**Effects of oxalic acid-enhanced lime on growth and cadmium** **accumulation of *Panax notoginseng* under cadmium stress**

Qi Li1, Yumeng Liao1#, Zuran Li2\*, Yanqun Zu1\*#, Na Li1, Xinyue Mei1, Li Qin1, Bo Li1

1 College of Resources and Environment, Yunnan Agricultural University, Kunming 650201, China

2 College of Landscape and Horticulture, Yunnan Agricultural University, Kunming 650201, China

\*For correspondence:zuyanqun@qq.com

**Abstract**

Under 6 mg·kg-1 Cd treatment, the contents of organic acids (oxalic acid, tartaric acid, acetic acid, citric acid, malic acid and succinic acid) in the plants and root exudates of two-year-old *Panax notoginseng* were analyzed with 0, 750, 2 250, 3 750 kg·hm-2 lime application. Based on the changes of organic acids in root exudates of *P. notoginseng* under Cd stress with different lime application rates, field experiments were conducted with different lime application rates coupling with foliar spraying oxalic acid to understand the biomass, Cd content, Cd formation and accumulation characteristics of three-year-old *P. notoginseng*. The results showed that: (1) Under 6 mg·kg-1 Cd stress, the Cd content of *P. notoginseng* root was significantly reduced with lime application. Cd content in root decreased by 70.1% with 3 750 kg·hm-2 lime application. (2) Oxalic acid content was the highest low-molecular organic acids in the shoots of *P. notoginseng*, while oxalic acid and tartaric acid were the main acids in the roots. Compared to non-lime application under Cd stress, the oxalic acid contents increased by 33.59% and 76.90% in shoots and roots, respectively, with 750 kg·hm-2 lime application. The oxalic acid contents in the root exudates of *P. notoginseng* decreased with the increase of lime application rates. (3) The Cd contents of *P. notoginseng* decreased by 68.57% with 2 250 kg·hm-2 lime and 0.1 mol·L-1 oxalic acid application. (4) The Cd transformation of sodium chloride was promoted with oxalic acid spraying. The contents of sodium chloride extraction and acetic acid extraction formations were the highest. Therefore, it could be recommended that 2 250 kg·hm-2 lime application and 0.1 mol·L-1 oxalic acid spraying to make the Cd content of *P. notoginseng* met the standard value, ensured the quality and safety of *P. notoginseng* under Cd stress.

**Keywords:** *Panax notoginseng*; Cd morphology; lime; organic acid

**Introduction**

With the increase of environmental stress, the problem of soil environmental pollution becomes more and more prominent (Li et al., 2015a). According to the National Survey Bulletin of Soil Pollution in 2014, the Cd pollution of cultivated land and soil in China was serious, and the rate of exceeding the standard value of Cd was 7.0% (Zhuang, 2015). The spatial distribution of soil Cd content increased gradually from northeast to southwest in China. Wenshan prefecture of Yunnan Provinceis the main producing area of *Panax notoginseng,* a traditional Chinese medicinal material. Because of the mining of lead and zinc ore, the local Cd soil background value was higher (Zu et al., 2017). *P. notoginseng* is a herb of the genus Panax with its root as the main medicinal part (Zu et al., 2016). *P. notoginseng* has a certain enrichment ability to Cd. Soil Cd pollution not only caused the Cd contents of *P. notoginseng* exceeded the standard value, but also hindered the growth of plant roots (Li et al., 2015b). It had toxic effect on physiological metabolism of *P. notoginseng* and reduced the yield of *P. notoginseng* (Zhu et al., 2014). The potential risk of soil Cd pollution in *P. notoginseng* planting area was not able to be ignored.

