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Effect of Lead on the Chlorophyll and Antioxidant Response in the Leaves of *Arabidopsis thaliana*

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Abstract: Lead (Pb) is a non-essential heavy metal element for plants. In this study, the effect of 0.025, 0.05, 0.1, 0.25, and 0.5 g/L Pb on the chlorophyll and antioxidant response was analyzed in the leaves of *Arabidopsis thaliana*. The results indicate that 0.025 g/L Pb had no significant effect on the fluorescence intensity of chlorophyll. However, 0.05, 0.1, 0.25, and 0.5 g/L Pb decreased the fluorescence intensity of chlorophyll in the leaves of *A. thaliana*. Additionally, 0.05, 0.1, 0.25, and 0.5 g/L Pb significantly decreased the content of chlorophyll-a and chlorophyll-b in the leaves of *A. thaliana*. The activities of Glutathione peroxidase (Gpx), Superoxide Dismutase (SOD), and Catalase (CAT) were also reduced by 0.05, 0.1, 0.25 and 0.5 g/L Pb in the leaves of *A. thaliana*. The effect of Pb on the chlorophyll and antioxidant response was revealed, which will provide a reference for studying the impact of Pb on the plants.

Keywords: Lead, Leaves, Chlorophyll, Laser Scanning Confocal Microscopy, Antioxidant Response

Introduction

With the development of agriculture, industry, and technology, heavy metals have been widely used in the industries, agriculture, and other fields (Arti and Mehra, 2023; Chen *et al*., 2022; Manwani *et al*., 2023; Rajmohan *et al*., 2014; Sarker *et al*., 2022; Ullah *et al*., 2022). Thus, the environmental pollution induced by various heavy metals has become increasingly serious (Chen *et al*., 2021; Esfandiari and Hakimzadeh, 2022; Kumar *et al*., 2019). Recently, the harm of heavy metals to the environment and human beings has aroused people's deep concern (Mukherjee *et al*., 2022; Perrelli *et al*., 2022; Pratush *et al*., 2018). Lead (Pb) is one of the most severely polluted heavy metals (Cheng and Hu, 2010; Järup, 2003; Kayiranga *et al*., 2023).

Soil contaminated by heavy metals threatens crops and human health, which is a widely concerned environmental issue nowadays (Hsuang *et al*., 2021; Yang *et al*., 2019; Zhao *et al*., 2022). Of the hidden and difficult-to-eliminate nature of soil pollution, the ecological environment has unknowingly deteriorated, resulting in serious consequences such as grassland degradation, farmland damage, forest decline, and human and animal diseases (Ghafouri-Fard *et al*., 2021; Mazurek *et al*., 2017; Sun *et al*., 2020; Xiaojun *et al*., 2010). Soil is the material foundation for humans to obtain food and other renewable resources. Once the soil is contaminated by heavy metals, it can cause serious consequences (Aljerf and Choukaife, 2018). Heavy metal pollutants in soil are difficult to degrade by plants and microorganisms, the retention of heavy metal pollutants can affect the biochemical activity of soil, which affects the growth and quality of crops (Chen *et al*., 2021; Rai *et al*., 2019; Wu *et al*., 2022). If heavy metal pollutants enter the food chain, the safety of human health will be threatened (Islam *et al*., 2007; Kladsomboon *et al*., 2020; Rai *et al*., 2023).

Mild harm of lead inhibits plant growth. However, Pb pollution may cause plant death in severe pollution cases (Bindler, 2011; Küpper, 2017; Zheng *et al*., 2019). The plasma membrane is the interface between the organism and the external environment. When heavy metal Pb enters the plant body, it is the first membrane to be poisoned by lead (Pourrut *et al*., 2011; Sengar *et al*., 2008). Afterward, lead ions enter into cells through the plasma membrane, which influences the physiological processes in cells and causes metabolic disorders (Gupta *et al*., 2013; Wang *et al*., 2023).

Reactive Oxygen Species (ROS) refer to several metabolic products of oxygen with high chemical reactivity, which are formed by oxygen (Apel and Hirt, 2004; Choudhury *et al*., 2017; Mittler *et al*., 2022; Waszczak *et al*., 2018). Under normal circumstances, the level of ROS in plants is in a balanced state. However, under the stresses, the balance is disrupted and the level

of ROS increases. The over-production of ROS is harmful to the normal metabolism, cellular and organelle structures, and the stable structures of biological macromolecules. There are various antioxidant enzymes that can remove ROS. Antioxidant enzymes are a collective term for Superoxide Dismutase (SOD), thioredoxin peroxidase, Glutathione peroxidase (Gpx), and Catalase (CAT) (Bela *et al*., 2015; Liu *et al*., 2018; Logan *et al*., 2006; Pourrut *et al*., 2011). Antioxidant enzymes have the ability to convert peroxides into less toxic or harmless substances.

