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Cadmium-induced physiological response and antioxidant enzyme changes in the novel cadmium accumulator, *Tagetes patula*

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ABSTRACT

The accumulation and effect of cadmium (Cd) on the growth and enzymatic activities changes of antioxidants in *Tagetes patula*, French marigold, were investigated to reveal the physiological mechanisms corresponding to its Cd tolerance and accumulation. Hydroponically grown *T. patula* plants were treated with different concentrations of Cd (0, 10, 25, 50 μM $CdCl_2$) at various regime of times. *T. patula* accumulated Cd to a maximum of 450 mg Cd kg^{-1} dry weight (DW) in shoot and 3500 mg Cd kg^{-1} DW in root after 14 days' exposure at 10 and 50 μM $CdCl_2$, respectively. The translocation factors of Cd were greater than 1 in plants exposed to 10 μM $CdCl_2$. Toxic effects were gradually observed with increasing Cd concentration (25 and 50 μM) accompanied with the reduction of biomass, chlorophyll content, decrease of cell viability and the increase level of lipid peroxidation. In leaves of *T. patula*, the activities of ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) were induced by Cd. However, in roots, activities of APX, GR, SOD and catalase (CAT) were significantly reduced by 25 and 50 μM Cd treatment but not 10 μM Cd. In-gel zymography analysis revealed that Cd induced the enzymatic activities of APX, MnSOD, CuZnSOD and different isozymes of GR in leaves. These results indicate that *T. patula* is a novel Cd accumulator and able to tolerate with Cd-induced toxicity by activation of its antioxidative defense system.

1. Introduction

Cadmium (Cd) is a non-essential trace element and is highly toxic to humans, animals and plants. Cadmium contamination of agricultural soils via anthropogenic emissions, phosphate fertilizer, and sewage sludge is a big concern for environmental issue [1]. Excessive amount of Cd to sensitive plants has long been known to reduce growth, damage of the photosynthetic apparatus [2,3], reduce chlorophyll content [4], and inhibit stomatal opening [5].

Cd is known to stimulate the formation of free radicals and disrupt the plant defense system against reactive oxygen species (ROS). To ameliorate the effect of Cd-induced oxidative stress, plants have developed a ROS scavenging system to remove free radicals and to retain the cellular steady-state level of ROS. This system consists of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and several non-enzymatic antioxidants, including ascorbic acid, glutathione (GSH), tocopherol, and carotenoids [6,7]. SOD catalyzes the dismutation of 2 molecules of superoxide into O_2 and H_2O_2. H_2O_2 can be converted to H_2O and O_2 by the action of CAT and APX. GR catalyzes the conversion of oxidized glutathione to a reduced form (GSH) to control endogenous H_2O_2 through an oxido-reduction cycle involving GSH and ascorbate [8].

In plants, the metal-induced antioxidative defense system is activated to buffer oxidative stress, but the response of antioxidative enzymes to metal stress varies among species [9]. In *Crotalaria juncea* leaves, Cd increases the activity of CAT and GR [10]. While in *Thlaspi caerulescens*, the CAT activity was enhanced [11]. In *Arabis paniculata* F. leaves, the enzymatic activities of SOD, CAT, guaiacol peroxidase, and APX were induced by Cd [12]. In *Sedum alfredii* Hance, the accumulation of ascorbic acid and GSH was increased to enhance its tolerance to Cd [13]. In *Spartina densiflora*, Cd-induced oxidative stress modulated the capacity of almost all antioxidative enzymes; however, Cd-induced ROS accumulation and oxidative damage was alleviated only under the lowest Cd concentration (10 μM), not the highest (100 μM) [14].

Recently, the *Tagetes* genus was reported to have the potential for the application of removing Cd by phytoextraction. This annual cut-flower *T. erecta* can accumulate 12.8 mg Cd kg^{-1} DW from Cd-contaminated soil [15]. The garden flower *T. patula*, was found to be another potential Cd accumulator when grown either in a Cd-contaminated field or artificially Cd-contaminated soils [16,17]. *T. patula* is one of the easiest annual flowers to cultivate and has been widely adapted to different soils and climate con-

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ditions [18]. In addition, *T. patula* is a beneficial plant for crop production. It secretes the chemical alpha-terthienyl from root tissue [19], which is an effective nematicide for root-knot nematodes and lesion nematodes control. Because of its allelopathic effect, *T. patula* was used as a companion plant or cover crop in cropping systems to protect crops against nematodes [20].

Although *T. patula* was recognized as an accumulator for its capability of Cd remediation, the biochemical and physiological responses to elevated levels of Cd in *T. patula* are largely unknown. Thus, in this study, we aimed to explore the effects of Cd on the growth, physiological performance, and changes in antioxidative defense systems of *T. patula* under hydroponic cultivation.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *T. patula* were sterilized with 2% sodium hypochlorite for 30 min and washed extensively with distilled water, then germinated in Petri dishes on moist filter papers at 25 °C in the dark. After 48 h incubation, uniformly germinated seeds were grown in a 0.2 L pot containing a mixture of vermiculite and organic matter (1:1). Two weeks later, seedlings were moved to a 5 L hydroponic tank containing half-strength Kimura B solution [21] for 3 weeks. The seedlings were grown in a Phytotron with natural light at 30 °C day/25 °C night and 90% relative humidity. The 35-day-old plants were used for Cd treatment in all experiments. Different concentrations of $CdCl_2$ were added directly to the culture solution during the experiment.

