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Effect of Mercuric Ion on the Oxidant Response and Calcium Ion in the Protoplasts of *Arabidopsis Thaliana*

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Abstract: As a non-essential element for plant growth and development, the heavy metal mercury (Hg) has significant toxicity to plants. The aim of this study was to investigate the effect of mercuric ion (Hg^{2+}) on the oxidant response and calcium ion (Ca^{2+}) in the protoplasts of *Arabidopsis thaliana*. The protoplast cells were exposed to 5.0, 10.0, and 20.0 mmol/L Hg^{2+} for 0.5 and 1.0 h, respectively. To detect the effect of Hg^{2+} on the Reactive Oxygen Species (ROS) with the laser scanning confocal microscopy, 2' 7'-Dichlorofluorescent yellow Diacetate (DCFH-DA) was used to label ROS in protoplast cells of *Arabidopsis thaliana*. The result of confocal microscopy analysis showed that the level of ROS was increased in protoplast cells after the treatments of 5.0, 10.0, and 20.0 mmol/L Hg^{2+}. However, the activity of antioxidant enzymes Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GSH-PX) in protoplasts was decreased by 5.0, 10.0, and 20.0 mmol/L Hg^{2+} treatments. In addition, ion chromatography was used to study the effect of Hg^{2+} on the content of Ca^{2+} in the protoplasts of *Arabidopsis thaliana*. It showed that the content of Ca^{2+} in the outer cell of protoplasts was increased after the treatments of 5.0, 10.0, and 20.0 mmol/L Hg^{2+}. In conclusion, Hg^{2+} decreases the activity of antioxidant enzymes and induces the elevation of ROS in the protoplast cells. Mercuric ion inhibits the absorption of Ca^{2+} by protoplast cells and the change of Ca^{2+} content may affect ROS level in the protoplasts of *Arabidopsis thaliana*.

Keywords: Mercury, Protoplasts, Reactive Oxygen Species, Calcium Ions, *Arabidopsis Thaliana*

Introduction

As one of the heavy metal elements, mercury is toxic to plants and humans (Rahman and Singh, 2019; Tiodar *et al*., 2021; Zhao *et al*., 2022). The forms of mercury mainly include organic mercury (methylmercury, ethylmercury, vinyl mercury, propyl mercury, and mercury acetate, etc.,) and inorganic mercury (HgS, $HgSO_4$, $Hg(NO_3)_2$ and $HgCl_2$, etc.,) and the organic mercury is more toxic than inorganic mercury for animals (Camacho *et al*., 2022; Kim *et al*., 2016). In addition, inorganic mercury is easily accumulated in plants after mercury exposure. The accumulation of inorganic mercury in plant cells not only damages the cellular structure but also affects the physiological metabolism of plant cells (Kim *et al*., 2017; Uraguchi *et al*., 2019b). And, it will finally lead to metabolic disorders in plants and affect the development and growth of plants (Uraguchi *et al*., 2019a; Wang *et al*., 2021). If the pollution of inorganic mercury is serious, it may cause plant wilt and aging. Moreover, plant death may occur in severe cases of mercury pollution (Rahman and Singh, 2019; Regier *et al*., 2013). Inorganic mercury mainly enters plants through the pollution of water and soil sources (Raj and Maiti, 2020; Wagner-Döbler, 2003). The inorganic mercury that is absorbed through the food chain is easily accumulated in the human body, which may cause damage to the physiological metabolism and cell function (Du *et al*., 2021; Fu and Xi, 2020; Yang *et al*., 2020). The severe pollution of inorganic mercury may lead to the occurrence of cancer in humans (Skalny *et al*., 2022).