Plants could form some avoidance and resistance mechanisms to environmental Cd2+ (Sun et al., 2018). Avoidance mechanism included that the root system prevents Cd from being absorbed by the root system through root exudates and morphological changes (Choppala et al., 2014). Cd in plant stimulated chelate (Anjum et al., 2015), antioxidant enzyme system (Zhou, 2018), osmotic regulation and other resistance mechanisms to inhibit Cd toxicity. Low molecular weight organic acids (LMWOAs) played an important role. LMWOAs is a class of compounds containing carboxyl groups (-COOH) with molecular weight less than 300 (Kou et al., 2019), which include oxalic acid, acetic acid, citric acid, tartaric acid, succinic acid and malic acid found in plants and root exudates. Plant roots can affect the absorption of Cd through secretion LMWOAs, such as the increase of root secretion with the increase of stress degree (Yuan et al., 2019). Many experiments show that these small molecular compounds can be activated by soil Cd and promote the absorption of Cd by roots, which mainly affect Cd migration through acid dissolution and reduction (Wang et al., 2017). LMWOAs in plants can induce stress resistance, and their chelation was an important way of intracellular detoxification (Verbruggen et al., 2009). LMWOAs precipitate or chelate heavy metals on the cell wall, change the form of heavy metals, and improve plant resistance to stress. Oxalic acid, acetic acid, citric acid could reduce toxicity by directly binding to Cd, such as oxalic acid chelation Cd2+ in tobacco. The formation of crystals containing Cd and Ca, which are excreted through the apical cells of the trichomes, reduced Cd2+ content in plants (Choi et al., 2001). In addition, citric acid could reduce the toxicity of heavy metals by increasing the antioxidant enzyme activity of duckweed (Sallah-Ud-Din et al., 2017).

Lime is a commonly used passivator for remediation of soil heavy metal pollution, which was widely used because of its low cost and high efficiency (Hu et al., 2017), whose application rates mainly ranged in 750~6000 kg·hm-2 (Zeng et al., 2017). Many studies have shown that the bioavailability of Cd in soil reduced with lime application and increasing soil pH values (Tian et al., 2016). Ca2+ has a very complex regulatory mechanism in plants, which participates in the regulation of plant absorption and transport of various ions(White and Broadley, 2016), act as a messenger Cd transmit stress signals (Fu, 2015), promote hormone synthesis in plants and regulate plant stress resistance (Subramanian and Viswanathan, 2007). Ca2+ obviously promoted the accumulation of organic acids in Tartary buckwheat (Lu et al., 2020). Organic acids play an important role in regulating plant stress resistance under Cd stress, so the application of lime may be due to Ca2+ regulating plant organic acid metabolism and reduce Cd toxicity. Nowadays, a great deal of research had focused on the passivation and remediation of Cd in soil with lime application. There were few studies on the specific regulation process of Ca2+ by promoting the biosynthesis of organic acids, thereby changing the formation of Cd and improving plant resistance, especially the effects of organic acids on plant growth and Cd accumulation.

The regulation of heavy metal accumulation in crops by foliar spraying chelating agent is a new research direction of heavy metal control in safe agriculture in recent years (Li, 2019). Compared with traditional soil fertilization, it is not only low cost and easy to operate, but also greatly improves the absorption efficiency of crops, so it has a wide application prospect (Li et al., 2020). Therefore, the response of growth and cadmium accumulation characteristics of *P. notoginseng* to Cd stress mediated by oxalic acid and lime could provide a theoretical and practical basis for the safe production of *P. notoginseng*.

**Materials and Methods**

**Experimental design**

The field experiment was conducted in in Lannizhai, Qiubei County, Wenshan Prefecture, Yunnan Province (N 24°11′, E 104°3′, 1 446 m above sea level). The average annual temperature was 17 ℃ and the annual average precipitation was 1250 mm. The background values of the tested soil were TN 0.57 g·kg-1, TP 1.64g·kg-1, TK 16.31 g·kg-1，OM 31.86 g·kg-1, alkali hydrolyzed N 88.82 mg kg-1, available P 18.55mg kg-1, available K 100.37 mg kg-1, total Cd 0.3 mg kg-1 and pH 5.4.

The 6 mg·kg-1 Cd2+ (CdCl2·2.5H2O) and the lime treatments (0, 750, 2250 and 3750 kg·hm-2) were applied and mixed with the surface 0~10 cm soil layer of each plot on December 10, 2017. Each treatment was repeated for 3 times.The experimental plots were arranged randomly, and the area of each plot was 3 m2. One-year-old *P. notoginseng* seedlings were transplanted after 15 days of soil culture. The shading net was used and the light intensity of *P. notoginseng* in the shading shed was about 18% of the normal natural light intensity. It was cultivated according to the local conventional cultivation and management. Ten plants were collected in each cell at the mature stage of biennial *P. notoginseng* (October 20, 2018) for the extraction and determination of organic acids in roots, shootsand root exudates.