2.2. Biomass analysis

At the end of treatment, seedlings were divided into shoot and root. The fresh weight (FW) of the shoot and root was then measured. For DW estimation, the shoots and roots were dried at 65 °C for 48 h.

2.3. Cd determination

Root tissues were washed for 15 min in 5 mM $CaCl_2$ for exchange of apoplastic Cd at the end of treatment [22]. Root and shoot were dried at 65 °C for 48 h. Dry material was ashed at 550 °C for 20 h. The ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72 °C for 2 h, then dissolved in distilled water. Cd was subsequently quantified by use of an atomic absorption spectrophotomer (Model AA-6800, Shimadzu, Kyoto, Japan). The amount of Cd was expressed on a DW basis.

2.4. Determination of chlorophyll, protein, and malondialdehyde (MDA)

The chlorophyll content was determined as described [23] after extraction in 96% (v/v) ethanol. MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid, and content was determined as described [24]. For protein determination, leaves were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 × *g* for 20 min, and the supernatants were used for determination as described [25].

2.5. Analysis of cell death

Cell death was quantified by staining with Evans blue (Sigma, USA) [26]. Roots were harvested from 10 randomly selected seedlings. The roots were stained in 0.25% aqueous Evans blue solution for 15 min at room temperature and then washed twice

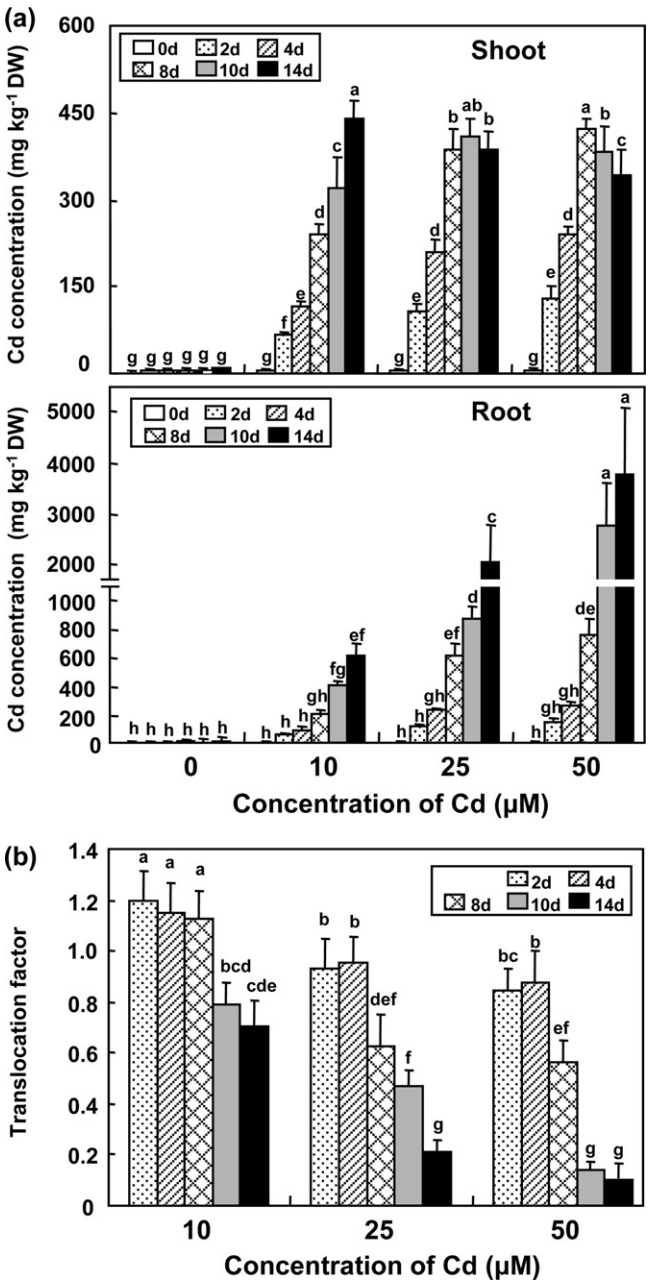

Fig. 1. Accumulation of Cd by *Tagetes patula* exposed to different concentrations of $CdCl_2$. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$. Cd concentrations in root and shoot were determined after plants grew for 2, 4, 8, 10, and 14 days. (a) Cd concentrations in shoot (upper panel) and root (lower panel). (b) Translocation factors (ratio of shoot to root concentration) of *T. patula* exposed to different concentrations of $CdCl_2$ for different times. Data are mean ± SD. ($n = 4$). Values with the same letter are not significantly different at $p < 0.05$.

for 15 min in distilled water to remove any excess stain. Finally, the roots were left in the distilled water overnight. For quantitative assessment, the extreme 5 mm of root tips were excised from 10 roots, followed by extraction of dye in a solution of 50% methanol/1% SDS for 1 h at 50 °C and subsequent quantification by monitoring the A_{595}.