Excessive heavy metals in soil can have a serious impact on plant physiology, ROS metabolism, and growth status (El Amine *et al*., 2023; Riyazuddin *et al*., 2021; Sameena *et al*., 2021). Confocal microscopy has been used to study the fluorescence correlation and molecular interactions (Pack, 2021). Here, we studied the effect of different concentrations of heavy metal Pb on the chlorophyll and antioxidant response in *Arabidopsis thaliana*. The effect of heavy metal Pb on the chlorophyll of *A. thaliana* was revealed, which could provide a reference for studying the impact of Pb on plants.

Materials and Methods

Materials and Instruments

Standard Pb solution (Beijing Puxi Standard Technology Co., Ltd.). 0.1 moL/L phosphate buffer solution (PBS, pH 7.4). A Confocal Microscope (LSCM, Leica TCS SP2) and a UV Spectrophotometer (SPECTRA MAX M5) were used for this experiment.

Plant Cultivation

The seeds of *A. thaliana* (Columbia-0) were disinfected with 75% alcohol for 1 min, soaked in 2% sodium hypochlorite for 10 min, washed with sterilized water, and seeded on 1/2 MS medium (0.6 agar, 3% sucrose, pH 5.8). After the seeds were placed at 4°C for 3 days, they were cultured with the light/dark cycle 14/10 h at 23°C. After 15 days of cultivation in 1/2 MS medium (0.6 agar, 3% sucrose, pH 5.8) in a culture bottle, it was transferred to soil cultivation. The soil cultivation medium included nutrient soil, vermiculite, and perlite (proportion: 4:2:1). Nutrient soil was purchased from Shandong Shouguang Shenghe Agricultural Technology Co., Ltd. Then, the leaves were collected for the subsequent experiments (Fig. 1).

Leaf Treatment

The plants with appropriate leaf sizes were put in a culture dish containing PBS buffer solution. The leaves were washed with PBS for usage. Then the leaves were collected to determine the effect of Pb on chlorophyll.

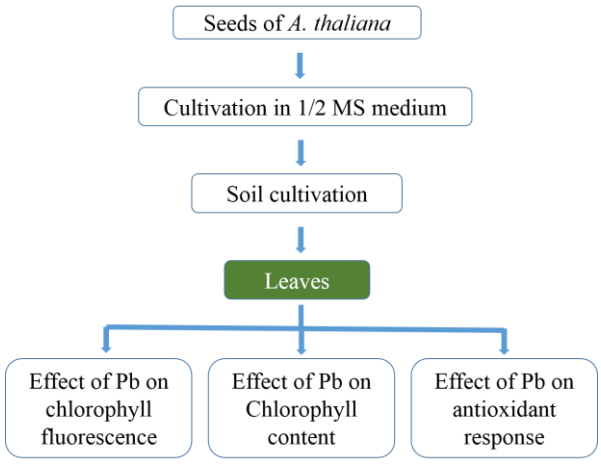


Fig. 1: The cultivation of *A. thaliana* and leaf treatments

Determination of the Effect of Pb on Chlorophyll Fluorescence Intensity by Laser Scanning Confocal Microscopy at Different Time

0.025, 0.05, 0.1, 0.25, and 0.5 g/L Pb were prepared by using Pb standard solution and PBS buffer. The leaves were washed with PBS buffer and then placed in a 35 mm confocal microscope culture dish. Subsequently, the different concentration of Pb was added, and the fluorescence images of chloroplasts were collected by using a laser scanning confocal microscopy at 0, 600, and 1200 sec, respectively. There were 3 replicates for each Pb treatment. Finally, Leica TCS-SP 2 software was used to analyze the fluorescence intensity of chlorophyll in *Arabidopsis* leaves.

Determination of the Effect of Pb on the Instant Changes of Chlorophyll Fluorescence Intensity with XYT Mode of Laser Scanning Confocal Microscopy

The leaves were placed in a 35 mm confocal microscope culture dish. Each lead treatment concentration has 3 replicates. The fluorescence images of chloroplasts were collected with XYT mode of laser scanning confocal microscopy. The different concentrations of lead ion solutions (0.025, 0.05, 0.1, 0.25, and 0.5 g/L Pb) were added after collecting the fourth image, respectively. Subsequently, Leica TCS-SP 2 software was used to statistically analyze the chlorophyll fluorescence intensity in *Arabidopsis* leaves. 20 chloroplast fluorescence images were collected with a 10 sec interval between each image acquisition.