Plant cells are prone to producing excessive Reactive Oxygen Species (ROS) under external stimulations (Choudhury *et al*., 2017; Mittler *et al*., 2022; Qi *et al*., 2018). It is known that ROS mainly includes superoxide anion, singlet oxygen, hydrogen peroxide, etc. In addition, ROS have high oxidation activity and easily cause harm to biological macromolecules (Devireddy *et al*., 2021;

Nadarajah, 2020; Rizhsky *et al*., 2002). Under normal physiological states, plants have low ROS levels due to the scavenging effect of various antioxidant enzymes (Liang *et al*., 2019; Liu *et al*., 2014). Thus, ROS cannot have damaging effects on cells. However, as plants are stressed by the external environment, such as salt, low temperature, and heavy metal pollution, it easily destroys the state of ROS balance in plant cells (Agurla *et al*., 2018; Baral, 2019; Cheng *et al*., 2021; Ding *et al*., 2022; Yang and Guo, 2018). Thus, it will lead to a sharp rise in ROS levels and damage the normal physiological function of plant cells (Gechev *et al*., 2006; Gomes *et al*., 2014). In addition, calcium ion (Ca^{2+}) is one of the signal molecules in plant cells and plays an important physiological role as a second messenger (Kudla *et al*., 2018; Ma and Berkowitz, 2011). Environmental stimuli such as low temperature, hypoxia, and heavy metals are easy to cause the fluctuation of Ca^{2+} content (Tian *et al*., 2020). The change in cellular Ca^{2+} content not only affects cell signaling but also leads to abnormal cell physiological function (Aldon *et al*., 2018; Park *et al*., 2022).

Laser scanning confocal microscopy is an imaging device that is developed on the basis of fluorescence microscope technology. In recent years, laser scanning confocal microscopy has become a key instrument for studying the structure and function of plant cells (French *et al*., 2008; Truernit and Haseloff, 2008; Webb, 1999). Moreover, an ion chromatograph is mainly used to study the content of alkali metal ions and anions and it is also used to analyze the dynamic changes of intracellular and extracellular Ca^{2+} in cells (Cataldi *et al*., 2003). Previously, more studies were performed on the effect of mercuric ion (Hg^{2+}) in plants. There were fewer studies on the effect of Hg^{2+} in the protoplast cells. Thus, the aim of this study was to investigate the effect of Hg^{2+} on the oxidant response and Ca^{2+} in the protoplasts of *Arabidopsis thaliana*. The laser scanning confocal microscopy was used to analyze the effect of Hg^{2+} on ROS level in the protoplasts and ion chromatography was used to analyze the extracellular Ca^{2+} content in the protoplasts of *Arabidopsis thaliana*. The results will provide a theoretical basis for studying the physiological regulation mechanism of heavy metal mercury in plant cells.

Materials and Methods

Materials and Instruments

Ethanol, Sodium Hypochlorite (NaClO), sucrose, agar, cellulase, pectinase, 2-Morpholino Ethanesulfonic Acid (MES), mannitol, Calcium Chloride ($CaCl_2$), Mercuric Chloride ($HgCl_2$) and 2' 7'-Dichlorofluorescent yellow Diacetate (DCFH-DA) were analytical reagents and purchased from Aladdin Reagent Co., Ltd (Shanghai, China). The commercial kits for detecting the activity of Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GSH-PX) were purchased from Nanjing Jiancheng bioengineering institute (Nanjing, China). Methylsulfonic Acid (MSA) and the standard solution of Ca^{2+} were purchased from the Shanghai Institute of measurement and testing technology (Shanghai, China). The On-Guard RP C18 column and 0.22 μm micropore filter were purchased from agela technologies (Tianjin, China). The instruments include laser scanning confocal microscopy (Leica TCS SP2, Germany) and ion chromatograph (DIONEX ICS-2000, USA).

The Culture of Arabidopsis Thaliana

Arabidopsis thaliana was the Columbia ecotype (Col-0). *Arabidopsis* seeds were sterilized with 75% ethanol for 1 min and soaked in NaClO (2%) for 10 min. Then, the seeds were washed 5 times with sterile water and the seeds were subsequently sown in 1/2 modified medium (MS medium: 3% Sucrose, 0.6% agar, pH 5.8). After 3 days of culture at 4℃, the plants were incubated in the culture chamber. The light/dark period was 14 h (h)/10 h, light intensity was 6000 Lux (l×) and the incubation temperature was 23℃.