2.6. Measurement of antioxidative enzyme activities

For extraction of enzymes, leaf tissue was homogenized with 0.1 M sodium phosphate buffer (pH 6.8) by use of a chilled pestle and mortar. CAT activity was assayed according to Kato and Shimizu

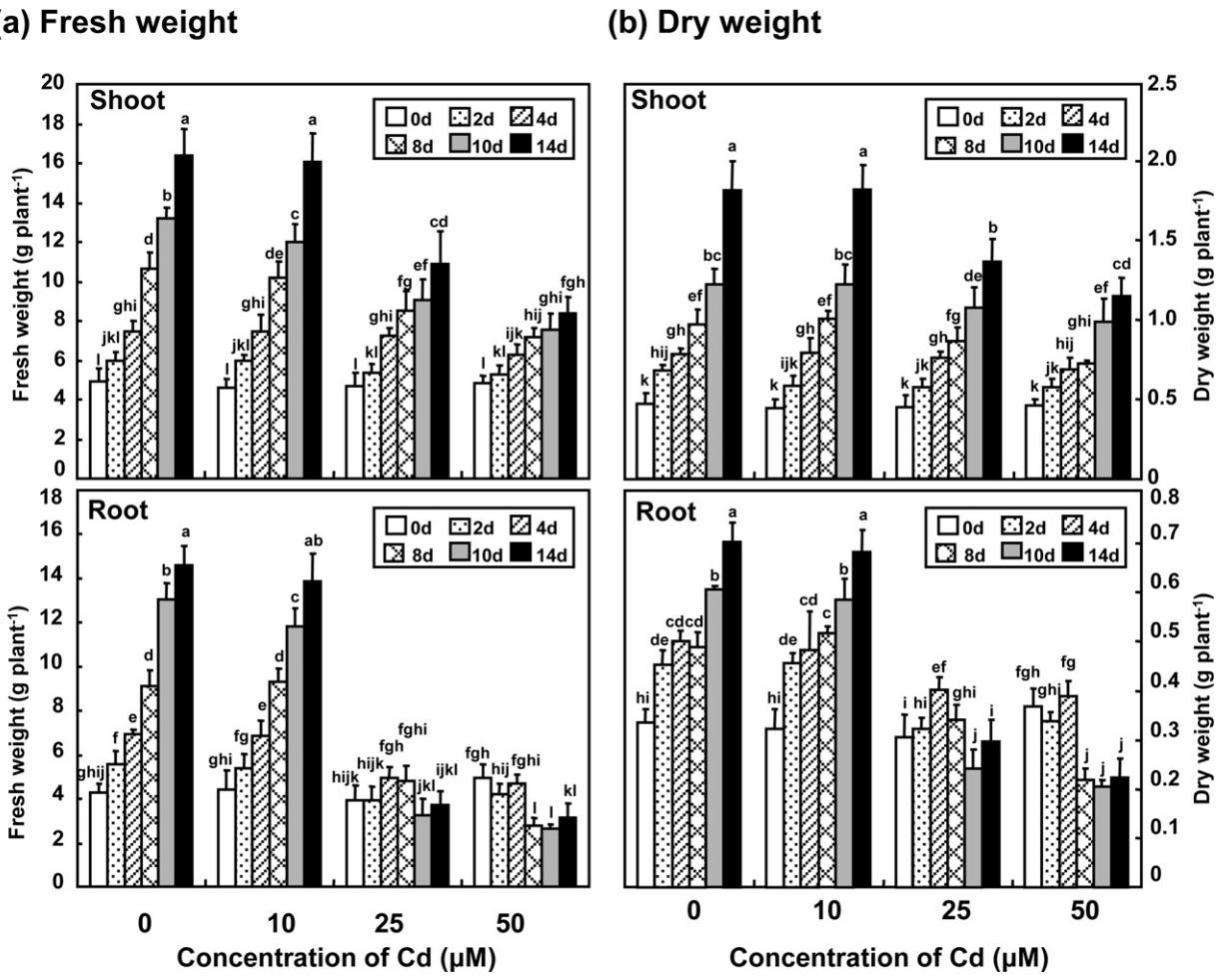

Fig. 2. Effect of Cd on root and shoot biomass of *T. patula*. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$. Fresh and dry weight were determined after plants grew for 2, 4, 8, 10, and 14 days. (a) Fresh weight. (b) Dry weight. Data are mean ± SD. ($n = 4$). Values with the same letter are not significantly different at $p < 0.05$.

[27]. One unit of CAT enzymatic activity was defined as the amount of enzyme that degraded 1 μmol H_2O_2 per minute. For analysis of APX activity, 2 mM ascorbate was added to the extraction buffer. The homogenate was centrifuged at 12,000 × *g* for 20 min, and the resulting supernatant was used for determining enzyme activity and protein content. The entire extraction procedure was carried out at 4 °C. APX activity was determined according to Nakano and Asada [28]. One unit of activity for APX was defined as the amount of enzyme that degraded 1 μmol of ascorbate per minute. SOD activity was determined by photochemical inhibition of nitro blue tetrazolium (NBT) as described [29]. One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NBT reduction observed in a blank sample. The SOD isoforms were identified by adding 3 mM KCN to inhibit CuZnSOD activity (i.e., to detect MnSOD and FeSOD) and 3 mM KCN and 5 mM H_2O_2 to inhibit CuZnSOD and FeSOD activities (i.e., to detect MnSOD). The CuZnSOD activity was subtracted from total SOD activity with MnSOD and FeSOD activity. The FeSOD activity was subtracted from the activity of both MnSOD and FeSOD with MnSOD activity. GR was determined as described [30]. One unit of GR was defined as the amount of enzyme that decreased 1 A_{340} per minute.