Analysis of the Content of Chlorophyll

The fresh weight of leaves was recorded and leaf samples were treated with 0.025, 0.05, 0.1, 0.25, and 0.5 g/L Pb for 1.0 h and collected for analysis. The acetone extraction method was used for the detection of

chlorophyll content. The leaves were soaked in an 80% acetone solution. The absorbance of different samples was analyzed by using a UV Spectrophotometer (SPECTRA MAX M5) at 663 and 645 nm. The chlorophyll contents were calculated using the following equations (Wang *et al*., 2016):

$$Chlorophyll-a = 12.72\,A_{663} - 2.59\,A_{645} \quad (1)$$

$$Chlorophyll-b = 22.88\,A_{654} - 4.67\,A_{663} \quad (2)$$

Analyzing the Effect of Pb on the Activity of Antioxidant Enzymes

The fresh weight of leaves was recorded and leaf samples were treated with 0.025, 0.05, 0.1, 0.25, and 0.5 g/L Pb for 1.0 h and collected for analysis. The leaves were broken and centrifuged at 3000 r/min for 10 min. Then the supernatant was collected to analyze the antioxidant enzyme activities. The activity of CAT, Gpx, and SOD and soluble protein content was measured with the kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Data Analysis

The effect of Pb on the Chlorophyll and enzyme activity was analyzed by using SPSS software and one-way ANOVA at $p<0.05$. The different letters indicate that $p<0.05$.

Results

Effect of 0.025 g/L Pb on the Fluorescence Intensity of Chlorophyll

The effect of 0.025 g/L Pb on the fluorescence intensity of chlorophyll was shown in Fig. 2. 0.025 g/L Pb had no remarkable effect on the fluorescence intensity of chlorophyll (Fig. 2). The fluorescence intensity of chloroplasts was slightly decreased after 0.025 g/L Pb added by using XYT mode of laser scanning confocal microscopy (Fig. 3).

Effect of 0.05 g/L Pb on the Fluorescence Intensity of Chlorophyll

The effect of 0.05 g/L Pb on the chlorophyll fluorescence intensity was shown in Fig. 4. 0.05 g/L Pb significantly decreased the fluorescence intensity of chlorophyll compared to that of control (Fig. 4). The fluorescence intensity of chloroplasts were decreased after 0.05 g/L Pb added by using XYT mode of laser scanning confocal microscopy (Fig. 5).

Effect of 0.1 g/L Pb on the Fluorescence Intensity of Chlorophyll

Compared to the control, 0.1 g/L Pb significantly decreased the fluorescence intensity of chlorophyll (Fig. 6). The fluorescence intensity of chloroplasts was decreased after 0.1 g/L Pb added by using XYT mode of laser scanning confocal microscopy (Fig. 7).

Effect of 0.25 g/L Pb on the Fluorescence Intensity of Chlorophyll

Compared to the control, 0.25 g/L Pb significantly decreased the fluorescence intensity of chlorophyll (Fig. 8). The fluorescence intensity of chloroplasts was decreased after 0.25 g/L Pb added by using XYT mode of laser scanning confocal microscopy (Fig. 9).

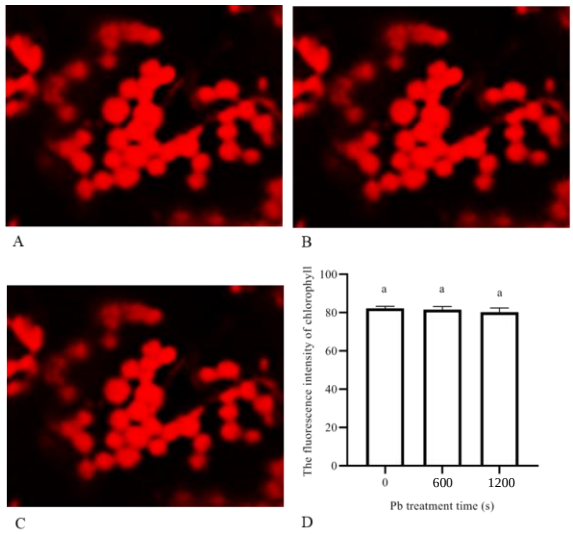


Fig. 2: Effect of 0.025 g/L Pb on the chlorophyll fluorescence intensity in the leaves of *A. thaliana*; (A) The chlorophyll fluorescence at 0 s; (B) The chlorophyll fluorescence at 600 s; (C) The chlorophyll fluorescence at 1200 s; (D) Effect of 0.025 g/L Pb on the chlorophyll fluorescence intensity (n = 3). The different letters showed that $p<0.05$ compared to the chlorophyll fluorescence intensity at 0 s

