BIOACCUMULATION, SUBCELLULAR DISTRIBUTION AND CHEMICAL MORPHOLOGY OF CADMIUM IN *SCUTELLARIA BAICALENSIS* GEORGI BASED ON ICP-MS

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(Received 19th Jan 2024; accepted 4th Jun 2024)

Abstract. *Scutellaria baicalensis* Georgi has a long history of use and multiple applications worldwide owing to its medicinal and health benefits. However, the safety of its use is seriously affected by its high Cd content. To regulate and reduce the Cd content, it is necessary to understand the accumulation, absorption patterns, and detoxification mechanisms in response to Cd stress. In this study, we analyzed Cd bioaccumulation, subcellular distribution, and chemical morphology of *S. baicalensis* after Cd exposure using inductively coupled plasma–mass spectroscopy. Cd mainly accumulated in the root tissues and mainly bound to the cell wall (49.40%–61.71%). Cd mainly existed in low-mobility and low-toxicity chemical forms, and the proportion of the soluble fraction increased with the duration of Cd stress. Therefore, Cd retention in root tissues, cell wall deposition, vesicle sequestration, and the formation of low-mobility and low-toxicity chemical forms may have been the tolerance and detoxification strategies of *S. baicalensis* in response to Cd stress. The results of this study provide a theoretical basis for studying Cd accumulation and detoxification mechanisms in *S. baicalensis*, which will assist in future cultivation of high-quality and low-contamination products. This study also provides important information for regulating and reducing Cd accumulation in other medicinal plants.
Keywords: *heavy metal, Scutellaria baicalensis, medicinal plant, accumulation, detoxification*

Introduction

Owing to rapid industrialization and urbanization, the global environment is under considerable pressure (Mustafa and Komatsu, 2016; Su and Liang, 2015). Anthropogenic activities release large amounts of pollutants, including heavy metals (Rahimi et al., 2021). Various heavy metal pollutants produced by anthropogenic industrial activities enter ecosystems and have toxic effects on water, soil, and air, causing irreversible damage throughout ecosystems. Cd is one of the most harmful heavy metals (Huihui et al., 2020; Zhang et al., 2020; Chen et al., 2019). The Cd spatial distribution in nature is mainly influenced by geological conditions and the natural environment (Birke et al., 2017). However, Cd distribution and content in the environment are increasingly closely related to anthropogenic activities. Numerous Cd compounds in the ecological environment are mostly associated with industrial and agricultural modernization and interventions, such as mining, metal smelting, chemical fertilizer use, and automobile exhaust (Bigalke et al., 2017; Wang et al., 2018 a; Xia et al., 2011). Cd is a widespread and highly toxic heavy metal for plant growth and development (Li et al., 2017). Once absorbed by a plant, heavy metals are transferred from the roots to aboveground parts through various proteins in the xylem (White,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

2017) and phloem (Page and Feller, 2015), disrupting the physiological activity of plants and even causing plant death. Excessive levels of Cd damage plants as it accumulates in plant tissues, producing toxic effects (Gonçalves et al., 2012), such as reducing the chlorophyll content and cell membrane permeability, disturbing the water balance, inhibiting antioxidant enzyme activity, causing accumulation of reactive oxygen species, leading to cell damage and significantly inhibiting plant growth (Sun et al., 2022; Jia et al., 2020). Cd accumulated in plants can enter the human body through the food chain (Chen et al., 2020), posing a significant threat to human health and safety. Cd disrupts the human metabolism (Fu and Xi, 2020) and causes injuries to the respiratory (Fatima et al., 2019), urinary, and reproductive systems (Rzymski et al., 2015) and may even be carcinogenic (Valko et al., 2006; Filipic et al., 2006).

The plant root system is the first plant organ exposed to Cd from the external environment. Studies have proven that Cd in plant tissues tends to accumulate in the roots, with only a small portion being translocated to the aerial parts of most plants (Shaari et al., 2022; Hasan et al., 2009; Kuppusamy et al., 2016). Aboveground parts are more sensitive than roots, suggesting that plants limit Cd^{2+} translocation to the aboveground parts by limiting Cd entry into the xylem and phloem of roots, which contain water and nutrients (Zhang and Shu, 2006). After Cd enters a plant, it is selectively distributed in the subcellular fractions and binds to certain substances in the plant cells (Xin et al., 2014; Xin and Huang, 2014). The subcellular structure of plants plays an important role in heavy metal detoxification. As a measure to mitigate Cd toxicity, the cell wall and soluble fractions, such as hemicellulose, protein, and lignin, contained in the cell wall reduce the toxic effects on the plant body through complexation with metal cations, which immobilize heavy metal ions and render them inactive (Zhu et al., 2013). Dong et al. (2019) showed that when *Arabidopsis thaliana* was subjected to Cd stress, there was a highly significant correlation between the cell wall fixed and protection mechanisms (Meng et al., 2019). Vacuoles contain large amounts of organic acids that can chelate heavy metal ions, reducing plant Cd damage (Xu et al., 2018). Heavy metals that enter plants exist in various forms, and to some extent, the magnitude of the toxic effects of Cd in plants is determined by its chemical form in plant tissues (Li et al., 2016). This is because the chemical form of Cd determines its migratory capacity in plant tissues. Ethanol-, H_2O-, and NaCl-extracted Cd may have greater toxicity than other forms of Cd because of the migration capabilities, which enable Cd to penetrate the cell symplasm more easily, bind to relevant cellular organelles, and disrupt the metabolic processes of the cells and the plant (Weng et al., 2012). The bioaccumulation, chemical form, and subcellular distribution of heavy metals in plants determine heavy metal transportation and accumulation, leading to differences in heavy metal mobility and toxicity in plants. In this way, all three processes are closely related to alleviating damage to plants facing Cd stress. Therefore, exploring the bioaccumulation, chemical morphology, and subcellular distribution of Cd in plant tissues is important for understanding the mechanisms of heavy metal detoxification and accumulation in plants (Cao et al., 2018; Yang et al., 2019).

Scutellaria baicalensis Georgi is a medicinal plant that is widely used worldwide. In China, *S. baicalensis* has a long history of medicinal use. It is a fundamental herb in traditional Chinese herbal medicine, known as Huang Qin, and is cultivated in various regions of China, mainly in the northeast and northwest. It was also used by Native Americans as an herbal remedy (Lawson et al., 2021). Over 40 compounds have been

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

isolated from *S. baicalensis*, including flavonoids (Ciocrlan et al., 2021), terpenoids, volatile oils, and polysaccharides (Lin et al., 2016). The pharmacological activities of *S. baicalensis* extract are mainly antibacterial (Qian et al., 2015; Choi et al., 2010), organ-protective (Wang et al., 2015), and immunomodulatory (Zhao et al., 2019). *S. baicalensis* is widely used in the clinical treatment of bacterial viruses that cause hepatitis (Chun et al., 2020), diarrhea, vomiting (Olennikov et al., 2008), and cancer (Xu et al., 2013). Because of its favorable biological activities, *S. baicalensis* has been developed into various products such as health foods (Shen et al., 2020) and herbal tea (Kim et al., 2014), and its extract is also used in cosmetics (Makino et al., 2008). For example, in Japan, *S. baicalensis* is used as an herbal tea and dietary supplement (Ordon et al., 2021). In addition, *S. baicalensis* extract is used as an active coating in food packaging materials (Tian et al., 2020). As the use of *S. baicalensis* has become increasingly widespread, its safety has come under scrutiny, and the high Cd content is an important factor affecting its safety. Diverse application pathways also increase the likelihood of Cd passing through the food chain and harming the human body. Strict regulations on Cd content in plants have been laid to prevent damage to human health due to excessive Cd intake. The World Health Organization (WHO) and Green Trade Standards of Importing & Exporting Medicinal Plants & Preparations has set a clear limit on the Cd level in medicinal plants ($\leq$ 0.3 mg/kg) (Li et al., 2022). High Cd concentrations in *S. baicalensis* have been reported, with the highest level reaching 1.2 mg/kg, exceeding the WHO regulation by a factor of 4; thus, the high Cd content in *S. baicalensis* seriously jeopardizes its safe use as a food and medicine (Huang et al., 2019).