Acquisition and Processing of Arabidopsis Protoplasts

Fresh *Arabidopsis* leaves were placed on the clean petri dishes and leaves were cut into filaments about 0.5 mm wide and 2 mm long with a sterilized scalpel. The leaves were weighed to obtain 0.2 g in total. Then, 0.2 g leaves were placed in 1.5 mL centrifuge tubes with 1.0 mL enzyme solution (2% cellulase, 1% pectinase, 50 mol/L 2-Morpholino Ethanesulfonic acid (MES), 0.2 mol/L $CaCl_2$, 0.6 mol/L mannitol). The centrifuge tube was tightly wrapped with tin foil and dissociated for 24 h at room temperature.

The enzymatically digested protoplast suspension was filtered through a 300-mesh screen. Then, the filtered suspension was transferred to another centrifuge tube and centrifuged at 600 rpm for 5 min and repeated twice. After the supernatant was removed, 0.2 mol/L $CaCl_2$ was added to the centrifugal tube to suspend the solid protoplast, and wrapped the centrifugal tube with tin foil. Finally, the protoplast was placed in the refrigerator and stored at 4℃ for later usage.

Exposure Mercuric Ion

The protoplast suspensions were placed in twelve 1.5 mL centrifuge tubes. The unequal amounts of $HgCl_2$ were added to make the concentration of Hg^{2+} in the protoplast suspension 0, 5.0, 10.0, and 20.0 mmol/L, respectively. The exposure time of different concentrations of Hg^{2+} on protoplasts was 0.5 h and 1 h, respectively. The experiments were performed in triplicate for the following analysis.

Analyzing the Effect of Mercury Ions on the Fluorescence Intensity of ROS in the Protoplasts with Confocal Microscopy

After the treatment of different concentrations of Hg^{2+}, 10 μg/mL DCFH-DA was added and stained for 10 min to label ROS in the protoplasm. Then, the protoplast cells were aspirated to slides using a pipette, and the coverslips were covered to obtain fluorescence images. A laser confocal microscopy (Leica TCS SP2) was used to obtain fluorescence images of *Arabidopsis* protoplast cells with a laser excitation wavelength of 488 nm. Subsequently, the fluorescence statistical analysis was performed using the Leica TCS SP2 software.

Analyzing the Effect of Mercuric Ion on the Activity of Antioxidant Enzymes in the Protoplasts

After Hg^{2+} treatment, the tubes were centrifuged at 3000 r/min for 10 min and the supernatant was acquired to determine the activity of antioxidant enzymes in protoplasts. The activity of CAT, SOD, and GSH-PX and the content of soluble protein in the supernatant was measured by the CAT, SOD, and GSH-PX, soluble protein kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Analyzing the Effect of Mercuric Ion on the Extracellular Calcium Ion in Protoplasts with Ion Chromatography

After Hg^{2+} treatment, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant was acquired to determine the extracellular Ca^{2+} content in protoplasts. The content of Ca^{2+} was analyzed by using a DIONEX ICS-2000 ion chromatograph. The chromatographic conditions were as follows: DIONEX Ionpac CS12 A-4 mm column; mobile phase: 20 mm MSA; flow rate: 1.0 mL/min. Finally, the On-Guard RP C18 column was used to remove organic matter from the sample to avoid contaminating the column. The diluted sample was filtered by a 0.22 μm micropore filter for analytical testing.

Statistical Analyses

The results were expressed as mean ± Standard Error (SE). One-way ANOVA and Tukey test were performed for post hoc comparisons at $p<0.05$ by using SPSS 16.0.