2.7. Gel activity analysis

Leaves (0.1 g) were homogenized in 50 mM potassium phosphate (pH 7). The homogenate was centrifuged for 15 min at 13,000 × *g* at 4 °C. Samples containing 50 μg protein with the addition of bromophenol blue and glycerol were subjected to non-denaturing PAGE, with SDS omitted. For the analysis of APX activity, 2 mM ascorbate was added to the extraction buffer, the carrier buffer, and the gel was pre-run for 10 min before the samples were loaded [31]. The gel, equilibrated with 50 mM sodium phosphate (pH 7) containing 2 mM ascorbate for 30 min, was incubated in a solution of 50 mM sodium phosphate (pH 7), 2 mM ascorbate and 2.3 mM H_2O_2 for 20 min. The gel was washed in the buffer with gentle agitation for 1 min and submerged into a solution of 50 mM sodium phosphate buffer (pH 7) containing 28 mM TEMED and 2.45 mM NBT. CAT activity was detected by incubating the gels in 3.27 mM H_2O_2 for 5 min, rinsing in water, and staining with a solution of 1% potassium ferricyanide and 1% ferricchloride for 5 min [32]. GR activity was detected by incubating the gels in a reaction medium containing 0.25 M Tris–HCl buffer (pH 7.5), 2 mM 5′,5′-dithiobis (2-nitrobenzoic) acid, 4 mM glutathione disulfide and 1.5 mM NADPH [33]. SOD isoenzymes were separated by isoelectric focusing on a 10% non-denaturing polyacrylamide gel containing 2% ampholine (pH 3–10, Amersham, NJ) as described [34]. Aliquots of enzyme extract (50 μg protein) were loaded in the gel. Loading buffers were 0.01 M phosphoric acid for the anode and 0.02 M NaOH for the cathode. Gels were pre-run for 20 min at 60 V before sample loading and then run overnight at 200 V. After electrophoresis, the gel was soaked in a solution containing 2.45 mM NBT for 15 min, then incubated in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM riboflavin and 28 mM TEMED under dark conditions for 15 min. The gel was then exposed

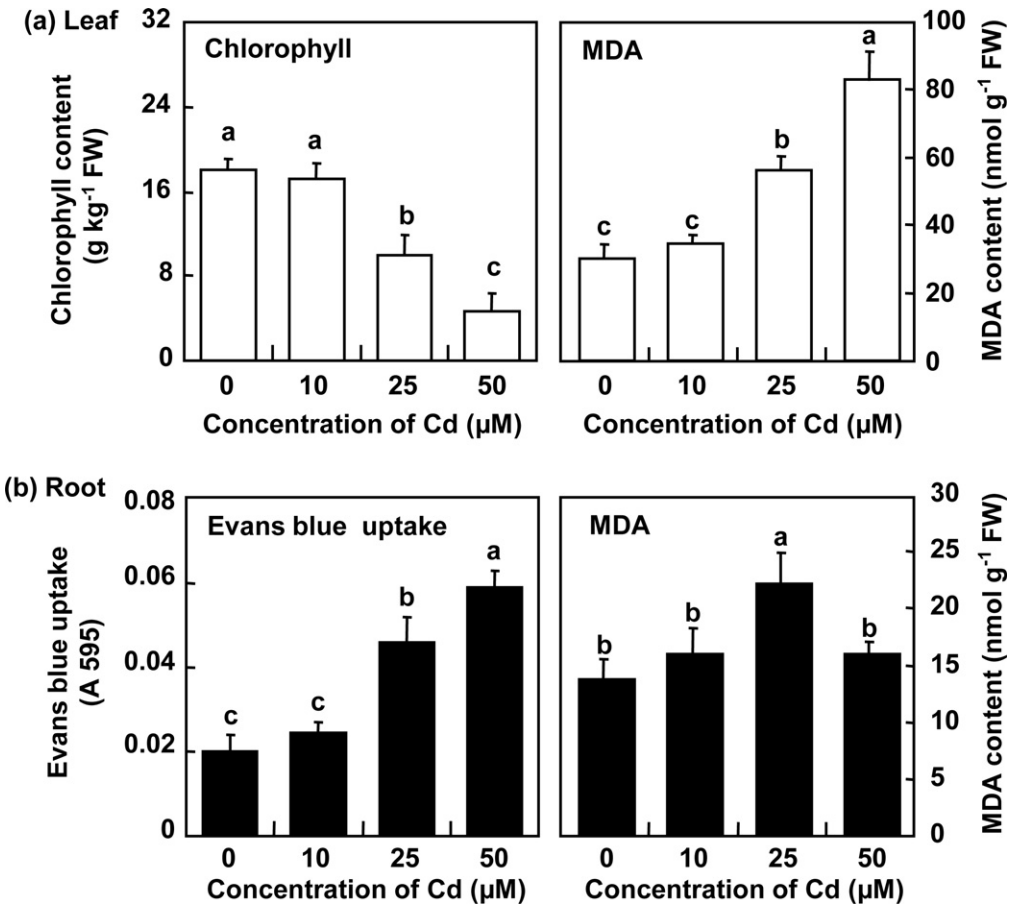

Fig. 3. Effect of Cd on chlorophyll content, malondialdehyde (MDA) content, and viability of roots in *T. patula*. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$. MDA content was determined after plants grew for 2, 4, 8, 10, and 14 days. (a) Left: chlorophyll content in leaves. Right: MDA content in leaves. (b) Left: viability of roots. Right: MDA content in roots. Data are mean ± SD. ($n = 4$). Values with the same letter are not significantly different at $p < 0.05$.

