Cadmium Stress in Halophyte *Thellungiella halophila*: Consequences on Growth, Cadmium Accumulation, Reactive Oxygen Species and Antioxidative Systems

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Abstract. Cadmium (Cd) stress on the growth and antioxidant system of the halophyte *Thellungiella halophila* is studied to explore heavy metal tolerance and the role of antioxidants in Cd tolerance. Biomass production of both leaves and roots of four leaves *T. halophila* seedlings significantly decreased under 0, 0.3, 0.6, and 0.9 mmol L⁻¹ CdCl₂ for 14 days compared to no-Cd control. Meanwhile, the photosynthetic oxygen evolution rate significantly decreased and the chlorophyll content slightly decreased. The MDA and hydrogen peroxide (H₂O₂) contents of leaves increased as the Cd concentrations increased, and the oxidative damage in Cd-treated seedlings was greater under Cd stress than under the no-Cd controls. Though the translocation factor (TF) was always under 1, *T. halophila* can tolerate and accumulate more than 100 mg kg⁻¹ Cd in shoot dry biomass. *T. halophila* mitigated the oxidative damage by increasing enzymatic components, glutathione (GSH), and ascorbic acid (AsA). In Cd-treated seedlings, both the leaf GSH and AsA and the activities of both peroxidase (POD) and catalase (CAT) were significantly greater under Cd stress than under no-Cd control. No significant differences were found in the activity of ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD). These results critically summarize the effects of Cd stress in *T. halophila* and these results above indicate that the antioxidants are responsible for Cd tolerance in *T. halophila*.

1. Introduction

Heavy metal pollution in soils, particularly in agricultural soils has been gaining ever-increasing attention worldwide, though more studies on the mechanisms of plant adaptations to heavy metals are required [1]. And cadmium (Cd) is terrifically poisonous to plants in those heavy metals. Studies have shown that Cd can decrease plant growth. Cd can lead to physiological and metabolic disturbances in plants by affecting chlorophyll (Chl) production and the activities of photosynthetic carbon reduction cycle enzymes [2]. High Cd stress resulted in accumulation of ROS and leading to serious plant damage [3]. High Cd concentrations in plants also led to lipid peroxidation and cell membrane fluidity in addition to permeability changes [4].

Plant cells have evolved enzymatic antioxidant (peroxidase, POD; superoxide dismutase, SOD, etc) and non-enzymatic antioxidant (glutathione, GSH; ascorbic acid, AsA, etc) to avoid the harmful effects
of ROS [5]. However, the pattern of each antioxidant enzyme differs in different plant species under Cd stress. For example, Cd stress increased the activity of POD and SOD, but decreased the catalase (CAT) activity in *Bacopa monnieri* leaves [6]. In tobacco leaves, Cd also increased the activity of POD and SOD, but not the ascorbate peroxidase (APX) activity [7]. In metal-accumulator plants, for example, *Brassica juncea*, the activity of APX, CAT and glutathione reductase (GR) were significantly enhanced by Cd [8].

Soil salinization is also a global environmental problem and is attracting increasing attention. Halophytes can complete their life cycle in environment containing more than 200 mmol L$^{-1}$ NaCl [9]. *Thellungiella halophila*, which belongs to the Brassicaceae family and relates to the model plant *Arabidopsis*, has come to the forefront in studying abiotic stress resistance. In China, *T. halophila* grew in the coastal areas along the mouth of the Yellow River Delta, where were predominated by highly saline soils and heavy metals accumulate [10]. Studies have showed that salt stress led to proline accumulation of *T. halophila* and affected K$^+$ uptake [11]. *T. halophila* is more tolerant to salt than *Arabidopsis thaliana* [12]. However, studies on effects of Cd on the growth and physiological characteristics of *Thellungiella* species are limited. Thus, to explore the Cd tolerance potential of native *T. halophila*, the objectives of the present study are to address the responses of both antioxidant enzyme activity as well as non-enzymatic AsA and GSH of shoots and roots in alleviating oxidative damage induced by Cd stress. The generated results could help to improve the understanding of the physiological and biochemical mechanisms of *T. halophila* tolerance to heavy metal stress.