Therefore, to facilitate the regulation and reduction of Cd uptake and accumulation in *S. baicalensis*, it is important to study the Cd tissue distribution, subcellular distribution, and chemical morphology. This information is essential for clarifying the accumulation and detoxification mechanisms of heavy metals in plants, which is important for reducing heavy metal accumulation in plants and ensuring the quality of medicinal plants. However, limited information is available on the Cd distribution patterns and chemical forms in *S. baicalensis*. This study aimed to address these important knowledge gaps. We used inductively coupled plasma–mass spectroscopy (ICP-MS) to determine the tissue accumulation pattern of Cd in *S. baicalensis* and investigated its subcellular distribution and chemical forms. The results provide a theoretical basis for understanding Cd accumulation and detoxification mechanisms in *S. baicalensis*, valuable information for cultivating high-quality *S. baicalensis* with low contamination, and a theoretical reference for determining standardized limits for Cd in medicinal plants.

Materials and methods

Plant cultures

Plants were cultivated in a glasshouse (natural light; 20°C–25°C; 70%–85% relative humidity). Seeds (collected from *S. baicalensis* Planting Base in Chun hua County, Shaanxi Province) were soaked for 24 h, spotted in black plastic pots (0–5-mm diameter) filled with humic soil (pH 5.0–6.0), and incubated for 1 month. Seedlings were transplanted into plastic containers and filled with full strength Hoagland's nutrient solution. There were 12 seedlings per container with 3 replicates per treatment. The nutrient solution was maintained at pH 6.0 and renewed every 3 d. Plants were acclimatized for 8 weeks and were then exposed to Cd concentrations of 0 μM Cd (CK)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

and 200 μM Cd (as Cd $[NO_3]_2$). The treatment concentrations were based on preliminary studies, in which no symptoms of plant growth defects were observed following exposure to 0, 50, 100, or 200 μM Cd; therefore, we selected the high Cd concentration (200 μM) for use in the present study. Plants were harvested after exposure to Cd at 0 h (Cd0h), 24 h (Cd24h), and 10 d (Cd10d) to determine the subcellular distribution and chemical forms of Cd in *S. baicalensis* during the treatment.

Tissue fractionation

To investigate the subcellular distribution of cadmium in different tissues, measurement of Cd concentration in subcellular using ICP-MS technique. The cells were divided into three fractions: cell walls, organelles, and soluble fractions (including vacuoles). According to Huang et al.'s method, the tissue fractionation analysis was carried out utilizing the differential centrifugation technique (Huang et al., 2018; Gao et al., 2021). All experimental manipulations were performed at 4°C. A prechilled extraction solution containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 1.0 mM $C_4H_{10}O_2S_2$ (DTE, Aladdin, USA) was used to homogenize freshly frozen roots, stems, and leaves. After homogenates were transferred to centrifuge tubes, they were spun at 3000 rpm for 15 min to produce a pellet that was primarily made up of cell walls and related debris, known as the cell wall fraction (Fcw). The pellet and supernatant produced by further centrifuging the supernatant at 12,000 rpm for 45 min are known as the soluble fraction (Fs) and the fraction containing organelles (Fco), respectively. ICP-MS was used to measure the levels of Cd in the three fractions and the various tissues.

Extraction of Cd in different chemical forms

The chemical forms of Cd were analyzed by the method of Xin (Xin et al., 2014). The following solutions were used in the following order to extract six different types of Cd: (1) 80% ethanol for extracting inorganic Cd (FE), including nitrate/nitrite, chloride, and aminophenol Cd; (2) deionized water (d-H_2O) for extracting water-soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$ (FW); (3) 1 M NaCl for extracting pectate- and protein-integrated Cd (FNaCl); (4) 2% acetic acid (HAc) for extracting insoluble Cd phosphate (FHAc), including $CdHPO_4$ and $Cd_3(PO_4)_2$; (5) 0.6 M HCl for extracting Cd oxalate (FHCl); and (6) the residual (FR) The extraction solution (w/v = 1/10) was used to homogenize frozen root, stem, and leaf tissues while they were shaken for 22 h at 25°C. After centrifuging the homogenate at 5000 × g for 10 min, the first supernatant was removed and put in a beaker. The same extraction solution was used again to extract the sediment, and it was agitated for 2 h at 25°C. The sediment was extracted using the subsequent solution in accordance with the solvent order after the three supernatants were combined. The same extraction methods, procedures, and times as for the first extraction solution were employed. ICP-MS (iCAP RQ, Thermo Scientific, USA) was used to determine the Cd concentration after each fraction of the pooled supernatant solution and the remainder fraction were dried to a constant weight and digested in a microwave with HNO_3/H_2O_2 (v/v = 3/1).

QA/QC control

A certified garlic powder reference material (GBW10022) was purchased from the Institute of Geophysical and Geochemical Exploration in China and was used for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

quality assurance. It contained 0.0611 ± 0.0001 mg/kg Cd dry weight, which was in line with the certified value of 0.062 ± 0.003 mg/kg Cd.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL, USA, Version 23.0). Bioaccumulation of Cd were analyzed using TWO-WAY analysis of variance (3 types of tissues × 3 different times after initiation of the experiment); Subcellular and chemical forms of Cd were analyzed using THREE-WAY analysis of variance (3 types of tissues × 3 subcellular fractions × 6 treated times) and (3 types of tissues × 6 chemical forms × 6 treated times). Post hoc tests are validated using the LSD method. Figures were generated using GraphPad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA).

Results

Cd bioaccumulation in S. baicalensis

Cd accumulation in *S. baicalensis* is shown in *Tables 1* and *A1*. After Cd treatment, Cd accumulation in different *S. baicalensis* tissues increased, and there was an increasing trend in the Cd content in tissues with increasing treatment time. However, the degree of Cd accumulation in different tissues differed after the same treatment time. Cd was mainly enriched in *S. baicalensis* roots. The Cd accumulated by the roots in all groups accounted for 46.84%–78.4% of the total Cd content in the whole plant. Moreover, Cd enrichment in the root tissues became more pronounced as the Cd treatment duration increased. Cd content in root tissues after 10 d of exposure increased 14.98-fold from 0 h to 10 d, whereas that in the stem and leaf only increased by 7.68- and 5.82-fold, respectively.

Table 1. *Accumulation of cd in different S. baicalensis tissues*

Tissues	Cd concentration (mg/kg)		
	Cd 0h	Cd 24h	Cd 10d
Root	0.340 ± 0.002^{Aa}	0.949 ± 0.002^{Ba}	5.140 ± 0.060^{Cc}
Stem	0.090 ± 0.0008^{Ba}	0.346 ± 0.005^{Ca}	0.784 ± 0.002^{Ab}
Leaf	0.160 ± 0.1183^{Bb}	0.355 ± 0.002^{Cb}	1.340 ± 0.012^{Ac}

Mean ± S.D. (replicates = 3). Different capital letters in the rows and lowercase letters in the columns denote significant variations in the concentration of Cd at various sampling periods various sampling times following the start of treatment, and in the concentration of Cd by various tissues type at $p < 0.05$, respectively

Subcellular Cd distribution in S. baicalensis

The effects of Cd treatment time and exposure level on the Cd subcellular distribution in leaves and roots are shown (*Fig. 1; Table A2; Table A3*). Overall, the Cd content in the cell wall dominated the subcellular structure of the three tissues in all *S. baicalensis* control groups. Among the three subcellular distributions in *S. baicalensis* without Cd treatment, the Cd content enriched in the cell wall occupied 47.54%–63.07% of the total Cd concentration, followed by the organelle fraction

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

(29.00%–35.45%) and the soluble fraction (7.14%–17.63%). With Cd treatment, the Cd concentration in different subcellular of all *S. baicalensis* tissues showed an increasing trend. In particular, the amount of Cd10d treatment group Cd accumulated showed the best performance compared to the control group, and the total Cd accumulation of three subcellular structures in the root, stem, and leaf tissues increased by 18.58-, 7.98-, and 4.92-fold, respectively. Among the three types of *S. baicalensis* tissues, the cell wall structure was the most enriched in Cd; after different Cd treatment times, the Cd content enriched in the cell wall structure accounted for 49.40%–61.71% of the total Cd content. The remainder was distributed in the organelles (19.39%–30.52%) and the soluble fraction (vesicles) (9.67%–28.96%). The subcellular Cd distribution in the three tissues decreased from the cell wall > organelle > soluble fractions.