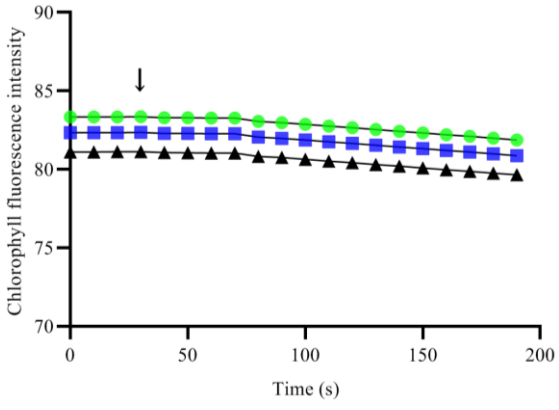


Fig. 3: Effect of 0.025 g/L lead on the instant change of chlorophyll fluorescence intensity in *A. thaliana*. The arrow indicates the time of adding lead solution. Different curves represent the changes in fluorescence intensity in the different leaves

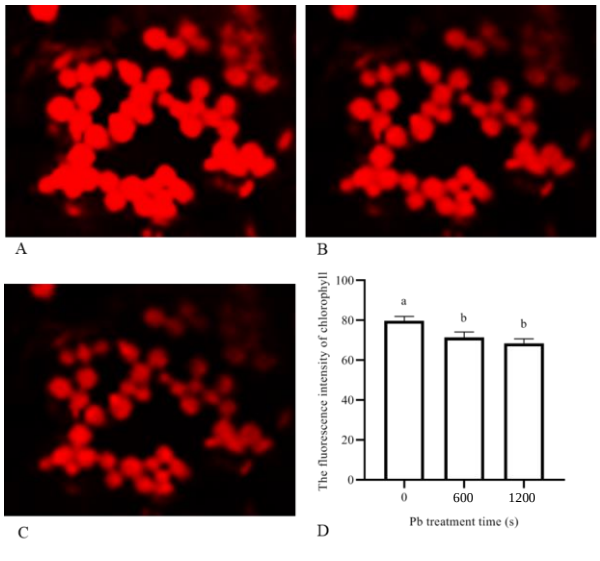


Fig. 4: Effect of 0.05 g/L Pb on the chlorophyll fluorescence intensity in the leaves of *A. thaliana*; (A) The chlorophyll fluorescence at 0 s; (B) The chlorophyll fluorescence at 600 s; (C) The chlorophyll fluorescence at 1200 s; (D) Effect of 0.05 g/L Pb on the chlorophyll fluorescence intensity (n = 3). The different letters showed that p<0.05 compared to the chlorophyll fluorescence intensity at 0 s

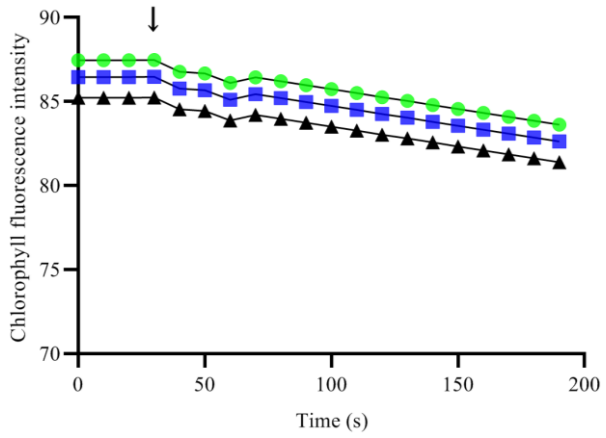


Fig. 5: Effect of 0.05 g/L lead on the instant change of chlorophyll fluorescence intensity in *A. thaliana*. The arrow indicates the time of adding lead solution. Different curves represent the changes in fluorescence intensity in the different leaves

Effect of 0.5 g/L Pb on the Fluorescence Intensity of Chlorophyll

Compared to the control, 0.5 g/L Pb significantly decreased the fluorescence intensity of chlorophyll (Fig. 10). The fluorescence intensity of chloroplasts was decreased after 0.5 g/L Pb added by using XYT mode of laser scanning confocal microscopy (Fig. 11).

