

## Effects of Cadmium Stress on Physiological Characteristics and Cadmium Accumulation in Alfalfa Seedlings (Postprint)

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### Abstract

Using “Gannong No. 3” alfalfa seedlings as experimental material, we investigated under hydroponic conditions the effects of different concentrations (0–2.0 mmol · L<sup>-1</sup>) of cadmium (Cd) stress within 10 days on root length, stem length, biomass, chlorophyll and malondialdehyde (MDA) content, superoxide dismutase (SOD) and peroxidase (POD) activities, Cd accumulation, and its subcellular distribution, to provide a theoretical basis for elucidating the toxic mechanism of plants under Cd stress and the phytoremediation of Cd-contaminated soils. The results showed that low concentration (0.125 mmol · L<sup>-1</sup>) Cd promoted root and stem growth and increased leaf chlorophyll content, whereas higher concentrations (0.5–2.0 mmol · L<sup>-1</sup>) significantly inhibited root and stem growth and markedly reduced chlorophyll content and biomass; Cd stress significantly increased MDA content while enhancing SOD and POD activities, which peaked at 0.5 mmol · L<sup>-1</sup> Cd concentration, likely representing a stress-protective response to environmental stress; Cd content in subcellular fractions followed the order: cell wall > cytoplasm > mitochondria > chloroplast, increasing with Cd stress concentration. When Cd stress concentration was 0.125 mmol · L<sup>-1</sup>, the Cd removal rate by the shoot of a single alfalfa seedling reached up to 0.214% after 10 days of hydroponic culture, while the removal rate per unit volume by the whole pot of plants reached up to 15.5%; when Cd stress concentration was 2.0 mmol · L<sup>-1</sup>, Cd content in alfalfa seedling shoots reached 89.36 g · g<sup>-1</sup>. These results indicate that alfalfa has strong Cd accumulation capacity, and although it does not meet the threshold criteria for Cd hyperaccumulator plants, comprehensive consideration of plant biomass, Cd tolerance, Cd accumulation amount, and Cd removal rate suggests that alfalfa has good application value in phytoremediation of Cd-contaminated soils.

## Full Text

### Effects of Cadmium Stress on Physiological Characteristics and Cadmium Accumulation in Alfalfa Seedlings

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#### Abstract

This study investigated the effects of cadmium (Cd) stress on root length, stem length, biomass, chlorophyll and malondialdehyde (MDA) contents, superoxide dismutase (SOD) and peroxidase (POD) activities, Cd accumulation, and subcellular distribution in “Gannong No. 3” alfalfa seedlings under hydroponic conditions over a 10-day period. Cd concentrations ranged from 0 to 2.0 mmol · L<sup>-1</sup> to elucidate the toxic mechanisms of Cd stress in plants and provide a theoretical basis for phytoremediation of Cd-contaminated soils.

The results demonstrated that low Cd concentrations (0.125 mmol · L<sup>-1</sup>) promoted root and stem growth while increasing leaf chlorophyll content. In contrast, higher concentrations (0.5-2.0 mmol · L<sup>-1</sup>) significantly inhibited root and stem growth and markedly reduced chlorophyll content and biomass. Cd stress caused a significant increase in MDA content while enhancing SOD and POD activities, which peaked at 0.5 mmol · L<sup>-1</sup> Cd concentration—likely representing a protective stress response. Subcellular Cd distribution followed the pattern: cell wall > cytoplasm > mitochondria > chloroplast, with all components increasing with Cd concentration. At 0.125 mmol · L<sup>-1</sup> Cd, the purification rate reached 0.214% per plant shoot and 15.5% per unit volume for the entire pot after 10 days. At 2.0 mmol · L<sup>-1</sup> Cd, shoot Cd content reached 89.36 g · g<sup>-1</sup>.

These findings indicate that alfalfa possesses strong Cd accumulation capacity. Although it does not meet the critical criteria for Cd hyperaccumulator plants, alfalfa demonstrates promising application value for phytoremediation of Cd-contaminated soils when considering biomass, Cd tolerance, accumulation capacity, and purification efficiency.

**Keywords:** Cadmium stress, alfalfa, physiological characteristics, cadmium accumulation, cadmium pollution

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## Introduction

Cadmium (Cd) occurs naturally as greenockite, but industrial activities including mining, electroplating, and metallurgy have released substantial Cd into the

environment, resulting in widespread soil contamination [Hawrylak et al., 2014; Reiser et al., 2014; Chen et al., 2015]. Cd exhibits high toxicity, mobility, and persistence, with an extremely long degradation cycle. When absorbed by crops, Cd disrupts plant physiological processes, reducing yield and quality, and poses serious health risks to humans through the food chain [Li et al., 2015; Shen et al., 2015]. Consequently, remediation of Cd-contaminated soils has become a critical research focus in soil science, ecology, and environmental science.