In addition to the enrichment of the cell wall structure with a large amount of Cd, changes in the Cd content of the soluble fraction of *S. baicalensis* after Cd stress were also considered. With prolonged treatment, the change rate in Cd's soluble fraction was greater than in the other two subcellular structures. Taking Cd10d as an example, the Cd-soluble fraction in the three tissues in the control group accounted for the total Cd content as follows: 24.7% in roots, 10.85% in stems, and 15.85% in leaves, respectively, whereas in the Cd-treated group, the soluble fraction of Cd in these three tissues increased by 13.00-, 11.90-, and 6.51-fold, respectively.

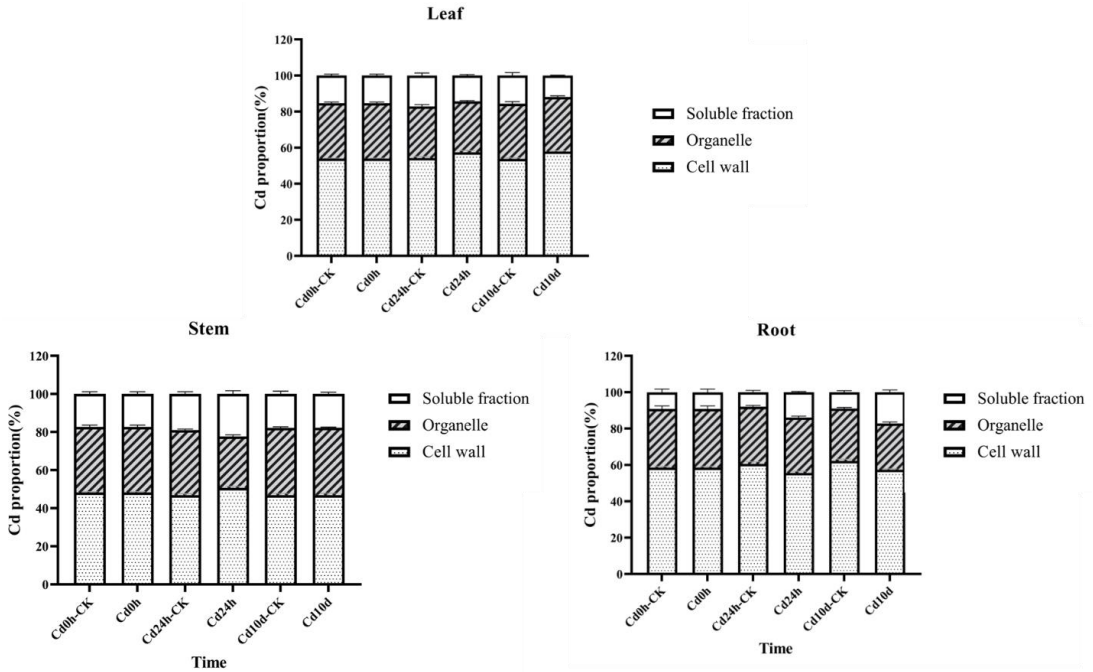

Figure 1. *Effects of the length of Cd exposure on the distribution of Cd subcellularly in different tissue*

Chemical forms of Cd in S. baicalensis

The ratios of the six Cd chemical forms in the root stems, and leaves of *S. baicalensis* are shown (*Fig. 2; Table A4; Table A5*). Overall, the chemical forms of Cd in these three tissues were mainly dominated by residual, HCl-extracted, and HAc-extracted forms. In the roots, stems, and leaves of all control groups, residual form

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

accounted for 41.38%–49.65%, the HCl-extracted 40.80%–47.77%, and HAc-extracted forms occupied 8.60%–9.98% of the six Cd chemical forms, respectively. With the Cd intervention in the treatment group and the increase in treatment time, the percentages of the three main chemical forms in roots, stems, and leaves were 39.73%–46.72%, 35.82%–46.29%, and 10.80%–16.92%, respectively. Thus, the Cd content of the above three chemical forms accounted for the largest percentage of the chemical forms of Cd in *S. baicalensis*. The 0.20% d-H_2O and ethanol-extracted fraction was only detected in the roots of the 10-d treatment group, accounting for only 0.45% of the total, and was not seen in the other tissue treatment groups.

Cd stress resulted in changes in the proportion of the Cd content in different Cd chemical forms in the three types of *S. baicalensis* tissue. The three main Cd chemical forms showed different trends in different tissues. After Cd stress, the residual form of the Cd content in the root and stem tissues showed a decreasing trend; in the Cd24h group, the residual state content decreased by 1.41- and 1.67-fold in root and stem tissues, compared to that in the control group. The HCl-extracted fraction increased 1.87- and 1.79-fold in root and stem tissues after 24 h treatment with 200 μL Cd. The HAc-extracted fraction increased the most in stem tissues (by 2.41-fold).

The proportions of the Cd content in the three main chemical forms in the root and stem tissues changed with further extension of the Cd treatment time. The ratio of residual Cd and HCl- and HAc-extracted fractions was regressed compared to that of the Cd24h group. Although the proportions of HCl- and HAc-extracted fractions in the Cd10d group tended to decrease compared to those in the Cd24h group, they remained higher than those in the control group. However, there was minimal change in the concentration of each chemical form of Cd in *S. baicalensis* leaves with increasing Cd treatment time.

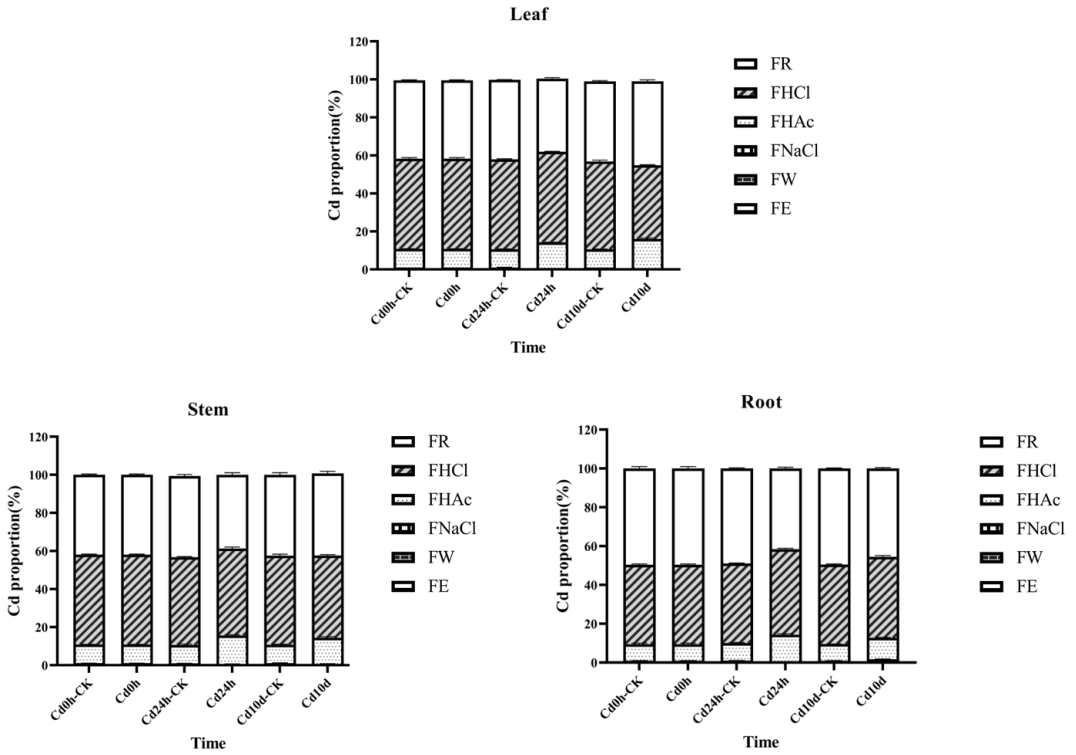

Figure 2. *Effects of the time spent exposed to Cd on the chemical forms of Cd in different tissues*