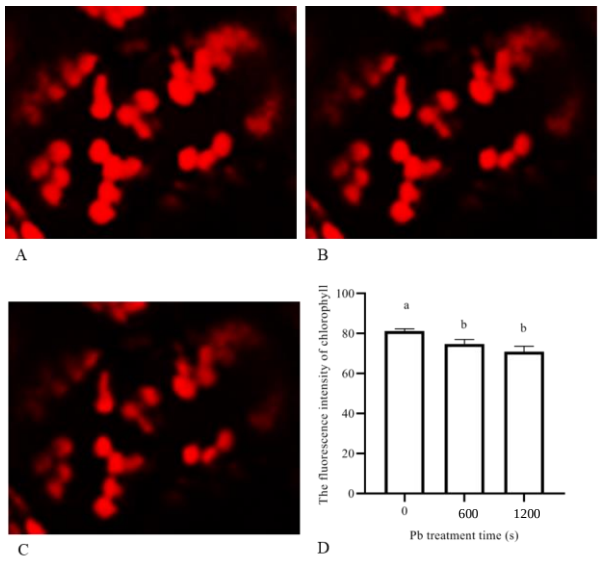


Fig. 6: Effect of 0.1 g/L Pb on the chlorophyll fluorescence intensity in the leaves of *A. thaliana*; (A) The chlorophyll fluorescence at 0 s; (B) The chlorophyll fluorescence at 600 s; (C) The chlorophyll fluorescence at 1200 s; (D) Effect of 0.1 g/L Pb on the chlorophyll fluorescence intensity (n = 3). The different letters showed that p<0.05 compared to the chlorophyll fluorescence intensity at 0 s

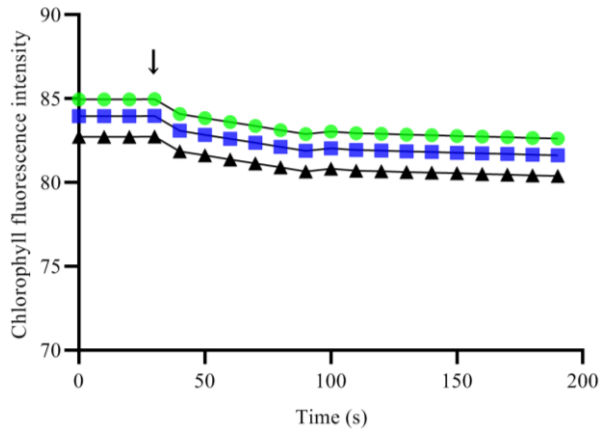


Fig. 7: Effect of 0.1 g/L lead on the instant change of chlorophyll fluorescence intensity in *A. thaliana*. The arrow indicates the time of adding lead solution. Different curves represent the changes in fluorescence intensity in the different leaves

Effect of Pb on the Content of Chlorophyll-a and Chlorophyll-b

0.05, 0.1, 0.25, and 0.5 g/L Pb significantly decreased the content of chlorophyll-a (Fig. 12A). In addition, 0.05, 0.1, 0.25 and 0.5 g/L Pb significantly decreased the content of chlorophyll-b (Fig. 12B).

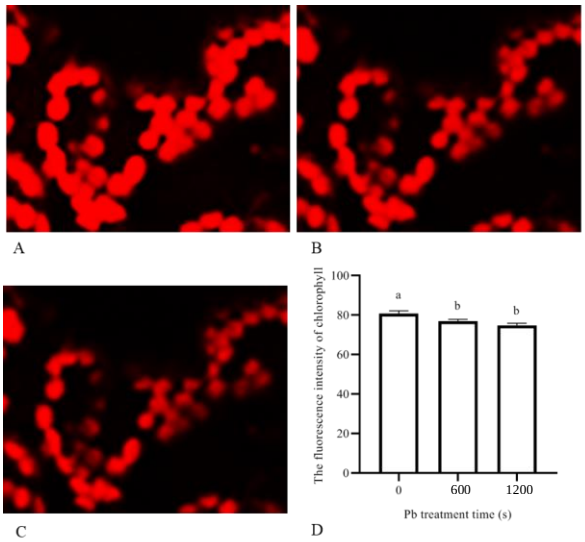


Fig. 8: Effect of 0.25 g/L Pb on the chlorophyll fluorescence intensity in the leaves of *A. thaliana*; (A) The chlorophyll fluorescence at 0 s; (B) The chlorophyll fluorescence at 600 s; (C) The chlorophyll fluorescence at 1200 s; (D) Effect of 0.25 g/L Pb on the chlorophyll fluorescence intensity (n = 3). The different letters showed that p<0.05 compared to the chlorophyll fluorescence intensity at 0 s

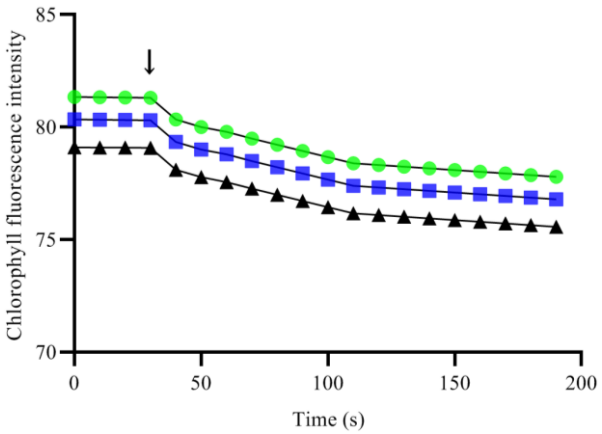


Fig. 9: Effect of 0.25 g/L lead on the instant change of chlorophyll fluorescence intensity in *A. thaliana*. The arrow indicates the time of adding lead solution. Different curves represent the changes in fluorescence intensity in the different leaves