to light for 15 min. For identification of individual SOD isoenzymes, the gel was treated with 8 mM KCN or H_2O_2 in 50 mM potassium phosphate buffer (pH 7) for 30 min before staining for SOD activity.

2.8. Statistical analysis

Data are expressed as mean ± SE. Statistical differences between measurements ($n = 4$) for different treatments or different times were analyzed by Duncan's multiple range test. A $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Cd accumulation

To study the uptake of Cd in *T. patula*, Cd concentration was examined over time in roots and shoots. In the shoots of *T. patula*, Cd concentrations accumulated up to 450, 390, 345 mg Cd kg^{-1} DW under the treatment of 10, 25, 50 μM $CdCl_2$ for 14 days, respectively (Fig. 1a). With 10 μM $CdCl_2$, maximal Cd uptake was observed at day 14, while with 25 or 50 μM $CdCl_2$, maximal Cd uptake was observed at day 8 and then slightly declined. No Cd was detected in control plants. In roots, 10 μM $CdCl_2$ treatment for 14 days gradually led to the increase of Cd concentration till 600 mg Cd kg^{-1} DW. Meanwhile, with 25 or 50 μM $CdCl_2$, Cd concentrations were continuously elevated in root tissue. At day 8 of exposure with 25 or 50 μM $CdCl_2$, Cd concentration greatly increased to 620 or 770 mg Cd kg^{-1} DW in root tissue, respectively. After 14 days' treatment, Cd concentration in roots treated with 25 or 50 μM $CdCl_2$ was 1900 and 3500 mg Cd kg^{-1} DW, respectively. No Cd was detected in control plants (Fig. 1a). With 10 μM Cd, translocation factors (TFs; ratio of root to shoot Cd concentration) were >1 at days 2, 4, and 8 and greater than with 25 and 50 μM Cd: 0.2–0.9 and 0.1–0.8, respectively (Fig. 1b).

3.2. Effect of Cd on biomass

To examine the effect of Cd on plant growth, 35-day-old *T. patula* was exposed to different concentrations of $CdCl_2$ for 2 weeks and the gain or loss of biomass was recorded. Fig. 2 shows the effect of $CdCl_2$ on FW and DW of roots and shoots over time. Treatment with both 25 and 50 μM $CdCl_2$ but not 10 μM $CdCl_2$ significantly decreased root and shoot FW and DW as compared with control plants (Fig. 2a and b).

Although Cd hyperaccumulators have Cd phytoextraction capability, most show low biomass and slow growth rate, which limits their application to phytoextraction [35]. Shoot biomass of *S. alfredii* Hance [13], *A. paniculata* F. [36], and *T. caerulescens* [11] is 669, 100, and <100 mg $plant^{-1}$ DW, respectively. In contrast, shoot biomass of *T. patula* can reach to 1800 mg $plant^{-1}$ DW (Fig. 2). Thus, *T. patula* shows promised potential as a Cd-accumulator for phytoremediation in Cd-contaminated sites.

3.3. Effect of Cd on chlorophyll content, MDA content, and root viability

One of the most apparent symptoms of Cd toxicity in plants is chlorosis of leaves. After 14 days, chlorophyll content in *T. patula*