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DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Discussion

Bioaccumulation

Roots can effectively hinder Cd transfer. In most plants, only a small proportion of accumulated Cd is transferred to the aboveground parts. Gao et al. studied Cd uptake and distribution in different rice tissues and found that the root system was the main site of Cd uptake (Wang et al., 2018 b). Wang et al. found that two types of soybeans have different tolerances to Cd, although there are differences in the ability of root tissues to enrich Cd; in general, the root was the main site of Cd enrichment in the two studied soybean genotypes (Huang et al., 2023). The plant root tissues retain more heavy metals than other tissues, a phenomenon demonstrated in rice (Jia et al., 2021), tomatoes (Shenker et al., 2001), and other plants.

This study found that there was no significant difference in Cd content in stem and leaf tissues within the different Cd treatment groups, but there are significant differences ($p < 0.05$) between roots and the other two tissues (*Table 1*). And with the increase of Cd treatment time, the content of Cd in the same plant tissues showed significant differences ($p < 0.05$) between the group Cd10d and the other time-treated groups (*Table 1*). This not only suggests that the main site of Cd bioaccumulation is in the roots but also shows that the content of Cd in the tissues correlates with treatment time. There are several possible reasons for the mechanisms of root retention in response to Cd stress in plants. First, in the invitro process, the secretion produced by the plant root system can form metal ligand complexes with Cd in the environment, thus blocking heavy metal entry into the root cells and inhibiting Cd entry into the plant (Akhter et al., 2013). Additionally, elemental Cd tends to accumulate in the cell walls of the endodermis and xylem of roots, and relatively high Cd levels are present in the symplasts of the cortical cells (Dismukes., 2002). Within plant root cells, the binding sites on the cell membrane are capable of trapping heavy metal ions (Clemens, 2006; Vatamaniuk et al., 2000). The relevant ligands in plant cells also bind to heavy metal ions, allowing Cd ions to form chelates (Regvar and Vogel-Miku, 2008) and insoluble complexes (Rauser, 2003) in a low-mobility state, ensuring that Cd is immobilized in root tissues in a low-toxicity state (Rizvi, 2019).

As the first tissue and organ in contact with heavy metals, roots form a set of detoxification mechanisms to cope with Cd and become the first barrier for plants to cope with Cd stress. In this experiment, more Cd was accumulated in *S. baicalensis* roots than in aboveground parts, which confirms previous findings (Uddin et al., 2020). This indicated that the roots slowed the toxic effect of Cd on the plant by limiting Cd transfer to the plant aboveground parts of the plant and that *S. baicalensis* roots participated in the response to alleviate Cd stress, which was key to Cd detoxification in *S. baicalensis.*

Subcellular distribution

The selective distribution of heavy metals at the subcellular level can somewhat mitigate their toxic effects on organisms (Chen et al., 2013). At the subcellular level, the cell wall is the first barrier through which Cd enters plants (Li et al., 2019). In our study, Cd was always preferentially accumulated in the cell wall structures of the three tissues of *S. baicalensis*. In the different tissues the cell wall structures accounted for 48.13%–60.42% of the total Cd content in the three subcellular structures, and there was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

a significant difference ($p < 0.05$) in the Cd content of the cell wall structures between the other two subcellular structures (*Table A2*). By comparing the Cd contents of the three subcellular structures in different tissues of *S. baicalensis* under different treatment times, we found that the Cd concentrations of the different subcellular structures in the three tissues of *S. baicalensis* increased with the prolongation of the treatment time, and the Cd contents of the three subcellular structures in the different tissues of *S. baicalensis* under the Cd 10d treatment were the highest, and significant differences ($p < 0.05$) were found between the three subcellular of the different tissues with the other time-treated groups (*Table A2; Table A3*).

These results are consistent with studies on other plants. Yu et al. found that as the Cd concentration increased in the soil in which *Agrocybe aegerita* was grown, the Cd concentration enriched in subcellular structures increased in the stem and villous tissues, with approximately 19%~42% of the Cd stored in the cell wall structures (Yu et al., 2021). A study on the Cd accumulation capacity of different rice strains revealed that > 81% of the Cd in the root tissues of two rice varieties was enriched in the cell wall and soluble fraction and that the Cd accumulation capacity of high Cd-accumulating rice varieties was related to the pectin and polysaccharide contents in the cell wall (Qiu et al., 2011). The main function of the cell wall in reducing the harmful effects of Cd on the plant body is to limit Cd transport in the plant body to avoid its subsequent toxic effects. Plant cell walls contain proteins and polysaccharides such as cellulose, hemicellulose, lignin, and mucilage, which have a wide range of potential ligands such as hydroxyl, carboxyl, amino, aldehyde, phosphate, and thiol (Meychik et al., 2021; Wang et al., 2009 a). These ligands can be involved in ion exchange, adsorption, complexation, precipitation, and crystallization, leading to metal segregation under metal toxicity and ineffective Cd uptake (Zhou et al., 2015). Therefore, Cd enrichment in the cell wall was protective in *S. baicalensis*. Cd compartmentalization by the subcellular structures was an important detoxification mechanism in the plant's response to Cd stress.

In addition, the results showed that changes in the Cd content in the soluble fractions of *S. baicalensis* were more pronounced than those in the other two subcellular structures after Cd stress. Compared to the other two subcellular structures, the rate of change in the Cd content of the soluble fraction was greater after prolonged Cd treatment. This suggested that the soluble fraction may have been involved in alleviating Cd stress in plants and played an important role in plant stress caused by Cd. The soluble fraction may have been one of the important detoxification mechanisms in *S. baicalensis*. Vacuoles are important sites for mitigating plant Cd toxicity (Guan et al., 2018). Vacuoles are secondary sites other than the cell wall where plants reduce the toxic effects of Cd (Wójcik et al., 2005). Vacuoles are rich in proteins, organic acids, and polysaccharides, which can bind with Cd in tissues, resulting in Cd existing in plant tissues in low-toxicity and low-mobility forms, thus reducing the toxic effect of Cd on plants when it enters plant organelles. Some Cd tolerant plants rely mainly on cell wall structures to cope with the toxic effects of heavy metals, whereas Cd sensitive plants rely mainly on the separating ability of vacuoles (Verbruggen et al., 2009; Wu et al., 2022; Uraguchi et al., 2009). Yang et al. showed that high Cd concentration stress may cause damage to the plant cell wall and that to prevent further cell damage, Cd was gradually transferred to the soluble fraction, making the vesicle a secondary site for Cd enrichment in the soluble fraction (Yang et al., 2018). This indicates that cell wall fixation and vesicle compartmentalization are important self-protection mechanisms for *S. baicalensis* to reduce the damage caused by Cd stress.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Chemical forms

The chemical form of heavy metals determines their biological functions when they enter plants. Different forms of Cd are extracted by different solvents, reflecting their toxicity and mobility. The different forms of Cd extracted by different extracts directly relate to their toxicity and migration capacity. The inorganic salt form (80% ethanol extraction) and water-soluble form (d-H_2O extraction) had greater toxicity and transport capacity than pectinate and protein-chelated Cd (1 M NaCl extraction), insoluble Cd phosphate (2% HAc extraction), Cd oxalate (0.6 M HCl extraction), and residual Cd.