2. Materials and methods

2.1. Materials and Cd treatments

*Thellungiella halophila* seeds (stored at 4°C) were collected from a saline land of the Yellow River Delta, locating in Dongying, Shandong, China (N37°25′; E118°58′). The seed germination was on vermiculite soaked in 1/10 strength Hoagland’s solution in plastic pots. After germination, selected uniform *T. halophila* seedlings with four leaves were transplanted to 10×10 cm square flowerpots filled with vermiculite in a growth chamber. The chamber was maintained at 250 mol photon m$^{-2}$ s$^{-1}$, 14/10h and 24/18°C (day/night), and 65% relative humidity. After the plants grew to 8 or 9 leaves, they were watered once a day with 1/5 strength Hoagland’s solution containing different concentrations of CdCl$_2$ (0, 0.3, 0.6, and 0.9 mmol L$^{-1}$) for 14 days because leaf damage occurred after 16 days growing under more than 0.9 mmol L$^{-1}$ Cd in our preliminary experiments. Each treatment group contained five pots.

2.2. Determination of biomass, and tissue cadmium, leaf Chla, Chlb, carotenoids and photosynthetic oxygen evolution rate

After 14 days’ Cd treatment, we separated the seedlings into roots and shoots and rinsed, and then dried with blotting paper and weighed as fresh weight (FW). Fresh shoots and roots were firstly oven-dried at 105°C for 10 minutes, and then at 70°C until a constant dry weight (DW). Plant tissues were digested by a microwave decomposition, and tissue Cd (μg g$^{-1}$ DW) in the digestion extract was measured by an ICP-MS Nexion 300x (Perkin Elmer, USA).

The term of translocation factor (TF) defined as the ratio of shoot Cd content (mg kg$^{-1}$ DW) to root Cd content (mg kg$^{-1}$ DW) were determined [13].

After extraction with 80% acetone, leaf Chla, Chlb, and total carotenoids in the extracts were spectrophotometrically measured with a spectrophotometer (TU-1810D, Purkinje general instrument, Beijing, China) at 644, 663 and 452.5 nm, respectively. Meanwhile, to determine the leaf photosynthetic oxygen evolution rate, 1 cm$^2$ discs from the third leaves of *T. halophila* were cut into 1 mm$^2$ and placed in 2 mL of 0.1 mmol L$^{-1}$ NaHCO$_3$. The photosynthetic oxygen evolution rates were measured at 25°C by a Liquid-Phase Oxygen Measurement System (Chlorolab-2 type, Hansatech, UK) [14].

2.3. Measurement of lipid peroxidation, hydrogen peroxide (H$_2$O$_2$) and plasma membrane permeability

Measurement of malondialdehyde (MDA) was done using the 2-thiobarbituric acid assay. Concisely,
0.4 g FW *T. halophila* leaves were extracted by 5 mL 0.1 % trichloroacetic acid and then added 5 mL of 0.5 % 2-thiobarbituric acid. Leaf MDA contents were measured from *A*$_{532}$ and *A*$_{600}$ values, and then converted on the dry matter basis as nmol g$^{-1}$ DW.

To visualize the H$_2$O$_2$ in situ, the staining of leaves with 1 mg L$^{-1}$ 3,3-diaminobenzidine was performed. The H$_2$O$_2$ content (μmol g$^{-1}$ DW) in the leaves was determined by spectrophotometer [4].

The plasma membrane permeability was determined by the conductance method that was provided by the onductometer manual (Conductometer model DDS-11 A, Hongyi Analytic Equipment Corporation, Shanghai, China) [15].

2.4. Measurement of AsA and GSH contents

The AsA concentration (μmol g$^{-1}$ DW) was measured. Fe$_2$-BP complex consisted of 1 mL of sample extract, 1 mL of 5 % (w/v) trichloroacetic acid, 1 mL of ethanol, 0.5 mL of 0.4 % (v/v) H$_3$PO$_4$-ethanol, 1 mL of 0.5 % (w/v) bathophenanthroline (BP)-ethanol, and 0.5 mL of 0.03 % (w/v) FeCl$_3$-ethanol was prepared. After the color reaction, the absorbance of the colored solution was measured at 534 nm [16].