Results

Effect of Mercuric Ion on ROS in Protoplasts after 0.5 h Treatment

After obtaining the cell fluorescence images by laser confocal microscopy, the ROS fluorescence intensity in the protoplasts was analyzed (Fig. 1A-D). The results showed that the fluorescence intensity of ROS was significantly affected after 0.5 h Hg^{2+} treatment (Fig. 1 A-D). When the concentration of Hg^{2+} was 5.0, 10.0, and 20.0 mmol/L, the fluorescence intensity of ROS was higher than that of the control group (Fig. 1E). At 5.0 mmol/L Hg^{2+}, the fluorescence intensity of ROS in protoplasts was higher than that of the control group, but not significantly affected ROS level (Fig. 1E). However, at 10.0 and 20.0 mmol/L Hg^{2+}, the fluorescence intensity of ROS in protoplasts was significantly higher than that of the control group (Fig. 1E).

Effect of Mercuric Ion on the ROS in Protoplasts after 1.0 h Treatment

After 5.0, 10.0, and 20.0 mmol/L Hg^{2+} were added to the protoplast suspension, the cell fluorescence images were obtained by laser confocal microscopy. Then the fluorescence intensity of ROS in the protoplasts was analyzed (Fig. 2A-D). The results showed that the fluorescence intensity of ROS was significantly affected after 1.0 h Hg^{2+} treatment (Fig. 2A-D). When the concentration of Hg^{2+} was 5.0, 10.0, and 20.0 mmol/L, the fluorescence intensity of ROS was significantly higher than that of the control group (Fig. 2E).

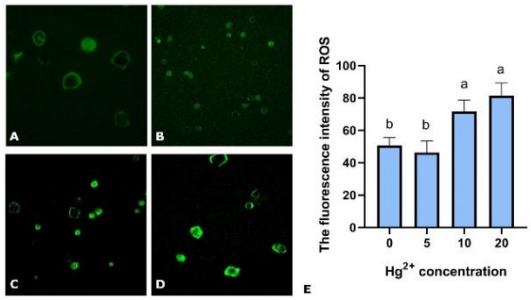


Fig. 1: Effect of mercury ion on the fluorescence intensity of ROS in protoplasts after 0.5 h treatment. (A) Control; (B) 5.0 mmol/L Hg^{2+}; (C) 10.0 mmol/L Hg^{2+}; (D) 20.0 mmol/L Hg^{2+}; (E) Effect of Hg^{2+} on the fluorescence intensity of ROS in protoplasts. Different letters indicate $p<0.05$

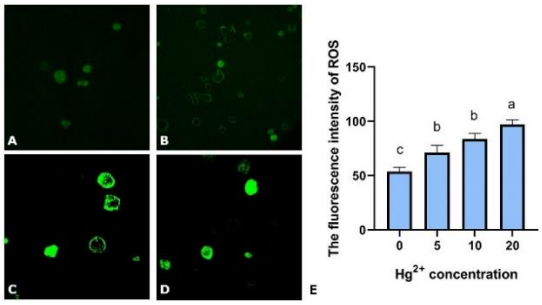


Fig. 2: Effect of mercury ion on the fluorescence intensity of ROS in protoplasts after 1.0 h treatment. (A) Control; (B) 5.0 mmol/L Hg^{2+}; (C) 10.0 mmol/L Hg^{2+}; (D) 20.0 mmol/L Hg^{2+}; (E) Effect of Hg^{2+} on the fluorescence intensity of ROS in protoplasts