The results of this study showed that the chemical forms of Cd in all three types of *S. baicalensis* tissue were dominated by the low-toxicity and low-mobility forms of Cd, which are the residual Cd, HCl-extractable, and 2% HAc-extractable forms. These three main chemical forms present in the three tissues of *S. baicalensis* showed significant differences ($p < 0.05$) in content compared to other chemical forms of Cd (*Table A4*). As the treatment time increased, the content of different chemical forms in the three tissues also showed an elevated trend of change, the significant differences ($p < 0.05$) in different chemical forms in different tissues of *S. baicalensis* under the different Cd treatment time groups (*Table A4; Table A5*).

Other plants showed similar results to the present study following Cd stress. After barley roots were stressed by Cd, analyses of the chemical forms of Cd in the root tissues of four different genotypes showed that the HAc- and HCl-extractable fractions occupied 36% and 33% of the total Cd, respectively (Wu et al., 2005). Huang et al. characterized the chemical forms of Cd in stems and leaves of *Morus alba L.* and showed that the chemical forms extracted using 2% HAc and 0.6 M HCl were dominant (Huang et al., 2018). Therefore, Cd in *S. baicalensis* was predominantly present in the low-toxicity, low-mobility form, which is one of the mechanisms underlying the mitigation of Cd toxicity.

After a short period of Cd stress in *S. baicalensis*, in the Cd24h treatment group, the root and stem tissues produced an upregulation of the proportion of the HCl- and HAc-extractable Cd fractions. This suggested that *S. baicalensis* responded to Cd stress within a short period by realizing a shift in the Cd chemical form to FHAc and FHCl, i.e., low-toxicity and low-mobility forms in the plant and that these changes in the chemical form of Cd were important processes in the detoxification mechanism. This suggested that the detoxification mechanism of *S. baicalensis* may have been related to the formation of complexes via Cd binding to phosphate and oxalate.

The increase in exogenous P allows Cd to form poorly mobile phosphate complexes (Wang et al., 2009 b), which are characterized by poor solubility, poor mobility, and low-toxicity, resulting in the presence of many Cd phosphate deposits in vacuoles and cell walls, thereby reducing the toxic effect of Cd on plant leaves (Ma et al., 2022). Therefore, to achieve stress relief when plants are subjected to Cd stress, researchers have increased the input of external P to plants to increase the proportion of insoluble Cd-containing salts, such as $CdHPO_4$, $Cd_3(PO_4)_2$, and other Cd phosphate complexes (Qiu et al., 2011). Similar to this detoxification mechanism, maize also relies on forming Cd phosphate complexes in plants to cope with Cd stress (Jiang et al., 2007). The involvement of exogenous P not only reduces the lipid oxidative damage caused by Cd in plants by decreasing the levels of superoxide dismutase, catalase, and peroxidase (Jia et al., 2020) but also leads to the deposition of Cd phosphates in plant vacuoles and

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DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

cell walls, suggesting that the involvement of phosphates is an important mechanism for regulating Cd toxicity in plants (Guo et al., 2018).

The acidic environment of plant cells favors the conversion of chemical forms of Cd to less toxic and less mobile forms (Jung et al., 2008). The numerous carboxyl groups (-COOH) present in organic acids can be complex with heavy metal ions, thereby reducing the damage to plants caused by heavy metal ions (Thakur et al., 2022). In plants, up to 80% of Cd ligands are provided by organic acids in cell walls and vacuoles; hence, the acidic environment in plants provides a favorable environment for the Cd complexations produced by the plant. Of the organic acids in plants, oxalic acid occupies a relatively large proportion, and Sedum alfredo ranks third among all organic acids (Tian et al., 2011). Moreover, the dicarboxylic anions in oxalic acid have a strong binding capacity for heavy metal cations (Nkoh et al., 2020). A study on the chemical form of Cd in *Triarrhena sacchariflora* seedlings revealed that Cd enriched in plant roots mitigated the detrimental effects of Cd on plants by complexing with oxalic acid in large quantities and excluding it in the form of Cd oxalate complexes (Xin et al., 2018). Duckweed, a Cd-hyperaccumulating plant, shows a significant increase in the proportion of HCl-extractable Cd after prolonged Cd stress, and the resulting low-toxicity Cd oxalate is the main coping strategy of plants under Cd stress (Zheng et al., 2023). Therefore, we hypothesized that *S. baicalensis* has a detoxification mechanism to cope with heavy metal stress, whereby oxalic acid in the plant reduces Cd mobility by forming complexes with Cd, thus reducing its toxic effects on the plant.

Conclusions

The Cd distribution and chemical forms determined in this study provide insights into Cd accumulation and detoxification mechanisms in *S. baicalensis.* The results showed that *S. baicalensis* coped with the negative effects of excessive Cd through strategies such as root sequestration, cell wall deposition, and transformation of heavy metals to low-toxicity and low-mobility forms. This study provides a theoretical basis for reducing Cd accumulation in *S. baicalensis*, which has important implications for cultivating high-quality *S. baicalensis* with low contamination and increasing the safety of its use. In the future, we will continue to study the molecular mechanisms of Cd uptake and transport in *S. baicalensis* to elucidate further the regulation and reduction of Cd accumulation in plant tissues.

Acknowledgements. We would like to publicly thank Shaanxi Dongzhuang Medicinal Botanical Garden Co., Ltd., for providing the experimental materials, and we thank Zebing Xia and Yuanrong Zou's help in this experiment.

Fundings. This research was funded by the Major Science and Technology Projects in Inner Mongolia Autonomous Region (No. 2019ZD005), the National Natural Science Foundation of China (No. 81903751), and the Natural Science Basic Research Project of Shaanxi Science and Technology Department (No. 2019JQ-877); Scientific Research Project of Shaanxi Administration of Traditional Chinese Medicine (No. 2019-ZZ-ZY018); and by the Research and Development Program of Shaanxi university of Chinese medicine (2023GP26, Shaanxi); Demonstration of Key Technology Integration and Application for Efficient and Ecological Cultivation of *S. baicalensis* in Jia County, Yulin City (23JC025); National Administration of Traditional Chinese Medicine high-level key discipline construction project (No. zyyzdxk-2023202).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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APPENDIX

Determination the concentration of Cd

The treatment concentrations of Cd in the experiments are based on the results of pre-experimental studies. By reviewing the relevant literature, several concentrations of Cd were used in the pre-experiments, i.e., 0, 50, 100 and 200 μM. Symptoms of growth defects in plants were not visible at all Cd concentrations tested. For studying the accumulation and transportation mechanisms of Cd in plants, understanding the localization and speciation that under excess Cd accumulation is important. Therefore, we selected the high concentration (200 μM) of Cd treatment.

ICP-MS measurement conditions

High-frequency power 1.2 kW, Plasma gas flow rate 8.0 L/min, Auxiliary gas flow rate 1.1 L/min, Carrier gas flow rate 0.7 L/min, Pool gas flow rate 6.0 mL/min, Fog chamber temperature 5°C, internal standard added online by the instrument.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Sample digestion

Precisely weigh 0.5 g of the sample into the digestion vessel. Next add 5 mL of concentrated HNO_3 and 1 mL of H_2O_2 (30%) and leave overnight. The vessel was closed and placed on the rotating turntable of the microwave digester and digested at 120°C for 5 min, 160°C for 5 min, 180°C for 25 min, ramped up for 15 min, maximum power 1000 W for 30 min and cooled for 15 min at 0 W. The digestion was repeated three times for each sample. After cooling, the solution was evaporated to a very small volume and could not be evaporated dry. The concentrated solution was then transferred to a 50 ml volumetric flask, deionized water and 0.5 mL HNO_3 were added, Sc was added to give a final concentration of 100 μg L^{-1} for evaluation as an internal standard and the final volume was fixed to 50 mL. The blank digestion was carried out in the same way.