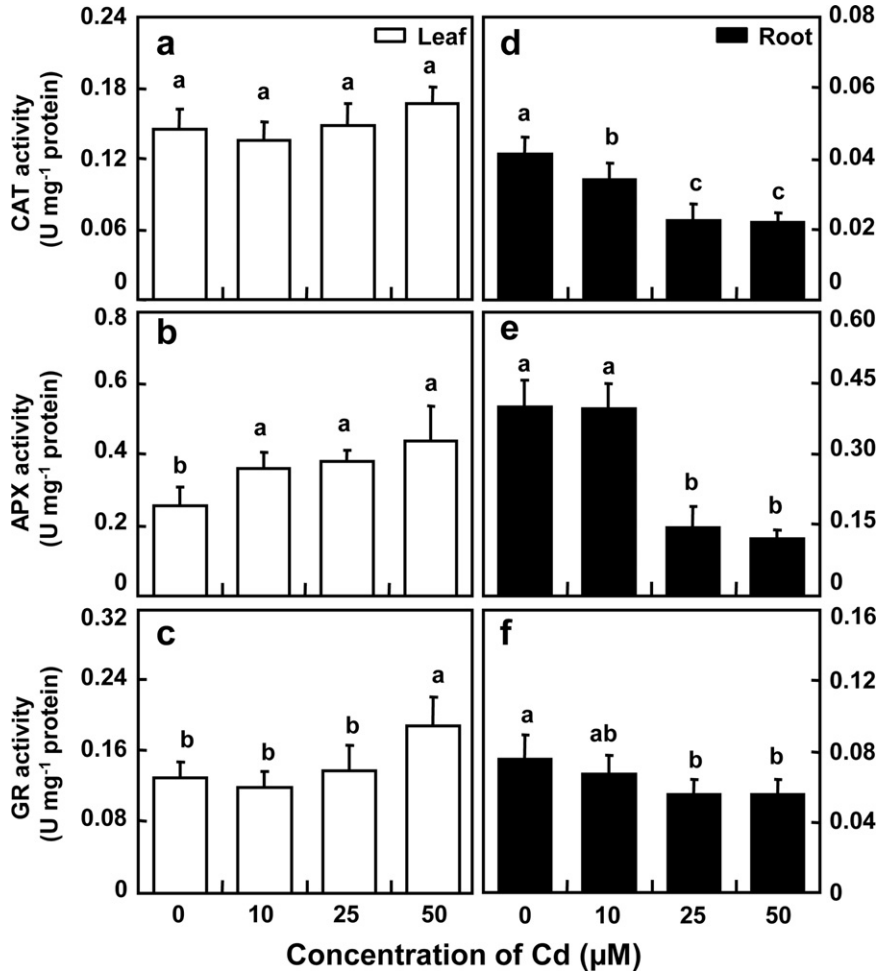

Fig. 4. Effect of Cd on catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activity in leaves and roots of *T. patula*. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$ for 14 days. (a, d) CAT activity in leaves and roots; (b, e) APX activity in leaves and roots; (c, f) GR activity in leaves and roots. Data are mean ± SD. ($n = 4$). Values with the same letter are not significantly different at $p < 0.05$.

leaves decreased by 45% with 25 μM $CdCl_2$ and by 74% with 50 μM $CdCl_2$ as compared with control leaves (Fig. 3a). In contrast, 10 μM $CdCl_2$-treated leaves and control leaves did not differ in chlorosis. Moreover, we determined the levels of lipid peroxidation in *T. patula* as the presence of MDA content. MDA content was significantly increased in leaves with 25 and 50 μM $CdCl_2$ treatment for 14 days but did not differ from control leaves with 10 μM $CdCl_2$ (Fig. 3a).

We further examined the viability of *T. patula* root cells by Evans blue staining assay: instead of 10 μM $CdCl_2$ treatment, 25 and 50 μM $CdCl_2$ obviously reduced root cell viability as compared with control plants (Fig. 3b). Under low amount of Cd (10 μM), the content of MDA did not change too much both in control or treated roots (Fig. 3b). In contrast, MDA content in the root was greatly increased with 25 μM $CdCl_2$ treatment for 14 days. Unexpectedly, MDA content did not increase when *T. patula* roots were treated with 50 μM $CdCl_2$. We can conclude that less than 10 μM of $CdCl_2$ do not cause lipid peroxidation in *T. patula* but high concentrations of Cd (i.e., 25 and 50 μM $CdCl_2$) can result in root cell death. We highly speculate that because high concentration of Cd treatment (50 μM $CdCl_2$) leads to the death of *T. patula* root cells, the MDA content was thus not increased (Fig. 3b). In addition, *T. patula* treated with 50 μM $CdCl_2$ for 14 days resulted in an accumulation of 3500 mg Cd kg^{-1} DW in roots. The super-accumulation of Cd in roots may have 2 explanations. The first is the death of root cells. Fig. 3b shows that Evans blue was highly absorbed by root tissue, which indicates that root viability was markedly reduced by 50 μM $CdCl_2$. The death of root tissue abolished the root-to-shoot translocation of Cd in *T. patula*. Therefore, the Cd translocation factor of plants treated with 50 μM $CdCl_2$ was greatly reduced (Fig. 1b), thus causing high accumulation of Cd in root tissue. The second explanation is that root has small dry weight as compared with shoot tissue. The determination of Cd concentration was based on dry weight. Therefore, a high accumulation of Cd in root tissue may result in super-accumulation of Cd.

3.4. Effect of $CdCl_2$ on antioxidative enzyme activities

$CdCl_2$ treatment did not affect CAT activity in leaves (Fig. 4a) but significantly decreased that in roots (Fig. 4d). APX activity increased with $CdCl_2$ treatment in leaves (Fig. 4b) but decreased to 36% and 30% with 25 and 50 μM $CdCl_2$ treatment, respectively, in roots (Fig. 4e). GR activity was affected by $CdCl_2$ only with 50 μM $CdCl_2$ treatment in leaves (Fig. 4c) and was slightly reduced by $CdCl_2$ in roots (Fig. 4f). Activity of total SOD progressively increased with increasing $CdCl_2$ concentration in leaves (Fig. 5a) but was significantly decreased in roots (Fig. 5e). APX, GR and SOD activity was significantly increased with high concentrations (25 and 50 μM $CdCl_2$) of Cd in leaves, with the reverse results in roots (Fig. 4). These findings are in agreement with earlier reports [10,12]. The response of antioxidative enzymes to Cd stress seems to vary among plant species and tissues.