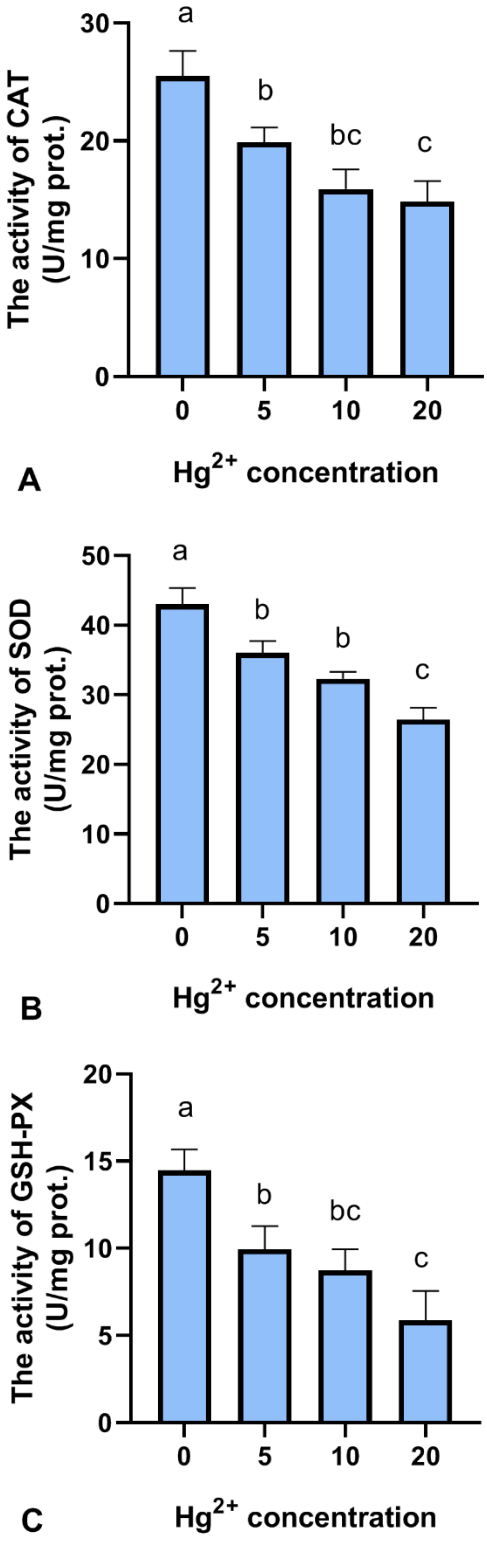


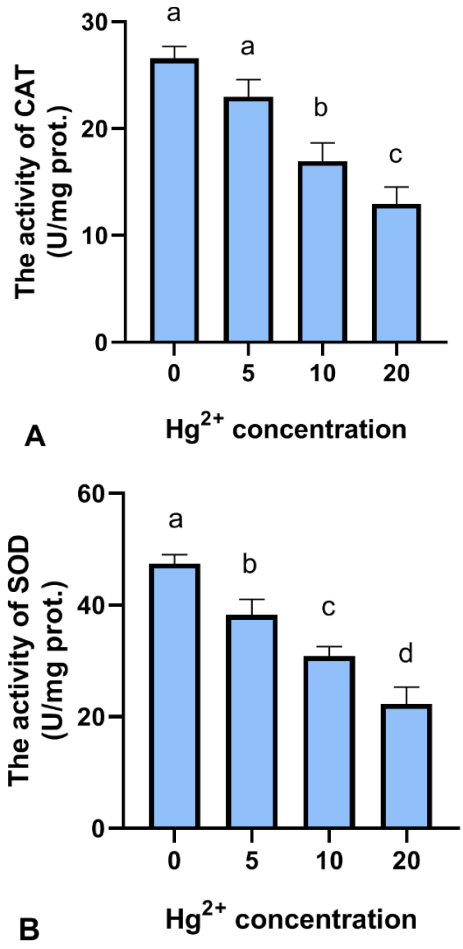
Fig. 3: Effect of mercury ion on the activities of CAT, SOD, and GSH-PX in protoplasts after 0.5 h treatment. (A) Effect of Hg^{2+} on the activity of CAT. (B) Effect of Hg^{2+} on the activity of SOD. (C) Effect of Hg^{2+} on the activity of GSH-PX

Effect of Mercuric Ion on the Activity of Antioxidant Enzymes in Protoplasts after 0.5 h Treatment