Table A1. *TWO-WAY analysis of variance results (3 types of tissues × 3 different times since the experiment)*

Tests of Between-Subjects Effects

Dependent Variable: Concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29.492[a]	8	3.686	2.157	.084
Intercept	30.156	1	30.156	17.648	.001
Treatment	9.479	2	4.739	2.774	.089
Tissue	16.117	2	8.059	4.716	.023
Treatment * Tissue	3.896	4	.974	.570	.688
Error	30.757	18	1.709		
Total	90.406	27			
Corrected Total	60.249	26			

a. R Squared = .489 (Adjusted R Squared = .263)

Post hoc tests of treatment time

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cd(0h)	Cd(24h)	.1810444	.59163886	.762	-1.0459395	1.4080283
	Cd(10d)	-1.1565556	.59163886	.063	-2.3835395	.0704283
Cd(24h)	Cd(0h)	-.1810444	.59163886	.762	-1.4080283	1.0459395
	Cd(10d)	-1.3376000*	.59163886	.034	-2.5645839	-.1106161
Cd(10d)	Cd(0h)	1.1565556	.59163886	.063	-.0704283	2.3835395
	Cd(24h)	1.3376000*	.59163886	.034	.1106161	2.5645839

Based on observed means.
The error term is Mean Square(Error) = 1.575.

*. The mean difference is significant at the .05 level.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Post hoc tests of different tissue

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Tissue	(J) Tissue	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Leaf	Stem	.2116222	.59163886	.724	-1.0153617	1.4386061
	Root	-1.5228667*	.59163886	.017	-2.7498506	-.2958828
Stem	Leaf	-.2116222	.59163886	.724	-1.4386061	1.0153617
	Root	-1.7344889*	.59163886	.008	-2.9614728	-.5075050
Root	Leaf	1.5228667*	.59163886	.017	.2958828	2.7498506
	Stem	1.7344889*	.59163886	.008	.5075050	2.9614728

Based on observed means.
The error term is Mean Square(Error) = 1.575.

*. The mean difference is significant at the .05 level.

Table A2. *Subcellular concentrations of Cd in different tissues of Scutellaria baicalensis Georgi*

Tissues	Subcellular fraction	Cd concentration (mg kg^{-1})					
		Cd0h-CK	Cd0h	Cd24h-CK	Cd24h	Cd10d-CK	Cd10d
Leaf	Cell wall	0.1027±0.004^{Bb}	0.1027±0.004^{Bb}	0.104±0.0029^{Bb}	0.7086±0.0017^{Ba}	0.1017±0.0032^{Bb}	0.5783±0.0012^{Ba}
	Organelle	0.0576±0.0007^{Cb}	0.0576±0.0007^{Cb}	0.057±0.0011^{Cb}	0.3563±0.0058^{Ca}	0.0576±0.0004^{Cb}	0.2683±0.0029^{Ca}
	Soluble fraction	0.0309±0.0004^{Db}	0.0309±0.0004^{Db}	0.0310±0.0006^{Db}	0.2013±0.0029^{Da}	0.0317±0.0003^{Db}	0.0906±0.0006^{Da}
	Total	0.1911±0.0035^{Ab}	0.1911±0.0035^{Ab}	0.1921±0.004^{Ab}	1.2662±0.003a^{Ac}	0.191±0.0034^{Ab}	0.9373±0.003^{Aa}
Stem	Cell wall	0.047±0.0008^{Bc}	0.047±0.0008^{Bc}	0.0463±0.0019^{Bc}	0.1801±0.0004^{Bb}	0.0464±0.0004^{Bc}	0.3827±0.006^{Ba}
	Organelle	0.0331±0.0005^{Cc}	0.0331±0.0005^{Cc}	0.0336±0.0009^{Cc}	0.0676±0.0049^{Cb}	0.0346±0.0024^{Cc}	0.2253±0.0017^{Ca}
	Soluble fraction	0.0166±0.0014^{Db}	0.0166±0.0014^{Db}	0.0171±0.0003^{Db}	0.101±0.0005^{Da}	0.0166±0.0003^{Db}	0.1667±0.0005^{Da}
	Total	0.0968±0.0014^{Ac}	0.0968±0.0014^{Ac}	0.097±0.0009^{Ac}	0.3486±0.005^{Ab}	0.0977±0.0018^{Ac}	0.7747±0.005^{Aa}
Root	Cell wall	0.1697±0.001^{Bc}	0.1697±0.001^{Bc}	0.16974±0.0024^{Bc}	0.1893±0.0012^{Bb}	0.1701±0.0026^{Bc}	2.903±0.045^{Ba}
	Organelle	0.0953±0.001^{Cb}	0.0953±0.001^{Cb}	0.0954±0.0005^{Cb}	0.1047±0.002^{Ca}	0.0782±0.0278^{Cb}	1.3±0.0082^{Ca}
	Soluble fraction	0.0217±0.0002^{Dc}	0.0217±0.0002^{Dc}	0.0204±0.0009^{Dc}	0.049±0.0002^{Db}	0.0214±0.0006^{Dc}	0.8103±0.0026^{Da}
	Total	0.2867±0.002^{Ac}	0.2867±0.002^{Ac}	0.2855±0.0025^{Ac}	0.343±0.001^{Ab}	0.2697±0.0284^{Ac}	5.014±0.039^{Aa}

Mean ± standard deviation (replicates = 3). The Cd concentration of each subcellular fraction is equal to the Cd content in each subcellular fraction divided by the tissues weight. Different capital letters in the rows and lowercase letters in the columns denote significant variations in the concentration of Cd at various sampling periods various sampling times following the start of treatment, and in the concentration of Cd by various Subcellular fraction at $p < 0.05$, respectively

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DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Table A3. *TWO-WAY analysis of variance results (3 types of tissues × 3 subcellular fractions × 6 treated times)*

Tests of Between-Subjects Effects

Dependent Variable: Concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	30.674[a]	53	.579	6639.431	<0.001
Intercept	6.393	1	6.393	73343.311	<0.001
Tissue	2.136	2	1.068	12254.742	<0.001
Subcellular	1.826	2	.913	10474.275	<0.001
Treatment	9.952	5	1.990	22835.223	<0.001
Tissue * Subcellular	.750	4	.187	2149.897	<0.001
Tissue * Treatment	10.117	10	1.012	11606.720	<0.001
Subcellular * Treatment	2.786	10	.279	3196.505	<0.001
Tissue * Subcellular * Treatment	2.904	20	.145	1665.729	<0.001
Error	.009	102	8.717E-5		
Total	37.528	156			
Corrected Total	30.682	155			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

Post hoc tests of different tissue

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Tissue	(J) Tissue	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
leaf	Stem	.0981463*	.00185209	<0.001	.0944727	.1018199
	Root	-.1771833*	.00185209	<0.001	-.1808569	-.1735097
Stem	Leaf	-.0981463*	.00185209	<0.001	-.1018199	-.0944727
	Root	-.2753296*	.00179679	<0.001	-.2788935	-.2717657
Root	Leaf	.1771833*	.00185209	<0.001	.1735097	.1808569
	Stem	.2753296*	.00179679	<0.001	.2717657	.2788935

Based on observed means.
The error term is Mean Square(Error) = 8.717E-5.

*. The mean difference is significant at the .05 level.

Post hoc tests of subcellular concentrations

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Subcellular	(J) Subcellular	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cell wall	Organelle	.1854865*	.00183101	<0.001	.1818547	.1891183
	Soluble fraction	.2681000*	.00183101	<0.001	.2644682	.2717318
Organelle	Cell wall	-.1854865*	.00183101	<0.001	-.1891183	-.1818547
	Soluble fraction	.0826135*	.00183101	<0.001	.0789817	.0862453
Soluble fraction	Cell wall	-.2681000*	.00183101	<0.001	-.2717318	-.2644682
	Organelle	-.0826135*	.00183101	<0.001	-.0862453	-.0789817

Based on observed means.
The error term is Mean Square(Error) = 8.717E-5.