Effect of Pb on the Activity of Antioxidant Enzymes

After the leaf samples were treated with 0.025, 0.05, 0.1, 0.25 and 0.5 g/L Pb, the activity of CAT was significantly decreased by 0.05, 0.1, 0.25 and 0.5 g/L Pb (Fig. 13A). The activity of SOD was significantly decreased by 0.05, 0.1, 0.25 and 0.5 g/L Pb (Fig. 13B). Additionally, 0.05, 0.1, 0.25 and 0.5 g/L Pb significantly decreased the activity of Gpx (Fig. 13C).

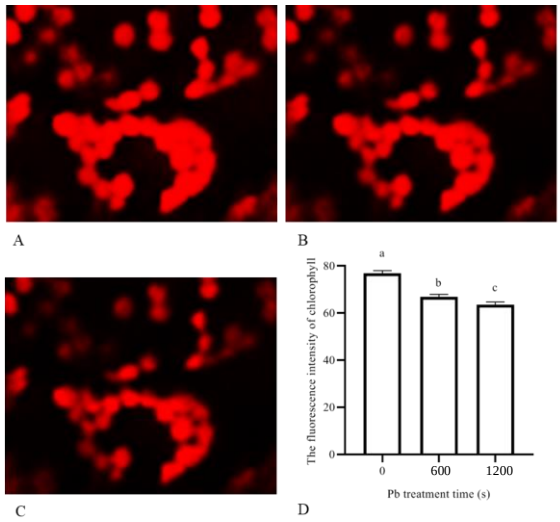


Fig. 10: Effect of 0.5 g/L Pb on the chlorophyll fluorescence intensity in the leaves of *A. thaliana*; (A) The chlorophyll fluorescence at 0 s; (B) The chlorophyll fluorescence at 600 s; (C) The chlorophyll fluorescence at 1200 s; (D) Effect of 0.5 g/L Pb on the chlorophyll fluorescence intensity (n = 3). The different letters showed that p<0.05 compared to the chlorophyll fluorescence intensity at 0 s

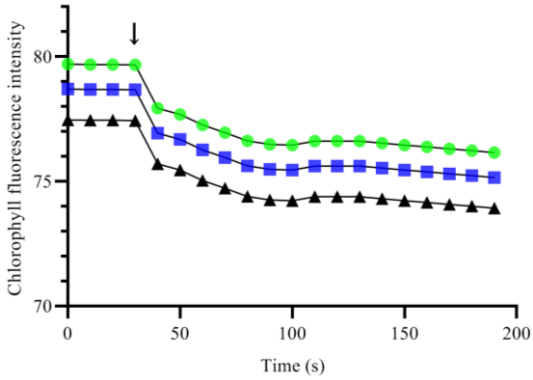
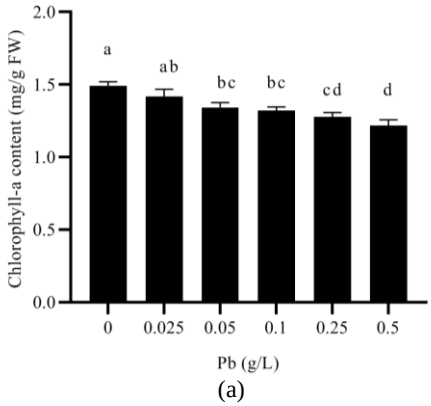
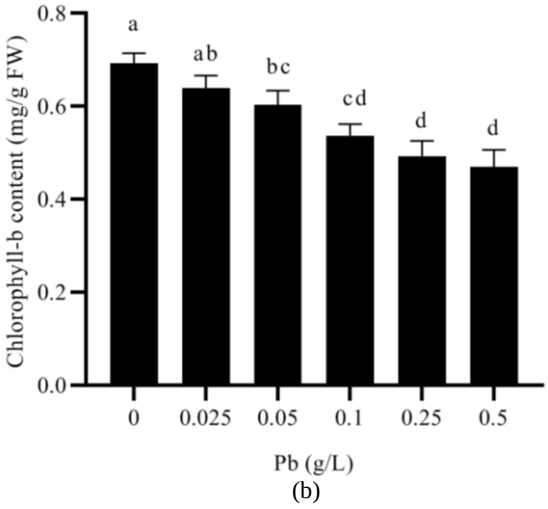