Phytoremediation using hyperaccumulator plants has gained global attention due to its operational simplicity, cost-effectiveness, environmental friendliness, and scalability [Ma et al., 2013]. However, naturally occurring hyperaccumulator species are rare, typically characterized by slow growth, low biomass, and limited geographic distribution. This has prompted a new approach: screening fast-growing, high-yield plants from existing resources for remediation applications.

Alfalfa (*Medicago sativa* L.) is a globally cultivated herbaceous plant with rapid growth, strong adaptability, extensive root systems, and high biological yield, making it a premier forage crop [Xu et al., 2008; Chen et al., 2015]. Previous studies have demonstrated alfalfa's ability to accumulate heavy metals such as Cu and Zn [Qu et al., 2016; Wang et al., 2006; Dai et al., 2014]. While most research has focused on alfalfa's responses to salt and drought stress [Li et al., 2010; Zhang et al., 2012] and heavy metal effects on seed germination and seedling physiology [Cao and Dai, 2014; Qu et al., 2016], studies on Cd accumulation characteristics and remediation potential remain limited.

Cd stress triggers a cascade of physiological and biochemical changes. Chronic Cd exposure leads to reactive oxygen species (ROS) accumulation, causing oxidative stress that reduces root viability, inhibits growth, degrades chlorophyll, and induces leaf chlorosis and desiccation [Smeets et al., 2005; Wang et al., 2011; Yin et al., 2013]. Antioxidant systems help regulate intracellular ROS levels and mitigate damage [Francisco and Joséluis, 2011; Jiang et al., 2014]. This study examined biomass, photosynthetic pigments, membrane lipid peroxidation, antioxidant responses, and Cd accumulation in alfalfa seedlings under varying Cd concentrations to assess growth performance and Cd uptake, providing insights into Cd toxicity mechanisms and remediation strategies.

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## Materials and Methods

### 1.1 Materials and Reagents

“Gannong No. 3” alfalfa seeds were obtained from the Gansu Academy of Agricultural Sciences. Cadmium stress was induced using CdCl<sub>2</sub> · 2.5H<sub>2</sub>O (Sinopharm Chemical Reagent Co.), with all other reagents being analytical grade.

## 1.2 Experimental Design

Uniform alfalfa seeds were selected and soaked in warm distilled water (50–60°C) for 30 minutes to enhance germination. After drying, seeds were sown in growth medium containing half-strength Hoagland's nutrient solution and cultivated indoors on growth racks (24 pots total), with regular medium replacement. After approximately 30 days, when seedlings developed three leaves, 21 uniform pots were selected and divided into seven groups. CdCl<sub>2</sub> was added to achieve concentrations of 0 (control), 0.125, 0.25, 0.5, 1.0, 1.5, and 2.0 mmol · L<sup>-1</sup>. Hydroponic culture continued for 3 days (500 mL volume) before sampling roots, stems, and leaves for analysis of chlorophyll a, chlorophyll b, carotenoids (Car), MDA content, POD and SOD activities, and Cd subcellular distribution. Final sampling occurred at 10 days to measure root length, stem length, fresh and dry weights, and Cd content.

## 1.3 Analytical Methods

**1.3.1 Physiological and Biochemical Indices** MDA content was determined using the thiobarbituric acid method [Zhao et al., 2002]. Chlorophyll content was measured by acetone extraction [Zhang and Zhai, 2004]. SOD activity was assayed via nitroblue tetrazolium (NBT) reduction [Li, 2000], and POD activity by guaiacol colorimetry [Li, 2000].