The GSH (μmol g$^{-1}$ DW) was quantified using LC-MS/MS (Agilent 1200 Series Rapid Resolution LC system, USA) coupled with a detector Agilent 6410B Triple quadrupole. Separation was performed using a C18 column (150×2.1 mm, 5μm, Acchrom, China) and mobile phase consisting of acetonitrile and water (5:95). The quantification of the compounds was based on appropriate MRM of ion pairs at *m/z* values 308/76 [17].

2.5. Determination of antioxidant enzymes and protein

3.0 g fresh leaves were homogenized in 2 mL 0.1 mol L$^{-1}$ phosphate buffer (the homogenized leaves were put in 2 mL 4 mmol L$^{-1}$ AsA when determining APX activity) (pH 7.8) containing 10 mmol L$^{-1}$ dithiothreitol, 2 mmol L$^{-1}$ EDTA, 1 mmol L$^{-1}$ PMSF, and 2% (w/w) insoluble polyvinyl-polypyrrolidone. Then centrifuged at 12,000g for 15 min and filtered the supernatant for the determination of the activity (U mg$^{-1}$ protein) of SOD, POD, CAT, GR and APX [18, 19]. One unit of SOD was defined as the quantity of enzyme caused a 50% inhibition in the reduction of nitrotetrazolium blue chloride as monitored at 560 nm. The reaction mixture to measure POD activity included 0.6 mL of guaiacol and 0.6 mL of 2% H$_2$O$_2$ in 1.74 mL of 100 mmol L$^{-1}$ phosphate buffer (pH 7.0) and the absorbance change caused by guaiacol was measured at 470 nm. One unit of CAT was determined as 1 μmol of H$_2$O$_2$ consumed in the absorbance at 240 nm per minute. One unit of GR was determined as a change of 1 μmol NADPH oxidation per minute, and the oxidation of NADPH depended on the change in absorbance at 340 nm. One unit of APX activity was considered as 1 μmol of ascorbate consumed per minute, and the change of absorbance measured at 290 nm indicated the ascorbate oxidation.

Protein in extracts was measured by Coomassie Brilliant Blue G-250 and albumin as dye and standard respectively. All procedures were performed under 4°C.

In addition, native polyacrylamide gel electrophoresis was done while the quantitative analysis of isoforms was performed with a Gel-Pro Analyzer (Media Cybernetics, USA). To separate SOD isoforms, 20 mg soluble proteins was applied to the gel and run at 4°C for 2 h, then stained. After performing non-denaturing electrophoresis at 4°C for 1.5 h, the gels were stained for analyzing POD isoforms with 0.1 % benzidine in acetic buffer (100 mmol L$^{-1}$, pH 4.5) and 0.4 mL of 3% H$_2$O$_2$, rinsed with ultrapure water, and then dried at room temperature [20]. Following the non-denaturing electrophoresis at 4°C for 3.5 h, the gel was stained for CAT isoforms analysis.

2.6. Statistical analysis

ANOVA with Duncan’s test (*P* < 0.05) was used to measure the significance of the data (means ± SD, *n* = 5). All tests were performed with SPSS Version 16.0 for Windows (SPSS, Chicago, IL, USA).

3. Results

3.1. Plant Growth and Cd Content in Shoots and Roots
Table 1 Effects of Cd stress on biomass of *T. halophila* plants

| Cd (mmol L\(^{-1}\)) | Fresh weight (mg per plant) | Dry weight (mg per plant) |
|----------------------|-----------------------------|---------------------------|
|                      | Shoots                      | Roots                     | Shoots | Roots |
| 0                    | 66.8 ± 4.8a                 | 14.5 ± 0.7a               | 5.63 ± 0.40a | 1.1 ± 0.2a |
| 0.3                  | 44.9 ± 3.9b                 | 11.7 ± 0.7b               | 3.9 ± 0.37b | 0.94 ± 0.11a |
| 0.6                  | 42.6 ± 5.7b                 | 9.9 ± 1.3c                | 3.2 ± 0.4bc | 0.7 ± 0.09b |
| 0.9                  | 34.9 ± 1c                   | 6.3 ± 0.2d                | 3.0 ± 0.22c | 0.67 ± 0.05b |