Fig. 11: Effect of 0.5 g/L lead on the instant change of chlorophyll fluorescence intensity in *A. thaliana*. The arrow indicates the time of adding lead solution. Different curves represent the changes in fluorescence intensity in the different leaves

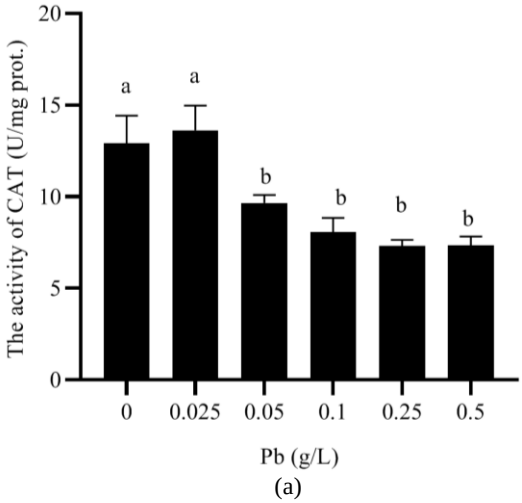


(a)

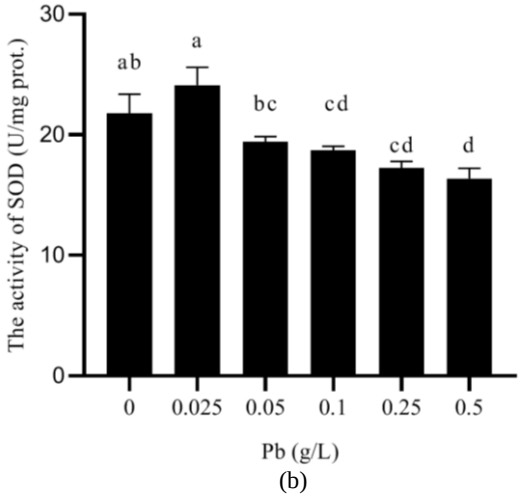


(b)

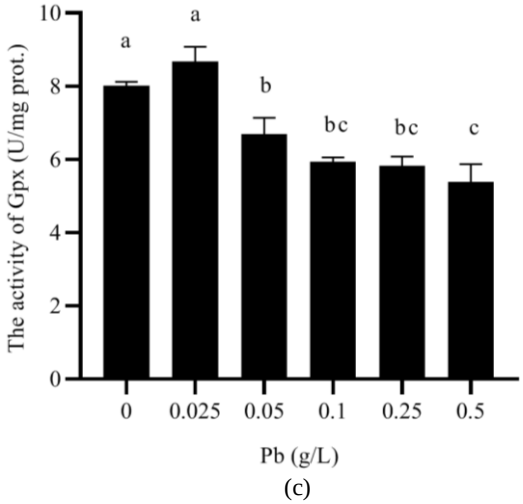
Fig. 12: Effect of Pb on the content of chlorophyll-a and chlorophyll-b; (A) Effect of Pb on the content of chlorophyll-a; (B) Effect of Pb on the content of chlorophyll-b. The different letters indicate that $p<0.05$ ($n = 3$)



(a)



(b)



(c)

Fig. 13: Effect of Pb on the activity of antioxidant enzymes; (A) Effect of Pb on the activity of CAT; (B) Effect of Pb on the activity of SOD; (C) Effect of Pb on the activity of Gpx. The different letters indicate that $p<0.05$ ($n = 3$)

Discussion

In recent years, due to the excessive discharge of industrial wastewater, it often causes water system pollution (Ahmed and Slima, 2018; Gemeda *et al*., 2021; Li *et al*., 2022). Lead is a pollution metal element in soil pollution and the content of lead in soil may exceed the normal value for its pollution (Cao *et al*., 2022; Charkiewicz and Backstrand, 2020; Kumar *et al*., 2022). Previous studies have shown that lead easily leads to damage to chloroplasts in plants, posing a threat to plant metabolism and affecting plant growth (Domínguez and Cejudo, 2021; Pourrut *et al*., 2011; Sengar *et al*., 2008). It has been found that the organs damaged by heavy metals in plants are primarily the roots, followed by the mesophyll and the chloroplasts in the leaves are highly sensitive to stress (Kushwaha *et al*., 2022; Ortiz-Luevano *et al*., 2021; Sengar *et al*., 2008; Zhang *et al*., 2020).

The effect of heavy metal lead on plants has been widely studied. When lead enters plant roots, stems, or leaves, it will accumulate affect plant growth, and cause harm to plants. Lead can reduce the rate of mitosis in root cells and inhibit plant growth. The accumulation of lead in plants can cause a decrease in root water absorption, an increase in oxygen consumption, hinder plant growth, and even cause plant death (Melese *et al*., 2023). The accumulation of lead can directly affect the metabolic function of cells and it may destroy the metabolic enzyme system.