After 5.0, 10.0, and 20.0 mmol/L Hg^{2+} were added to the protoplast suspension for 0.5 h, the effect of Hg^{2+} on the activity of antioxidant enzymes in protoplasts was shown in Fig. 3. At 5.0, 10.0 and 20.0 mmol/L Hg^{2+}, the activity of CAT in the protoplasts was significantly lower than that of the control group (Fig. 3A). At 5.0, 10.0 and 20.0 mmol/L Hg^{2+}, the activity of SOD and GSH-PX in the protoplasts was significantly lower than that of the control group (Fig. 3B-C).

Effect of Mercuric Ion on the Activity of Antioxidant Enzymes in Protoplasts after 1.0 h Treatment

After 5.0, 10.0, and 20.0 mmol/L Hg^{2+} were added to the protoplast suspension for 1.0 h, the effect of Hg^{2+} on the activity of antioxidant enzymes in protoplasts was shown in Fig. 4. At 5.0, 10.0 and 20.0 mmol/L Hg^{2+}, the activity of CAT in the protoplasts was remarkably decreased compared to the control group (Fig. 4A). Moreover, 5.0, 10.0 and 20.0 mmol/L Hg^{2+} significantly decreased the activity of SOD and GSH-PX in the protoplasts compared to that of the control group (Fig. 4, B, C).



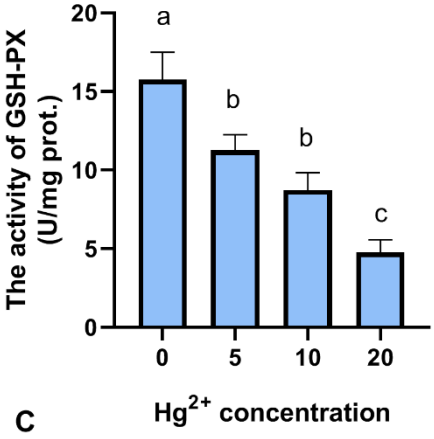


Fig. 4: Effect of mercury ion on the activities of CAT, SOD, and GSH-PX in protoplasts after 1.0 h treatment. (A) Effect of Hg^{2+} on the activity of CAT. (B) Effect of Hg^{2+} on the activity of SOD. (C) Effect of Hg^{2+} on the activity of GSH-PX

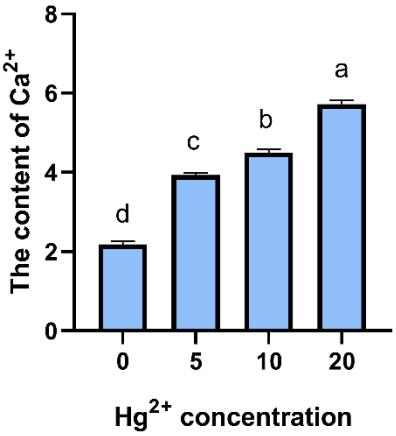


Fig. 5: Effect of mercury ion on the content of Ca^{2+} in protoplasts after 0.5 h treatment

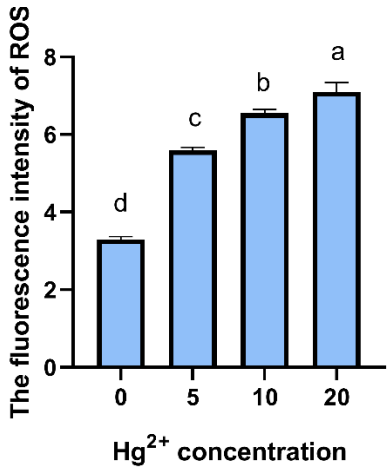


Fig. 6: Effect of mercury ion on the content of Ca^{2+} in protoplasts after 1.0 h treatment

Effect of Mercuric Ion on the Content of Ca^{2+} in Protoplasts after 0.5 h Treatment

After 5.0, 10.0, and 20.0 mmol/L Hg^{2+} were added to the protoplast suspension for 0.5 h, the extracellular Ca^{2+} content of protoplasts was determined by ion chromatography. The extracellular Ca^{2+} content of protoplasts was shown in Fig. 5. Compared with the control group, 5.0, 10.0, and 20.0 mmol/L Hg^{2+} significantly increased the extracellular Ca^{2+} content in the protoplasts compared to that of the control group (Fig. 5).