Different letters in same column show significant differences (means ± SD, *n* =5, Duncan’s test, *P* < 0.05)

Plant growth of *T. halophila* was significantly decreased with the increase of Cd concentrations (Table 1). Compared to the no-Cd control, biomass production of fresh roots and shoots was decreased by 43 to 50%, under 0.9 mmol L\(^{-1}\) Cd (Table 1). Accordingly, a decrease in root length was consistent with the decrease of root biomass (Fig. 1A).

Cd content in the shoots and roots of *T. halophila* seedlings both increased with increase of external Cd (Fig. 1B), and was generally obviously higher in roots than in shoots. The TF values were all less than 1 under both control and Cd treatments especially under 0.6 mmol L\(^{-1}\) Cd treatments (Fig. 1C).

Fig 1. Effects of Cd treatment on root length, TF value, Cd accumulation and photosynthetic oxygen evolution rate of *T. halophila* after being treated with 0, 0.3, 0.6, and 0.9 mmol L\(^{-1}\) Cd for 14 days. (A) Root length. (B) TF value. (C) Cd accumulation in the shoots and roots. (D) The photosynthetic oxygen evolution rate. Different letters show significant differences (means ± SD, *n* =5, Duncan’s test, *P* < 0.05)

3.2. Photosynthesis and Pigment Contents

Table 2 Leaf chlorophyll concentrations of *T. halophila* seedlings after 14-day 0, 0.3, 0.6 or 0.9 mmol L\(^{-1}\) Cd treatment

| Cd (mmol L\(^{-1}\)) | Chla (mg kg\(^{-1}\) FW) | Chlb (mg kg\(^{-1}\) FW) | Chla+b (mg kg\(^{-1}\) FW) | Carotenoids (mg kg\(^{-1}\) FW) |
|----------------------|--------------------------|--------------------------|-----------------------------|---------------------------------|
| 0                    | 7.94 ± 0.12a             | 2.61 ± 0.83a             | 10.55 ± 1.19a               | 2.97 ± 0.36a                    |
0.3 mmol L\(^{-1}\) Cd did not obviously affect the photosynthetic oxygen evolution rate compared with the control. However, after treating with 0.6 and 0.9 mmol L\(^{-1}\) Cd, the photosynthetic oxygen evolution rate dropped to 70.3 % and 47.1 % of the no-Cd control, respectively (Fig. 1D).

Chla content was significantly reduced by external Cd and decreased with increasing Cd concentration. Leaf Chlb and carotenoid contents were slightly reduced with increasing Cd concentration. In addition, Chla+b content was reduced by external Cd but little change was found under different Cd concentration (Table 2).

### 3.3. Leaf H\(_2\)O\(_2\) Accumulation, Membrane Lipid Peroxidation, and Permeability

Greater intensity of brown spots on *T. halophila* leaves was visualized under 0.9 > 0.6 > 0.3 ≥ 0 mmol L\(^{-1}\) Cd treatment (Fig. 2A). Significantly greater leaf H\(_2\)O\(_2\) concentrations among Cd treatments patterned as under 0.9 > 0.6 > 0.3 ≈ 0 mmol L\(^{-1}\) Cd treatment (Fig. 2B). Significantly greater leaf MDA concentrations or percentage of the plasma membrane permeability among Cd treatments ranked as 0.9 > 0.6 > 0.3 ≈ 0 mmol L\(^{-1}\) Cd treatment (Fig. 2C) or as under 0.9 > 0.6 > 0.3 > 0 mmol L\(^{-1}\) Cd treatment (Fig. 2D).