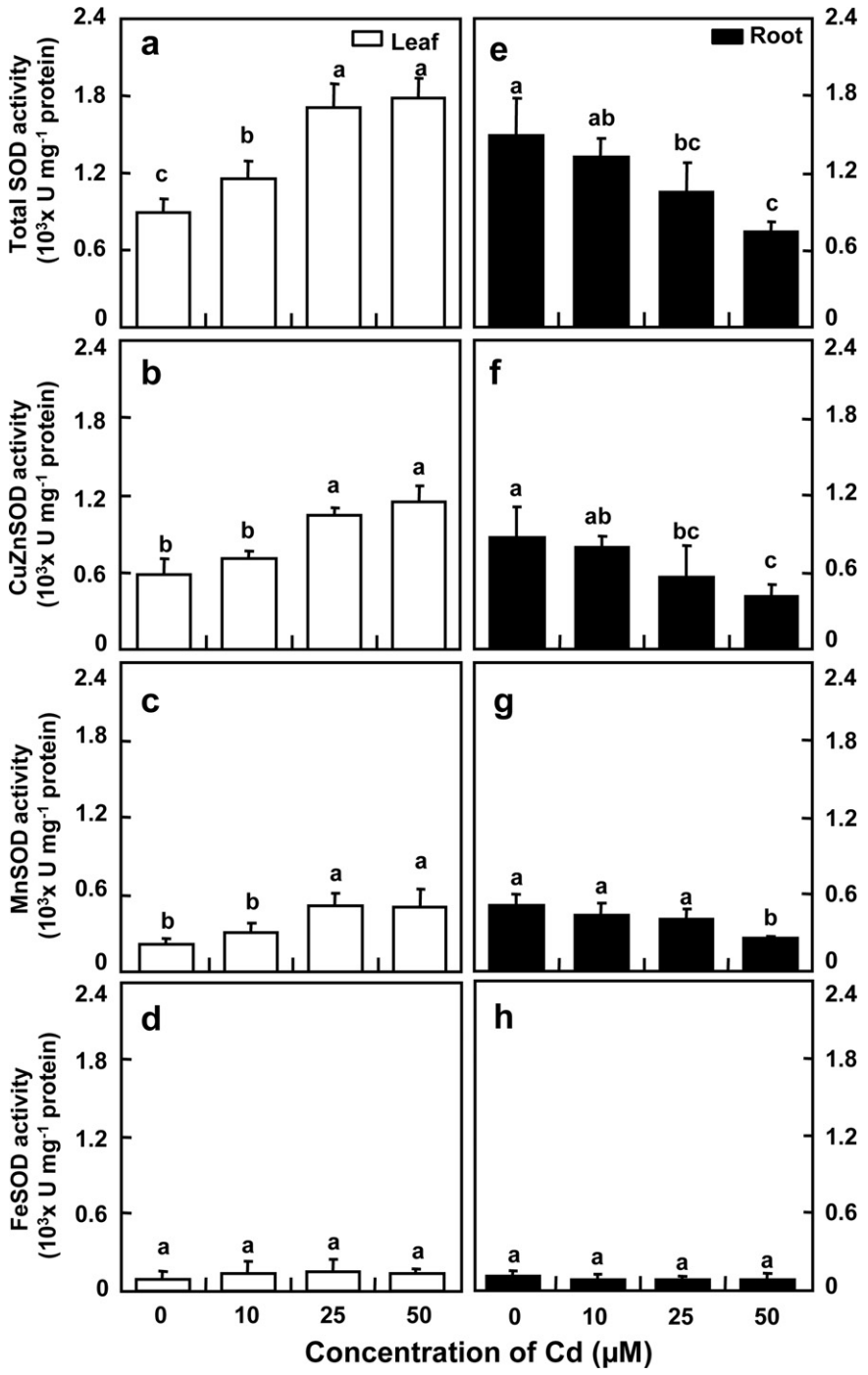

Fig. 5. Effect of Cd on superoxide dismutase (SOD) activity in leaves and roots of *T. patula*. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$ for 14 days. (a, e) Total SOD activity in leaves and roots; (b, f) CuZnSOD activity in leaves and roots; (c, g) MnSOD activity in leaves and roots; (d, h) FeSOD activity in leaves and roots. Data are mean ± SD. ($n = 4$). Values with the same letter are not significantly different at $p < 0.05$.

The activity of the CuZnSOD and MnSOD isoforms in leaves significantly increased with $CdCl_2$ treatment as compared with control leaves (Fig. 5b and c). In contrast, CuZnSOD and MnSOD activity decreased in root tissue (Fig. 5f and g). The activity of FeSOD did not change in leaves or roots after 14 days' Cd treatment (Fig. 5d and h). Our results suggest that the bioaccumulation of Cd in *T. patula* is often accompanied by cellular antioxidants enzymatic activities changes to protect itself against Cd-induced toxicity.

