2O_2 in wheat was related to Cd^{2+} concentration, because the generation of $\text{O}_2^{\bullet-}$ could promote the $\text{H}_2\text{O}_2 \rightarrow \cdot\text{OH}$ process [3]. In this study, the peroxidation of the spinach chloroplast membrane along with Pb^{2+} concentration was demonstrated by an enhancement in MDA content (Fig. 3).

Plants use a diverse array of enzymes like SOD, CAT, APX, and GPX, as well as nonenzymatic antioxidants like ascorbate and GSH to remove oxidative stress. SOD can convert

Fig. 9 Oxygen evolution rate ($\mu\text{mol mg}^{-1}$ chlorophyll h^{-1}) of chloroplast of spinach grown in the presence of Pb^{2+} for 4 weeks. The oxygen evolution of chloroplasts isolated from spinach was measured with an Oxygraph oxygen electrode. The concentration of the artificial electron acceptor was 2 mM for FeCy. The concentration of the chloroplast was equivalent to about 20 μg of chlorophyll every milliliter. Values represent means \pm SE, $n=5$

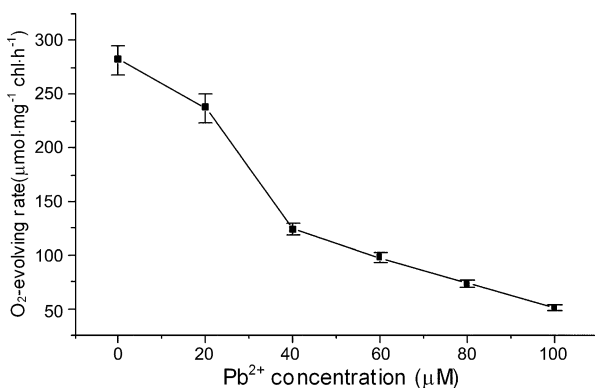
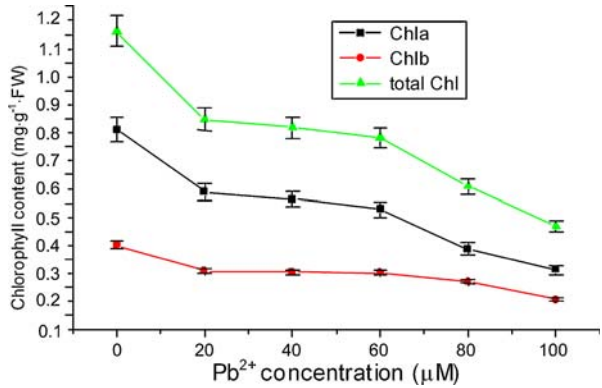


Fig. 10 Chlorophyll content (mg/g fresh weight) of spinach grown in the presence of Pb^{2+} for 4 weeks. The chlorophyll content was determined by Arnon's method. Values represent means \pm SE, $n=5$

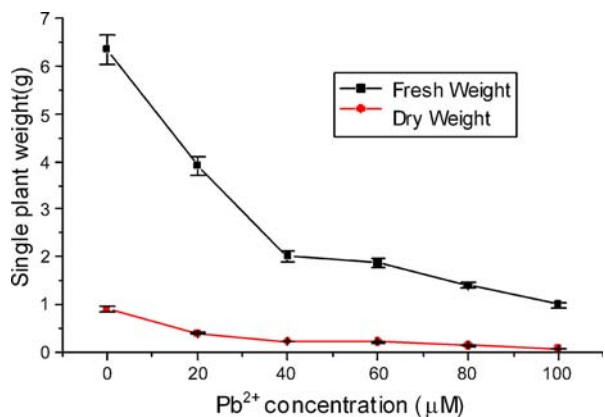


$O_2^{\cdot -}$ into H_2O_2 and O_2 ; moreover, CAT, APX, and GPX can reduce H_2O_2 into H_2O and O_2 [31]. Therefore, SOD, CAT, APX, and GPX can keep a low level of ROS and prevent ROS from poisoning cells [32]. In the experiments, we observed that the activities of SOD, CAT, APX, and GPX of spinach chloroplast grown in Pb^{2+} -contained media were significantly inhibited (Figs. 4, 5, 6, 7), suggesting that exposure to Pb^{2+} caused a strong oxidative stress in spinach chloroplast. Additional evidence pointing to the possibility of an oxidative stress was provided by a reduction in GSH content in spinach exposed to Pb^{2+} (Fig. 8).

Our data demonstrated that Pb^{2+} could enter spinach chloroplasts and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability. So, photosynthesis is inhibited. But, it needs to be further studied.

As a special organelle of plants, chloroplast is the main site for photosynthesis. Many studies indicated that lead and other heavy metals could evidently impair photosynthesis. Romanowska et al. indicated that growth under Pb^{2+} conditions led to a significant reduction in photosynthetic activity in the pea leaves [13]. Someone suggested that the effect of Pb^{2+} on PSII and PSI activities was profound [33, 34]. In the article, we proved that in spinach grown in the presence of Pb^{2+} , the oxygen evolution of chloroplast was inhibited (Fig. 9), chlorophyll content and plant weight were descending (Figs. 10, 11), which could ascribe to the oxidative stress of chloroplast by Pb^{2+} .

Fig. 11 Weight (g) of single spinach plant grown in the presence of Pb^{2+} for 4 weeks. The fresh weight and dry weight of single plant were weighted. Values represent means \pm SE, $n=10$



Conclusion

Lead applied to spinach cultures caused an oxidative stress status in chloroplast monitored by an increase in ROS accumulation. The enhancement of lipid peroxidation of chloroplast membrane in Pb^{2+} -treated spinach suggested an oxidative attack that was activated by a reduction in antioxidative defense mechanisms measured by analyzing the activity of SOD, CAT, APX, and GPX enzymes, as well as antioxidants such as GSH content. As the antioxidative response of chloroplast was reduced in spinach grown in a Pb^{2+} -contained medium, it caused the reduction in the chlorophyll content, oxygen evolution of chloroplast, and plant growth.

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