**1.3.2 Cadmium Content Analysis** Root, stem, and leaf samples were pooled, crushed, and sieved through a 0.425 mm mesh. Two-gram samples were digested in tubes with 20 mL concentrated HNO<sub>3</sub> and 4 mL HClO<sub>4</sub> at low heat. When reduced to ~4 mL, 10 mL ultrapure water was added, digestion continued for ~13 minutes, then transferred to 25 mL volumetric flasks, cooled, and diluted with 0.2% HNO<sub>3</sub>. Cadmium concentration was analyzed by atomic absorption spectrophotometry (Thermo Scientific 220FS) [Bao, 2007]. Cd translocation rate, absorption rate, and shoot purification rate were calculated [Ran et al., 2012]:

- **Translocation rate** = Cd accumulation in shoot / root dry weight / 10 days
- **Absorption rate** = Total Cd accumulation per plant / root dry weight / 10 days
- **Shoot purification rate (10 days)** = (Cd accumulation in shoot / total Cd in solution) × 100
- **Pot purification rate per unit volume (10 days)** = (Total Cd accumulation in pot / total Cd in solution / hydroponic volume) × 100, where total Cd in solution = solution volume × Cd concentration

**1.3.3 Subcellular Distribution of Cd** Fresh leaves (1 g) were homogenized in 10 mL ice-cold buffer (250 mmol · L<sup>-1</sup> sucrose, 50 mmol · L<sup>-1</sup> Tris-HCl [pH 7.4], 1 mmol · L<sup>-1</sup> dithioerythritol [C H O S]). Differential centrifugation was

performed: 600 rpm for 10 min (cell wall pellet), 1000 rpm for 15 min (chloroplast pellet), and 10000 rpm for 20 min (mitochondrial pellet). The supernatant represented cytoplasmic components (including vacuoles, ribosomes). Each centrifugation was repeated three times [Tang et al., 2008].

#### 1.4 Statistical Analysis

Data were processed using Excel for means and variance calculations. SPSS 22.0 was used for one-way ANOVA with Duncan's multiple comparison test ( $P = 0.05$ ).

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## Results

### 2.1 Effects of Cd Stress on Root Length, Stem Length, and Biomass

Alfalfa seedling root and stem lengths initially increased then decreased with rising Cd concentrations. At  $0.125 \text{ mmol} \cdot \text{L}^{-1}$  Cd, stem and root lengths increased significantly ( $P < 0.05$ ) by 7.35% and 11.00% compared to the control, respectively. Further concentration increases progressively reduced lengths, with  $2.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd decreasing stem and root lengths by 47.47% and 58.18% ( $P < 0.05$ ) [Figure 1: see original paper]A. While dry weight showed no significant difference from the control, fresh weight and water content decreased significantly ( $P < 0.05$ ) with increasing Cd concentration. At  $2.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd, fresh weight and water content declined by 59.91% and 24.1%, respectively [Figure 1: see original paper]B.

### 2.2 Effects of Cd Stress on Chlorophyll and Carotenoid Contents

At  $0.125 \text{ mmol} \cdot \text{L}^{-1}$  Cd, chlorophyll a, b, and carotenoid contents showed no significant difference from the control ( $P > 0.05$ ). However, higher Cd concentrations caused significant reductions, reaching minimum values at  $2.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd: 47.01%, 72.27%, and 51.28% of control levels, respectively ( $P < 0.05$ ). The chlorophyll a/b ratio decreased continuously with increasing Cd concentration.

### 2.3 Effects of Cd Stress on MDA Content and Antioxidant Enzymes

MDA content increased significantly across all Cd treatments ( $P < 0.05$ ). At  $0.125 \text{ mmol} \cdot \text{L}^{-1}$  Cd, root MDA content exceeded that in stems and leaves. Root and stem MDA peaked at  $1.5 \text{ mmol} \cdot \text{L}^{-1}$  Cd, increasing by 117.06% and 318.35% over control ( $P < 0.05$ ). Leaf MDA reached maximum at  $2.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd, showing a 732.57% increase—substantially higher than in roots and stems [Figure 2: see original paper].

SOD activity in roots, stems, and leaves followed a trend of initial enhancement followed by decline. At  $0.125 \text{ mmol} \cdot \text{L}^{-1}$  Cd, stem SOD activity slightly exceeded

root and leaf activities, while root SOD activity decreased significantly ( $P < 0.05$ ) and stem and leaf activities increased marginally ( $P > 0.05$ ). Maximum SOD activities occurred at  $0.5 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  in roots and stems (55.27% and 45.90% increases) and at  $0.25 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  in leaves (100.28% increase) ( $P < 0.05$ ). At  $2.0 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$ , stem and leaf SOD activities were significantly inhibited ( $P < 0.05$ ), while root activity remained similar to control [Figure 3: see original paper]A.