The results indicate that heavy metal ions can alter enzyme activity or function through several pathways (Bayrakli, 2023; Yang *et al*., 2022). They may bind to enzymes and the binding of metals to the active sites of enzymes can affect the combination between enzymes

and substrates (Bayrakli, 2023). Metals can also cause changes in cofactors and substrate by altering membrane permeability, indirectly affecting enzyme activity (Tang *et al*., 2020).

Chloroplasts are organelles in plants that contain chlorophyll used for photosynthesis (Yang *et al*., 2020). They are the "food manufacturing workshop" and "energy conversion station" of plants (Aljerf and Aljerf, 2023). As an important component of chloroplasts, chlorophyll is crucial for plants and can reflect the impact of lead on chloroplasts. Some results have shown that lead has a significant impact on plant chloroplasts (Pourrut *et al*., 2011). Lead stress causes damage to the chloroplast structure of leaves, leading to a decrease in chloroplast content, which hinders photosynthesis and affects leaf growth (Pourrut *et al*., 2011). Thus, it indicates that chloroplasts in leaves are highly sensitive to heavy metal hazards (Picault *et al*., 2006; Seneviratne *et al*., 2019).

In this study, under the action of heavy metal lead, the fluorescence intensity of chlorophyll in *Arabidopsis* leaves was decreased, indicating that heavy metal lead has a significant impact on *Arabidopsis* chlorophyll. The higher concentration of lead has a greater impact on chlorophyll. The heavy metal lead can damage the chloroplast lamellar structure (Pourrut *et al*., 2011).

Because chlorophyll is an important component of chloroplasts, the effect of lead on chlorophyll is roughly equivalent to that on chloroplasts (Domínguez and Cejudo, 2021). In this study, the heavy metal lead has a significant impact on the chlorophyll. 0.025 g/L lead ion solution had no significant effect on chlorophyll fluorescence intensity, while 0.05, 0.1, 0.25, and 0.5 g/L lead ion solution all decreased chlorophyll fluorescence intensity. Additionally, 0.05, 0.1, 0.25, and 0.5 g/L lead significantly decreased the content of chlorophyll-a and chlorophyll-b, while 0.025 g/L lead had no significant effect on the content of chlorophyll-a and chlorophyll-b. Therefore, the higher lead concentration has a greater degree of weakening of chlorophyll fluorescence intensity. Lead could accumulate in the leaves, which may damage the chlorophyll and decrease the chlorophyll fluorescence intensity and chlorophyll content. In the current study, two methods of detecting the change of chlorophyll are compared. Similar results have been obtained to show the effect of Pb on chlorophyll, which is helpful for future studies.

The over-production of ROS is harmful to the metabolism of plants. Antioxidant enzymes are a collective term for SOD, Gpx, and CAT (Bela *et al*., 2015; Liu *et al*., 2018; Logan *et al*., 2006; Pourrut *et al*., 2011). Antioxidants have the ability to convert peroxides into less toxic or harmless substances. In this study, the activities of SOD, Gpx, and CAT were decreased by 0.05, 0.1, 0.25, and 0.5 g/L Pb in the leaves of *A. thaliana*. The decreased activity of antioxidant enzymes may enhance the over-production of ROS, which could damage the chlorophyll in the leaves of *A. thaliana*. However, the detailed mechanism needs to be further studied in the future.

Conclusion

In summary, the effect of Pb on the chlorophyll and antioxidant response was analyzed in the leaves of *A. thaliana*. The results of confocal microscopy analysis indicate that 0.025 g/L Pb had no significant effect on the chlorophyll fluorescence intensity. However, 0.05, 0.1, 0.25, and 0.5 g/L Pb decreased the fluorescence intensity of chlorophyll. Additionally, 0.05, 0.1, 0.25, and 0.5 g/L Pb significantly decreased the content of chlorophyll-a and chlorophyll-b in the leaves of *A. thaliana*. The activities of SOD, Gpx, and CAT were also decreased by 0.05, 0.1, 0.25, and 0.5 g/L Pb in the leaves of *A. thaliana*. The effect of Pb on the chlorophyll and antioxidant response was revealed, which will provide a reference for studying the impact of Pb on the plants.

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Author’s Contributions

Jun Xing: Participated in all experiments and coordinated the data analysis of the manuscript.

Cailing Yuan and Hui Yu: Participated in all experiments and contributed to the writing of the manuscript.

Rui Wu: Participated in the writing of the manuscript.

Xue Yang: Designed the research plan and organized the study.

Ethics

All authors have read and approved the manuscript and no ethical issues involved.

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