![Fig 2. Effect of Cd treatment on leaf H\(_2\)O\(_2\) concentration, MDA and plasma membrane permeability of *T. halophila* after being treated with 0, 0.3, 0.6, and 0.9 mmol L\(^{-1}\) Cd for 14 days. (A) Diaminobenzidine-stained leaves photographed with a light microscope at 10x magnification. (B) H\(_2\)O\(_2\) quantity. (C) MDA content. (D) plasma membrane permeability. Different letters show significant differences (means ± SD, \(n=5\), Duncan’s test, \(P<0.05\))](image)

### Table 3 Activities of ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD) in leaf of *T. halophila* seedlings treated with 0, 0.3, 0.6, and 0.9 mmol L\(^{-1}\) Cd for 14 days

| Cd (mmol L\(^{-1}\)) | APX (U mg\(^{-1}\) protein) | CAT (U mg\(^{-1}\) protein) | GR (U mg\(^{-1}\) protein) | POD (U mg\(^{-1}\) protein) | SOD (U mg\(^{-1}\) protein) |
|----------------------|----------------------------|-----------------------------|---------------------------|--------------------------|-----------------------------|
| 0                    | 6.2 ± 0.6 a                 | 17.7 ± 0.3b                 | 10.4 ± 0.9a               | 143.4 ± 18.9d            | 208.0 ± 7.1a                |
| 0.3                  |                            |                             |                          |                          |                             |
| 0.6                  |                            |                             |                          |                          |                             |
| 0.9                  |                            |                             |                          |                          |                             |
### 3.4. Non-enzymatic and Enzymatic Antioxidants

Significantly greater leaf AsA or GSH concentrations among Cd treatments ranked as under 0.9 > 0.6 > 0.3 > 0 mmol L\(^{-1}\) Cd treatment (Fig. 3A) or as under 0.9 > 0.6 > 0.3 > 0 mmol L\(^{-1}\) Cd treatment (Fig. 3B).

The protective enzymes including POD, SOD, CAT, APX, and GR respond differently in *T. halophila* plants to Cd stress (Table 3). The enzyme activity of either leaf GR or SOD was not affected by a 2-week period Cd treatment, but a greater CAT activity was observed under 0.9 mmol L\(^{-1}\) Cd treatment (Table 3). In contrast, significantly greater leaf POD activity among Cd treatments ranked as 0.9 > 0.6 > 0.3 > 0 mmol L\(^{-1}\) Cd treatment (Table 3).

The different isozymes of different antioxidant enzymes displayed variously (Fig. 4). For instance, with the increase of external Cd addition, five POD isozymes were displayed with increased protein bands or contents (Fig. 4A). Four leaf SOD isozymes, Fe-SOD, Cu/Zn-SOD1, Cu/Zn-SOD2 and Cu/Zn-SOD3 appeared under Cd treatments (Fig. 4B). Interestingly, Fe-SOD was detected only under 0.9 mmol L\(^{-1}\) Cd treated plants, suggesting an inducible property under higher Cd concentrations. The protein band of Cu/Zn-SOD1 increased with increasing Cd concentrations, and was 3.4 times higher under 0.9 mmol L\(^{-1}\) Cd than under no-Cd control. By contrast, the relative activities of the protein band of either Cu/Zn-SOD2 or Cu/Zn-SOD3 decreased with the increase of Cd concentrations, and was 80% or 70% under 0.9 mmol L\(^{-1}\) Cd treatment, compared to the no-Cd control (Fig. 4B). Only two catalase isozymes were observed (CAT1 and CAT2). CAT2 increased with increasing Cd concentrations, and its protein band was 1.1, 1.2, and 2.5 times greater under 0.3, 0.6 and 0.9 mmol L\(^{-1}\) Cd than under no-Cd treatment. By contrast, CAT1 declined to nearly 70% of the no-Cd control value at 0.9 mmol L\(^{-1}\) Cd treatment (Fig. 4C).