*. The mean difference is significant at the .05 level.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Post hoc tests of treatment time

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cd0h-CK	Cd0h	.0000000	.00269518	1.000	-.0053459	.0053459
	Cd24h-CK	.0002241	.00261924	.932	-.0049712	.0054193
	Cd24h	-.1592204*	.00261924	.000	-.1644156	-.1540251
	Cd10d-CK	.0020019	.00261924	.446	-.0031934	.0071971
	Cd10d	-.6832426*	.00261924	<0.001	-.6884379	-.6780473
Cd0h	Cd0h-CK	.0000000	.00269518	1.000	-.0053459	.0053459
	Cd24h-CK	.0002241	.00261924	.932	-.0049712	.0054193
	Cd24h	-.1592204*	.00261924	<0.001	-.1644156	-.1540251
	Cd10d-CK	.0020019	.00261924	.446	-.0031934	.0071971
	Cd10d	-.6832426*	.00261924	<0.001	-.6884379	-.6780473
Cd24h-CK	Cd0h-CK	-.0002241	.00261924	.932	-.0054193	.0049712
	Cd0h	-.0002241	.00261924	.932	-.0054193	.0049712
	Cd24h	-.1594444*	.00254104	<0.001	-.1644846	-.1544043
	Cd10d-CK	.0017778	.00254104	.486	-.0032624	.0068179
	Cd10d	-.6834667*	.00254104	.000	-.6885068	-.6784265
Cd24h	Cd0h-CK	.1592204*	.00261924	<0.001	.1540251	.1644156
	Cd0h	.1592204*	.00261924	<0.001	.1540251	.1644156
	Cd24h-CK	.1594444*	.00254104	<0.001	.1544043	.1644846
	Cd10d-CK	.1612222*	.00254104	<0.001	.1561821	.1662624
	Cd10d	-.5240222*	.00254104	<0.001	-.5290624	-.5189821
Cd10d-CK	Cd0h-CK	-.0020019	.00261924	.446	-.0071971	.0031934
	Cd0h	-.0020019	.00261924	.446	-.0071971	.0031934
	Cd24h-CK	-.0017778	.00254104	.486	-.0068179	.0032624
	Cd24h	-.1612222*	.00254104	<0.001	-.1662624	-.1561821
	Cd10d	-.6852444*	.00254104	<0.001	-.6902846	-.6802043
Cd10d	Cd0h-CK	.6832426*	.00261924	<0.001	.6780473	.6884379
	Cd0h	.6832426*	.00261924	<0.001	.6780473	.6884379
	Cd24h-CK	.6834667*	.00254104	<0.001	.6784265	.6885068
	Cd24h	.5240222*	.00254104	<0.001	.5189821	.5290624
	Cd10d-CK	.6852444*	.00254104	.000	.6802043	.6902846

Based on observed means.
The error term is Mean Square(Error) = 8.717E-5.

*. The mean difference is significant at the .05 level.

Table A4. *The concentrations of different chemical forms of Cd (mg kg-1 fresh weight) in different tissues of Scutellaria baicalensis Georgi*

Tissues	Chemical forms	Cd concentration (mg kg^{-1})					
		Cd0h-CK	Cd0h	Cd24h-CK	Cd24h	Cd10d-CK	Cd10d
Leaf	80% ethanol	-	-	-	-	-	-
	d-H2O	-	-	-	-	-	-
	1M NaCl	0.0019±0.0001^{Ec}	0.0019±0.0001^{Ec}	0.0019±0.0001^{Ec}	0.0060±0.0003^{Eb}	0.00190±0.0012^{Ec}	0.0070±0.0001^{Ea}
	2% HAc	0.0185±0.0008^{Dc}	0.0185±0.0008^{Dc}	0.0182±0.0005^{Dc}	0.1135±0.0017^{Db}	0.0180±0.0003^{Dc}	0.2186±0.0054^{Da}
	0.6 M HCl	0.0898±0.0005^{Cc}	0.0898±0.0005^{Cc}	0.0887±0.0018^{Cc}	0.3367±0.0026^{Cb}	0.0883±0.0016^{Cc}	0.4627±0.0234^{Ca}
	Residual	0.0778±0.0585^{Bc}	0.0778±0.0585^{Bc}	0.0783±0.0035^{Bc}	0.3233±0.0052^{Bb}	0.0803±0.0009^{Bc}	0.6036±0.0154^{Ba}
	Total	0.188±0.0589^{Ac}	0.188±0.0589^{Ac}	0.1871±0.0063^{Ac}	0.7795±0.0109^{Ab}	0.1885±0.0026^{Ac}	1.2919±0.0182^{Aa}
Stem	80% ethanol	-	-	-	-	-	-
	d-H2O	-	-	-	-	-	-
	1M NaCl	0.0021±0.0002^{Ec}	0.0021±0.0002^{Ec}	0.0019±0.0002^{Ec}	0.0020±0.0001^{Eb}	0.002±0.0001^{Ec}	0.0074±0.0001^{Ea}
	2% HAc	0.0187±0.0003^{Dc}	0.0187±0.0003^{Dc}	0.0191±0.0004^{Dc}	0.0461±0.0008^{Db}	0.0188±0.0004^{Dc}	0.1229±0.0019^{Da}
	0.6 M HCl	0.0894±0.0012^{Cc}	0.0894±0.0012^{Cc}	0.0886±0.0006^{Cc}	0.1593±0.0040^{Cb}	0.0897±0.0002^{Cc}	0.4373±0.0054^{Ca}
	Residual	0.0799±0.001^{Bc}	0.0799±0.001^{Bc}	0.0817±0.0017^{Bc}	0.1367±0.0042^{Bb}	0.0795±0.0004^{Bc}	0.3927±0.0046^{Ba}
	Total	0.1901±0.0007^{Ac}	0.1901±0.0007^{Ac}	0.1913±0.003^{Ac}	0.3441±0.0073^{Ab}	0.19±0.0005^{Ac}	0.9603±0.0076^{Aa}
Root	80% ethanol	-	-	-	-	-	0.0127±0.0003^{Fa}
	d-H2O	-	-	-	-	-	0.0087±0.0002^{Fa}
	1M NaCl	0.0018±0.0001^{Ec}	0.0018±0.0001^{Ec}	0.0019±0.0002^{Ec}	0.0021±0.0001^{Eb}	0.0019±0.0003^{Ec}	0.0801±0.0005^{Ea}
	2% HAc	0.0176±0.0004^{Dc}	0.0176±0.0004^{Dc}	0.0174±0.0007^{Dc}	0.0409±0.0012^{Db}	0.0172±0.0023^{Dc}	0.5063±0.0329^{Da}
	0.6 M HCl	0.0818±0.0006^{Cc}	0.0818±0.0006^{Cc}	0.0817±0.0020^{Cc}	0.1533±0.0052^{Cb}	0.0816±0.0025^{Cc}	1.9967±0.0330^{Ca}
	Residual	0.0981±0.0017^{Bc}	0.0981±0.0017^{Bc}	0.0985±0.0058^{Bc}	0.139±0.0081^{Bb}	0.0993±0.0004^{Bc}	2.1033±0.0704^{Ba}
	Total	0.1993±0.0009^{Ac}	0.1993±0.0009^{Ac}	0.1995±0.0037^{Ac}	0.3353±0.0075^{Ab}	0.2±0.0052^{Ac}	4.7078±0.0914^{Aa}

Mean ± standard deviation (replicates = 3). The Cd concentration of each chemical form is equal to the Cd content in each chemical form divided by the tissues weight. "-" indicates no detection, detection limit 0.001 mg kg^{-1} Different capital letters in the rows and lowercase letters in the columns denote significant variations in the concentration of Cd at various sampling periods various sampling times following the start of treatment, and in the concentration of Cd by various chemical forms at $p < 0.05$, respectively