Effect of Mercuric Ion on the Content of Ca^{2+} in Protoplasts after 1.0 h Treatment

After 5.0, 10.0, and 20.0 mmol/L Hg^{2+} were added to the protoplast suspension for 1.0 h, the extracellular Ca^{2+} content of protoplasts was determined by ion chromatography. The extracellular Ca^{2+} content of protoplasts was shown in Fig. 6. Compared with the control group, at 5.0, 10.0, and 20.0 mmol/L Hg^{2+}, the extracellular Ca^{2+} content of the protoplasts was significantly higher than that of the control group (Fig. 6).

Discussion

Mercury is one of the environmentally hazardous substances, which has high volatility and biological toxicity (Laacouri *et al*., 2013; Zhang *et al*., 2017). The main sources of Hg in agricultural soils derive from fertilizers and atmospheric deposition (Zhou *et al*., 2018). The contamination of Hg affects the development and growth of plants. It is observed that Hg inhibits photosynthesis, impairs protein function, and decreases biomass production (Chen and Yang, 2012). The physiological disorders were induced and photosynthetic light reactions were inhibited by Hg in plants (Asztalos *et al*., 2012; Deng *et al*., 2013; Pirzadah *et al*., 2018).

Laser scanning confocal microscopy is a generally used technology to study the fluctuation of signal molecules in plant cells (Liu *et al*., 2008; Liu, 2004). Using fluorescent probes to label the cellular components, the change of fluorescence signal of probe molecules can be observed. This technology can obtain the morphology of protoplasts and the fluorescence change of internal components (Blancaflor and Gilroy, 2000). The level of ROS plays a crucial role in plant cell development, differentiation, and apoptosis, which participate in regulating the physiological metabolism (Del Río, 2015; Del Río and López-Huertas, 2016; Li *et al*., 2022). The fluorescence probe that is generally used to detect intracellular ROS is DCFH-DA. In addition, DCFH is oxidized by ROS to produce the fluorescent substance DCF and the fluctuation of ROS can be investigated by detecting the fluorescence intensity of DCF (Dai *et al*., 2022). In this study, DCFH-DA was used to label ROS in

Arabidopsis protoplast cells. We observed that the low, medium, and high concentrations of Hg^{2+} could increase ROS levels in protoplast cells. However, the activity of antioxidant enzymes CAT, SOD, and GSH-PX in protoplasts was decreased by 5.0, 10.0, and 20.0 mmol/L Hg^{2+} treatments. The activities of antioxidant enzymes CAT, SOD, and GSH-PX play a key role in regulating the level of ROS in plant cells (Blokhina *et al*., 2003; Gzyl *et al*., 2009). Our results showed that Hg^{2+} decreased the activity of antioxidant enzymes, which further caused the elevation of ROS content in the protoplast cells. Previously, it is found that mercury affected the activities of antioxidant enzymes and induced oxidative stress in plants (Elbaz *et al*., 2010; Zhou *et al*., 2008) and our result is consistent with the previous studies. Thus, Hg^{2+} may damage the function of antioxidant enzymes, thereby causing the elevation of ROS in *Arabidopsis* protoplast cells. The biological toxicity of Hg^{2+} may be related to its inhibitory effect on the antioxidant enzyme activities, which may affect the development and growth of plants.