POD activity at  $0.125 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  was significantly higher than control in all tissues, with root POD activity exceeding stem and leaf activities ( $P < 0.05$ ). The POD trend mirrored SOD: maximum activities occurred at 0.5, 1.0, and  $0.25 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  in roots, stems, and leaves, respectively, showing 71.75%, 96.60%, and 54.13% increases over control ( $P < 0.05$ ). Despite subsequent declines at higher concentrations, POD activity remained significantly above control levels ( $P < 0.05$ ) [Figure 3: see original paper]B.

#### 2.4 Subcellular Distribution of Cd in Alfalfa Leaves

Cd content varied across subcellular fractions, with the highest concentration in cell walls (53.71–61.85% of total), followed by cytoplasm (19.51–31.68%), mitochondria (8.85–17.07%), and chloroplasts (2.44–7.87%). All fractions increased significantly with Cd concentration ( $P < 0.05$ ). Compared to control, total leaf Cd increased 43.17-fold, with maximum increases in cell walls, chloroplasts, mitochondria, and cytoplasm of 38.03, 100.52, 22.75, and 69.97 times, respectively ( $P < 0.05$ ).

#### 2.5 Cadmium Accumulation and Translocation in Alfalfa Seedlings

Both aboveground (stems, leaves) and belowground (roots) tissues accumulated Cd, with amounts increasing significantly as Cd concentration rose ( $P < 0.05$ ). Maximum accumulation occurred at  $2.0 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$ . However, purification rates per plant shoot and per unit volume decreased with increasing Cd concentration. The highest purification rates were observed at  $0.125 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$ : 0.214% per shoot and 15.5% per unit volume over 10 days. In contrast, Cd absorption and translocation rates increased progressively, peaking at  $2.0 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  at  $8.774$  and  $16.190 \text{ mol} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$ , respectively.

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## Discussion and Conclusion

Heavy metal accumulation disrupts normal plant physiological processes, inhibiting growth and reducing biomass [Xu et al., 2008]. Low Cd concentrations typically stimulate seed germination and seedling growth, while higher concentrations exert inhibitory effects [Zhang et al., 2004]. Our study showed that  $0.125 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$  significantly increased stem and root lengths, but further concentration increases progressively reduced growth, resulting in stunted

plants. Although dry weight remained unaffected, fresh weight and water content decreased significantly with Cd concentration, dropping to minimum values at  $2.0 \text{ mmol} \cdot \text{L}^{-1} \text{ Cd}$ . Since most metabolic activities occur in aqueous media and water serves as a crucial reactant, reduced water content impaired normal physiological metabolism, causing desiccation—consistent with previous findings [Adamakis et al., 2014] and confirming that excessive Cd uptake disrupts plant development.

Photosynthesis is fundamental for converting light energy to chemical energy, with chlorophyll content serving as a key indicator of growth capacity [Tang et al., 2008]. In our study, chlorophyll content in alfalfa leaves followed a similar trend to root and stem lengths: low Cd concentrations increased chlorophyll a, b, and carotenoid contents, while higher concentrations caused significant reductions, aligning with Tang et al. [2008]. Cd absorption triggers ROS accumulation [Sheng et al., 2009], which inhibits enzymes involved in chlorophyll and carotenoid synthesis and accelerates their degradation. While both chlorophyll a and b capture and transfer light energy, only specialized chlorophyll a molecules convert light energy to chemical energy [Sun et al., 2010]. The decreased chlorophyll a/b ratio indicates that Cd affected chlorophyll a more severely than chlorophyll b. Under our well-lit conditions, chlorophyll a content changes more profoundly impacted photosynthesis. Carotenoids perform light harvesting and photoprotection functions [Sun et al., 2010]; their reduction under Cd stress diminished light capture capacity and photoxidative defense, damaging photosynthetic apparatus [Demmig-Adams and Adams, 2000; Matsubara et al., 2008] and demonstrating that Cd stress weakened photosynthesis and inhibited growth.