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DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Table A5. *TWO-WAY analysis of variance results (3 types of tissues × 6 chemical forms × 6 treated times)*

Tests of Between-Subjects Effects

Dependent Variable: Concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	104.432[a]	125	.835	5290.278	<0.001
Intercept	11.348	1	11.348	71860.011	<0.001
Tissu	3.530	2	1.765	11176.546	<0.001
Treatment	15.469	5	3.094	19590.126	<0.001
Chemical	17.773	6	2.962	18757.107	<0.001
Tissu * Treatment	14.477	10	1.448	9166.848	<0.001
Tissu * Chemical	5.907	12	.492	3116.967	<0.001
Treatment * Chemical	24.217	30	.807	5111.524	<0.001
Tissu * Treatment * Chemical	23.060	60	.384	2433.676	<0.001
Error	.040	252	.000		
Total	115.820	378			
Corrected Total	104.472	377			

a. R Squared = 1.000 (Adjusted R Squared = .999)

Post hoc tests of different tissues

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Tissu	(J) Tissu	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Leaf	Stem	.069048*	.0015833	<0.001	.065930	.072166
	Root	-.161560*	.0015833	<0.001	-.164678	-.158442
Stem	Leaf	-.069048*	.0015833	<0.001	-.072166	-.065930
	Root	-.230608*	.0015833	<0.001	-.233726	-.227490
Root	Leaf	.161560*	.0015833	<0.001	.158442	.164678
	Stem	.230608*	.0015833	<0.001	.227490	.233726

Based on observed means.
The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Post hoc tests of treatment time

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cd 0h-ck	Cd 0h	.000000	.0022391	1.000	-.004410	.004410
	Cd 24h-ck	.002970	.0022391	.186	-.001440	.007380
	Cd 24h	-.120932*	.0022391	<0.001	-.125342	-.116523
	Cd 10d-ck	.025447*	.0022391	<0.001	.021037	.029856
	Cd 10d	-.546100*	.0022391	<0.001	-.550510	-.541690
Cd 0h	Cd 0h-ck	.000000	.0022391	1.000	-.004410	.004410
	Cd 24h-ck	.002970	.0022391	.186	-.001440	.007380
	Cd 24h	-.120932*	.0022391	<0.001	-.125342	-.116523
	Cd 10d-ck	.025447*	.0022391	<0.001	.021037	.029856
	Cd 10d	-.546100*	.0022391	<0.001	-.550510	-.541690
Cd 24h-ck	Cd 0h-ck	-.002970	.0022391	.186	-.007380	.001440
	Cd 0h	-.002970	.0022391	.186	-.007380	.001440
	Cd 24h	-.123903*	.0022391	<0.001	-.128312	-.119493
	Cd 10d-ck	.022477*	.0022391	<0.001	.018067	.026886
	Cd 10d	-.549070*	.0022391	<0.001	-.553480	-.544660
Cd 24h	Cd 0h-ck	.120932*	.0022391	<0.001	.116523	.125342
	Cd 0h	.120932*	.0022391	<0.001	.116523	.125342
	Cd 24h-ck	.123903*	.0022391	<0.001	.119493	.128312
	Cd 10d-ck	.146379*	.0022391	<0.001	.141969	.150789
	Cd 10d	-.425168*	.0022391	<0.001	-.429577	-.420758
Cd 10d-ck	Cd 0h-ck	-.025447*	.0022391	<0.001	-.029856	-.021037
	Cd 0h	-.025447*	.0022391	<0.001	-.029856	-.021037
	Cd 24h-ck	-.022477*	.0022391	<0.001	-.026886	-.018067
	Cd 24h	-.146379*	.0022391	<0.001	-.150789	-.141969
	Cd 10d	-.571547*	.0022391	<0.001	-.575956	-.567137
6.00	Cd 0h-ck	.546100*	.0022391	<0.001	.541690	.550510
	Cd 0h	.546100*	.0022391	<0.001	.541690	.550510
	Cd 24h-ck	.549070*	.0022391	<0.001	.544660	.553480
	Cd 24h	.425168*	.0022391	<0.001	.420758	.429577
	Cd 10d-ck	.571547*	.0022391	.000	.567137	.575956

Based on observed means.
The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2204_35393562

Post hoc tests of chemical forms

Multiple Comparisons

Dependent Variable: Concentration

LSD

(I) Chemical form	(J) Chemical form	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
80% ethanol	d-H2O	-.000439	.0024185	.856	-.005202	.004324
	1M NaCl	-.006903*	.0024185	.005	-.011666	-.002140
	2% HAc	-.055648*	.0024185	<0.001	-.060411	-.050885
	0.6 M HCl	-.169625*	.0024185	<0.001	-.174388	-.164862
	Residual	-.373555*	.0024185	<0.001	-.378318	-.368792
	Total	-.606396*	.0024185	<0.001	-.611159	-.601633
d-H2O	80% ethanol	.000439	.0024185	.856	-.004324	.005202
	1M NaCl	-.006465*	.0024185	.008	-.011228	-.001702
	2% HAc	-.055209*	.0024185	<0.001	-.059972	-.050446
	0.6 M HCl	-.169186*	.0024185	<0.001	-.173949	-.164423
	Residual	-.373116*	.0024185	<0.001	-.377879	-.368353
	Total	-.605957*	.0024185	<0.001	-.610720	-.601194
1M NaCl	80% ethanol	.006903*	.0024185	.005	.002140	.011666
	d-H2O	.006465*	.0024185	.008	.001702	.011228
	2% HAc	-.048745*	.0024185	<0.001	-.053508	-.043982
	0.6 M HCl	-.162722*	.0024185	<0.001	-.167485	-.157959
	Residual	-.366651*	.0024185	<0.001	-.371414	-.361888
	Total	-.599493*	.0024185	<0.001	-.604256	-.594730
2% HAc	80% ethanol	.055648*	.0024185	<0.001	.050885	.060411
	d-H2O	.055209*	.0024185	<0.001	.050446	.059972
	1M NaCl	.048745*	.0024185	<0.001	.043982	.053508
	0.6 M HCl	-.113977*	.0024185	<0.001	-.118740	-.109214
	Residual	-.317907*	.0024185	<0.001	-.322670	-.313144
	Total	-.550748*	.0024185	<0.001	-.555511	-.545985
0.6 M HCl	80% ethanol	.169625*	.0024185	<0.001	.164862	.174388
	d-H2O	.169186*	.0024185	<0.001	.164423	.173949
	1M NaCl	.162722*	.0024185	<0.001	.157959	.167485
	2% HAc	.113977*	.0024185	<0.001	.109214	.118740
	Residual	-.203930*	.0024185	<0.001	-.208693	-.199167
	Total	-.436771*	.0024185	<0.001	-.441534	-.432008
Residual	80% ethanol	.373555*	.0024185	<0.001	.368792	.378318
	d-H2O	.373116*	.0024185	<0.001	.368353	.377879
	1M NaCl	.366651*	.0024185	<0.001	.361888	.371414
	2% HAc	.317907*	.0024185	<0.001	.313144	.322670
	0.6 M HCl	.203930*	.0024185	<0.001	.199167	.208693
	Total	-.232841*	.0024185	<0.001	-.237604	-.228078
Total	80% ethanol	.606396*	.0024185	<0.001	.601633	.611159
	d-H2O	.605957*	.0024185	<0.001	.601194	.610720
	1M NaCl	.599493*	.0024185	<0.001	.594730	.604256
	2% HAc	.550748*	.0024185	<0.001	.545985	.555511
	0.6 M HCl	.436771*	.0024185	<0.001	.432008	.441534
	Residual	.232841*	.0024185	<0.001	.228078	.237604

Based on observed means.
The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

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