Recent studies have revealed that the capability of antioxidative defense system can be modulated by Cd in many plants [10–13]. In *C. juncea*, Cd induces the activity of CAT and GR in leaves though previous study showed no significant changes in SOD activity in roots and shoots under Cd treatment [10]. In *T. caerulescens* hairy roots, Cd increased the activity of CAT and APX [11]. Furthermore, the characterization of different types of SOD isozymes from our study by in-gel zymography analysis revealed that mainly CuZnSOD and MnSOD but not FeSOD contributed to the increase of Cd-induced SOD activity in *T. patula* leaves. Studies of *C. juncea* [10] also demonstrated that both CuZnSOD and MnSOD are major SOD isozymes responding to Cd. Several results have suggested that antioxidative enzyme activities are associated with Cd tolerance, assuming that altered antioxidative enzyme levels affect Cd tolerance [13,37–39].

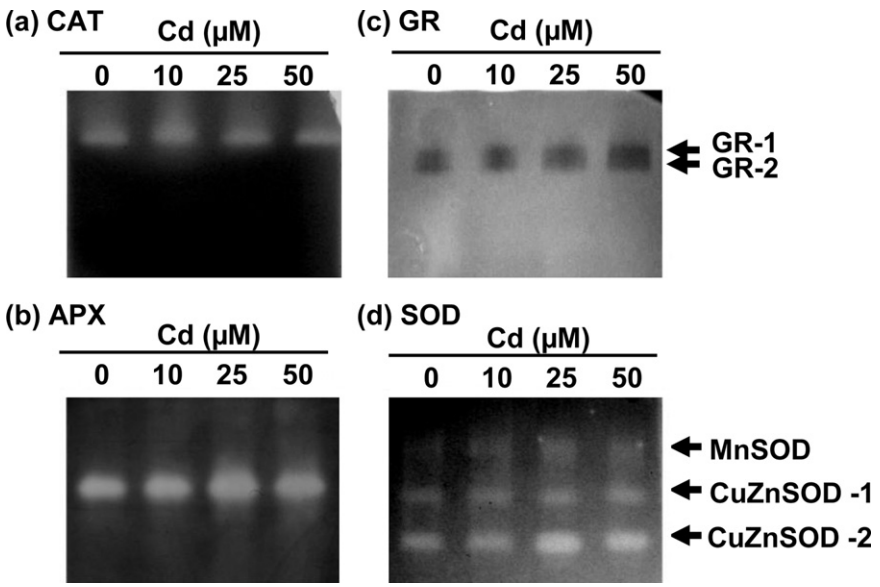

Fig. 6. Non-denaturing PAGE analysis of leaf antioxidant enzyme activities. The 35-day-old *T. patula* plants were exposed to 0, 10, 25, and 50 μM $CdCl_2$ for 14 days. A total of 50 μg proteins were loaded in each lane. (a) CAT isozyme; (b) APX isozyme; (c) GR isozymes; (d) SOD isozymes.

Further studies should determine whether antioxidative enzymes such as APX, GR, or SOD play a role in protecting *T. patula* against Cd toxicity.

3.5. In-gel activity assay of antioxidant enzymes in leaves

Shoot biomass is an important parameter for the indication of heavy metal tolerance in accumulator plants. In this study, leaf tissues of *T. patula* showed less effect of Cd toxicity than that of root tissues. To examine the isoforms of antioxidative enzymes which are involved in the protection of Cd toxicity in *T. patula*, we analyzed various APX, GR, CAT and SOD isovariants and activities in leaves by non-denaturing PAGE. CAT activity did not change significantly after 14 days of Cd treatment (Fig. 6a). Only a single band of APX could be detected in leaves, and the band intensity was greatly increased on exposure to 25 and 50 μM $CdCl_2$ (Fig. 6b). The band intensity of 2 GR isoforms increased with increasing Cd concentration (Fig. 6c). Three SOD isoforms exist in *T. patula* leaves (Fig. 6d). On preincubation with specific inhibitors, these isoforms could be distinguished from each other and be specified as one MnSOD and two CuZnSOD isoforms. FeSOD was not observed in leaves of *T. patula*, but the isoforms of MnSOD and CuZnSOD-2 were induced by $CdCl_2$ treatment (Fig. 6d). We conclude that mainly CuZnSOD and MnSOD but not FeSOD contributed to enhanced SOD activity in leaves.

4. Conclusions

In this study, we demonstrated that *T. patula* could accumulate at least 450 mg Cd kg^{-1} DW Cd in leaves, which is much higher than that of the standard Cd accumulator, that is, 100 mg Cd kg^{-1} DW of Cd in shoots. This result pointed out the great potential of *T. patula* as a Cd-hyperaccumlator which can be used in Cd phytoremediation. The biomass, chlorophyll level, and MDA content were not affected in 10 μM $CdCl_2$ hydroponically grown *T. patula*. Moreover, we showed that when exposed to 10 μM $CdCl_2$, the modulation of the capability of antioxidative defense system was correlated with Cd tolerance in *T. patula*. As mentioned before, advantages of *T. patula* for phytoremediation are as follows: (1) it is not an edible crop, which will prevents accumulated Cd from entering the food chain; (2) it grows with high shoot biomass when exposed to Cd; (3) it has wide adaptability to different soils and climate conditions; and (4) it secretes phytochemicals from root tissues and therefore can serve as a nematicide for the control of nematodes. Thus, using the garden flower *T. patula* may offer a new possibility for rendering agricultural soils or areas free of Cd contamination.

Acknowledgements

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