Consistent with our previous research [Qu et al., 2016], MDA content in roots, stems, and leaves increased significantly with Cd concentration, while antioxidant enzyme activities (SOD and POD) showed an initial increase followed by decline. At low Cd concentration ( $0.125 \text{ mmol} \cdot \text{L}^{-1}$ ), root MDA exceeded stem and leaf levels, indicating more severe membrane damage in directly exposed root tissues. Root SOD activity decreased significantly compared to control, while stem and leaf SOD activities remained unchanged, suggesting low Cd stress suppressed root SOD activity. However, POD activity in all tissues increased significantly, with root POD activity exceeding stems and leaves—indicating rapid, intense activation in Cd-exposed roots. At high Cd concentrations ( $1.5\text{--}2.0 \text{ mmol} \cdot \text{L}^{-1}$ ), root POD and SOD activities remained significantly above control levels despite declines from maxima, with root SOD significantly activated to scavenge ROS and reduce membrane lipid peroxidation, correspondingly decreasing root MDA content. This demonstrates that antioxidant enzymes function coordinately rather than individually [Gao et al., 2010]. Although stem SOD activity was lower than roots and leaves, moderate POD activity and high cell wall pectin/hemicellulose content facilitated Cd sequestration in cell walls, alleviating toxicity and resulting in lower MDA content [Zhou et al., 2017]. In contrast, leaf MDA content was significantly higher, causing membrane damage that reduced SOD and particularly POD activities—indicat-

ing that high MDA accumulation inhibits antioxidant enzyme activity [Zhu et al., 2013]. Thus, Cd-induced membrane lipid peroxidation varies among plant organs, and antioxidant enzyme activation speed and magnitude differ by tissue and Cd concentration, likely depending on organ type and enzyme species.

Plants can adapt to Cd toxicity by modulating subcellular distribution [Tang et al., 2008]. Our study showed Cd distribution ranked: cell wall > cytoplasm > mitochondria > chloroplast, consistent with Wang et al. [2008]. Most Cd was sequestered in cell walls, where polysaccharide and protein molecules bind  $Cd^{2+}$ , preventing entry into cells and reducing toxicity [Kupper et al., 2000; Li and Dong, 2015]. At high Cd concentrations, chloroplast and cytoplasmic Cd allocation increased while cell wall and mitochondrial allocation decreased, indicating saturated cell wall capacity and greater internal Cd influx. All subcellular Cd contents increased significantly with concentration, but chloroplasts showed the greatest increase (100.52-fold). As metabolically active organelles with double membranes, chloroplasts and mitochondria are functionally critical. Elevated Cd can disrupt thylakoid structure, cause matrix efflux, and induce chloroplast disintegration [Wu et al., 2016]. Since photosynthetic pigments reside in thylakoid membrane protein complexes [Sun et al., 2010], excessive chloroplastic Cd severely damages structure and function. Meanwhile, ROS accumulation inhibited chlorophyll and carotenoid synthesis enzymes while accelerating degradation, exacerbating Cd toxicity. Research indicates that when cell wall storage capacity saturates, Cd translocates to the cytoplasm [Allan and Jarrell, 1989]. The cytoplasm and cell wall are metabolically less active regions where small molecules can precipitate Cd, conferring some tolerance. Therefore, despite low absolute chloroplastic Cd content, its dramatic increase caused disproportionately severe damage compared to other components.

Cd content in alfalfa shoots and roots, along with absorption and translocation rates, increased significantly with Cd concentration, demonstrating dose-dependent accumulation and transport. However, excessive accumulation caused further damage. At low concentrations, root Cd exceeded shoot Cd due to direct contact and greater exchange adsorption, consistent with Kupper et al. [2000], with limited transport to stems and leaves resulting in lower shoot Cd content. After  $1.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd treatment, root adsorption approached saturation, absorption and translocation rates plateaued, and seedlings exhibited chlorosis and wilting, indicating physiological limits were reached. Baker and Brooks [1989] established  $100 \text{ g} \cdot \text{g}^{-1}$  shoot Cd as the hyperaccumulator threshold requiring robust growth resistance. In our study,  $2.0 \text{ mmol} \cdot \text{L}^{-1}$  Cd produced shoot Cd content of  $89.36 \text{ g} \cdot \text{g}^{-1}$ , while  $0.125 \text{ mmol} \cdot \text{L}^{-1}$  Cd yielded the maximum shoot purification rate of 0.214% per plant over 10 days. Although our 10-day alfalfa culture did not reach hyperaccumulator criteria, the Cd accumulation of  $89.36 \text{ g} \cdot \text{g}^{-1}$  is comparable to *Vallisneria spiralis* accumulating  $90 \text{ mg} \cdot \text{kg}^{-1}$  over 105 days [Qiao et al., 2016]. The maximum purification rate of 0.214% is similar to *Brassica juncea* achieving 0.23% Cr removal in 30 days and 0.29% Zn and 0.13% Cd removal in 66 days [Wang et al., 2011; Jiang et al., 2002]. Therefore, considering Cd accumulation capacity,

tolerance, and purification efficiency, alfalfa holds considerable potential for phytoremediation of Cd-contaminated soils.

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