### 4. Discussion

In this study, the growth and biomass of the halophyte *T. halophila* decreased under Cd stress, and Cd concentrations was generally higher in the roots than in the shoots. Root growth under all treatments was also markedly inhibited. It was reported that in most plant species Cd is primarily accumulated in...
roots and high Cd concentration in roots might be a way to deal with metal stress as previously studied [21]. When treated with Cd, the biomass production of wheat was inhibited [22]. Photosystem was very sensitive to heavy metal [14]. And this was also visible in *T. halophila* which reflected on the reduction of Chl and carotenoid contents under Cd treatment.

Researches have indicated that Cd hyperaccumulators can tolerate and accumulate more than 100 mg kg\(^{-1}\) Cd in shoot dry biomass and TF of the Cd hyperaccumulators should be greater than 1 [23]. For example, the typical Cd hyperaccumulator *Noccea caerulescens* contain as much as 1000 to 4000 mg kg\(^{-1}\) Cd in shoot dry biomass [24]. However, the TF values of *T. halophila* under Cd treatment were always less than 1. But the Cd content in shoot dry weight of *T. halophila* reached more than 100 mg kg\(^{-1}\). These indicate that though *T. halophila* are not Cd hyperaccumulators, they have certain ability of accumulating Cd in soil, and they accumulate Cd in the root to prevent excessive Cd accumulation in shoot.

It was found that Cd toxicity is partly related to oxidative stress, which is caused by ROS, especially H\(_2\)O\(_2\) accumulation [25]. MDA has been widely used as a lipid peroxidation indicator, and it therefore indicates oxidative damage in heavy metal-exposed plants. Lipid peroxidation caused by ROS produced under metal stress led to membrane destabilization. Our study showed that H\(_2\)O\(_2\) concentrations gradually increased in parallel with the increasing Cd concentrations, and MDA concentrations and the plasma membrane permeability also showed a significant increase under 0.6 and 0.9 mmol L\(^{-1}\) Cd. Therefore, H\(_2\)O\(_2\) accumulation induced by Cd in *T. halophila* might be one of the extremely important factors leading to lipid peroxidation and increased plasma membrane permeability. And the higher the Cd concentration, the stronger the oxidative damage to seedlings.

AsA and GSH concentrations in leaf of *T. halophila* seedlings treated with Cd increased with increasing Cd concentrations, which could partly help to suppress oxidative damage. When exposed to Cd, the activity of SOD and CAT of pea plants was decreased, and the activity of POD was slightly changed [26]. It was observed that Cd obviously decreased the activity of SOD and GR, and caused an increment in APX of *Arabidopsis thaliana* leaves [27]. However, in our study, the activity of APX, GR and SOD had no significant changes in *T. halophila* leaves treated with Cd, but the activity of POD was markedly elevated. A Cd-dependent enhancement in POD was also observed in *Brassica juncea* roots.
These results suggested that POD may serve as an important defense tool to resist Cd-induced increase of H$_2$O$_2$ in $T.$ halophila.

Isoforms of antioxidant enzymes respond differently to Cd stress in different plants. All SOD isoforms decreased with increasing of Cd concentration in pea plants [29], but POD isoforms increased in alfalfa roots and leaves [30]. Surprisingly, the four SOD isoforms in $T.$ halophila leaves responded differently to Cd treatments. The relative expression of Cu/Zn-SOD1 was increased, but the Cu/Zn-SOD2 and Cu/Zn-SOD3 declined. Particularly, leaf Fe-SOD was only visualized under 0.9 mmol L$^{-1}$ Cd. Similarly, the isozyme CAT2 increased, while the CAT1 decreased with increasing Cd concentrations. But the expression of all five POD isoforms increased with increasing Cd, the isozyme 5 in particular. It appears that with increase of Cd concentrations, $T.$ halophila leaves scavenged excess ROS via increasing of five POD isoforms, Cu/Zn-SOD1, CAT2 activity and inducing of Fe-SOD.

5. Conclusion

Cd tolerance in $T.$ halophila is related to a high ability to cope with oxidative stress via cooperative enhancement of GSH, AsA and antioxidant enzymes, particularly, increasing of POD expression. The ability of $T.$ halophila to accumulate a certain amount of Cd and tolerate metal and salt stress makes this species a candidate to remediate Cd-contaminated saline soils.

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