Calcium ion in plant cells is also an important signal molecule. Calcium ion participates in the signal transduction process that is caused by external environmental stimuli, such as low temperature, hypoxia, heavy metals, and other external factors (Pottosin and Schönknecht, 2007; Tian *et al*., 2020). These external environmental stimuli can cause sharp fluctuations of Ca^{2+} in plant cells (Pottosin and Schönknecht, 2007). The distribution of Ca^{2+} in plant cells is not uniform. The cytoplasmic Ca^{2+} in normal cells is 30 ~ 200 nmol/L, while the concentration of intracellular Ca^{2+} in the vacuole, endoplasmic reticulum, and mitochondria is much higher. Under the action of an abnormal external environment, the content of Ca^{2+} in the cytoplasm will increase, which mainly derives from Ca^{2+} in the extracellular environment and calcium reservoir (Toprak *et al*., 2021). Previously, it is found that heavy metals affected the function of calcium sensor calmodulins (Han *et al*., 2023) and cadmium affected guard cell regulation by entering the cytosol via Ca^{2+} channels (Han *et al*., 2023). In this study, ion chromatography was used to analyze the effect of heavy metal Hg^{2+} on the extracellular Ca^{2+} in the *Arabidopsis* protoplasts. We found that Hg^{2+} could increase the extracellular Ca^{2+} content in protoplasts and inhibit the absorption of Ca^{2+} by protoplasmic cells. The damage of Hg^{2+} to cells may further affect the signaling effect of Ca^{2+}. Mercuric ion inhibits the absorption of Ca^{2+} by protoplast cells and the change of Ca^{2+} content may affect ROS level in the protoplasts of *Arabidopsis thaliana*. However, the detailed mechanism of action needs to be further investigated.

Conclusion

In summary, we investigated the effect of Hg^{2+} on the oxidant response and Ca^{2+} in the protoplasts of *Arabidopsis thaliana*. The protoplast cells were exposed to 5.0, 10.0, and 20.0 mmol/L Hg^{2+} for 0.5 h and 1.0 h, respectively. To detect the effect of Hg^{2+} on ROS with laser scanning confocal microscopy, DCFH-DA was used to label ROS in protoplast cells of *Arabidopsis thaliana*. The result of confocal microscopy analysis showed that the level of ROS was increased in protoplast cells after the treatments of 5.0, 10.0, and 20.0 mmol/L Hg^{2+}. However, the activity of CAT, SOD, and GSH-PX in protoplasts was decreased by 5.0, 10.0, and 20.0 mmol/L Hg^{2+} treatments. In addition, ion chromatography was used to study the effect of Hg^{2+} on the content of Ca^{2+} in the protoplasts of *Arabidopsis thaliana*. It showed that the content of Ca^{2+} in the outer cell of protoplasts increased after the treatments of 5.0, 10.0, and 20.0 mmol/L Hg^{2+}. In conclusion, Hg^{2+} decreases the activity of antioxidant enzymes, which further causes the elevation of ROS content in the protoplast cells. Mercuric ion inhibits the absorption of Ca^{2+} by protoplast cells and the change of Ca^{2+} content may affect ROS level in the protoplasts of *Arabidopsis thaliana*.

Abbreviations

- 2’ 7’-Dichlorofluorescent yellow Diacetate (DCFH-DA)
- 2-Morpholino Ethanesulfonic acid (MES)
- Calcium Chloride ($CaCl_2$)
- Calcium ion (Ca^{2+})
- Catalase (CAT)
- Glutathione Peroxidase (GSH-PX)
- Mercuric Chloride ($HgCl_2$)
- Mercuric Ion (Hg^{2+})
- Methylsulfonic Acid (MSA)
- Modified medium (MS medium)
- Reactive Oxygen Species (ROS)
- Sodium Hypochlorite (NaClO)
- Superoxide Dismutase (SOD)

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Author’s Contributions

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

Rui Wu and Hui Yu: Participated in all experiments and contributed to the written of the manuscript.

Cailing Yuan: Participated in writing the manuscript.

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

Ethics

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

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