Until the maturity period of *P. notoginseng* in 2019, oxalic acid was sprayed in the form of sodium oxalate. The concentrations of oxalic acid were 0, 0.1 and 0.2 mol·L-1, and the pH was adjusted to 5.16 with NaOH, which was simulated the average pH of litter leaching solution. The upper and lower surfaces of leaves were sprayed at 8:00 am in the morning once a week. After 4 times spray, three-year-old *P. notoginseng* plants were collected in the 5th week for the determination of biomass, cadmium content and cadmium formation in plant. Plant samples were divided to roots, stems and leaves. Part of plants were dried 30 minutes at 105℃ after washing with tap water and to constant weight at 75℃, measured biomass, then grind samples in a mortar for Cd contents determination. Part of fresh roots, stems and leaves were used for determine Cd chemical formations.

**Extraction and concentration of root exudates**

The fresh *P. notoginseng* plants were carefully cleaned with clear water, then washed with deionized water once, then put roots in a container containing 250 mL of distilled water. The distilled water immersed the whole root system. After 24 h of continuous ventilation, the root system was removed and the root exudate was collected. Because the concentration of root exudates was low, it was necessary to concentrate in order to detect the content conveniently. The filtered collection solution was concentrated to 10 mL, at 30℃ and stored in -20℃ freezer.

**Parameters determination**

1. **Organic acids contents in root exudates**

Concentrated root exudates were filtered with 0.45 μm membrane. The organic acid secreted by root system was determined high performance liquid chromatograph (Thermo HPLC-ultimate 3000, Thermo Fisher Technology Co., Ltd.). The determination condition was ODS reverse phase column with a sample volume of 15 μL, and a column temperature of 35℃. 0.01 mol·L-1 KH2PO4 of mobile phase prepared with deionized water. pH value was adjusted to 2.65 with 20% of the H3PO4 with flow rate 1.0 mL·min-1 and UV detection wavelength 214 nm. The standard acids were oxalic acid, citric acid, acetic acid, malic acid, succinic acid and tartaric acid. The contents of organic acids was calculated by peak area method (Zhang et al., 2010).

1. **Organic acids contents in plants**

The sample solution was obtained by water bath filtration after grinding 5 g of fresh sample, and then determined by high performance liquid chromatograph (Thermo HPLC-ultimate 3000, Semerfishil Technology Co., Ltd.) after filtration with 0.45 μm membrane. The condition was the same as determination of organic acids contents in root exudates.

1. **Cd contents in plant**

Some 0.2 g of plant samples were accurately weighed and put into a triangular flask with a few glass beads, added 10 mL of mixed acid (HNO3: HClO4 = 4:1), and covered overnight. A small funnel was added to digest on the electric furnace until the white smoke arisen, and the digestive solution was colorless or transparent or slightly yellow. After cooling to room temperature, the mixture was moved to a 10 mL volumetric flask. The cadmium content was determined by a flame atomic absorption spectrophotometer (Thermo ICETM 3300 AAS, USA) (GB/T 23739-2009). Referring to the "Geographical Products Wenshan Sanqi" GB/T 19086-2008, the Cd limit standard should be ≤0.5 mg kg-1.

1. **Contents of Cd chemical formation**

0.5000 g fresh plant sample was weighted and transferred to 50 mL plastic centrifuge tube after grinding homogenate. The mixed solution was oscillated 22 h under 25℃, then centrifuged 10 min at 5000 r·min-1. The supernatant was poured out. 10 mL 80% ethanol was added and oscillated 1 h under 25℃, then centrifuged 10 min at 5000r·min-1. The supernatant was poured out. The two supernatants were merged to determined amino acid and alcohol-soluble protein bound formation (FF). Deionized water was added to the precipitate obtained from the second centrifugation to extract soluble organic bound and heavy metals dihydrogen phosphate [M(H2PO4)2] formation (FW). 2% acetic acid was added for extraction of insoluble heavy metal phosphates formation (FHAc); and 0.6 mol·L-1 Hydrochloric acid added mainly for extraction heavy metal oxalate formation(FHCl). The Cd chemical formations were measured with a flame atomic absorption spectrophotometer (Thermo ICETM 3300AAS, Thermo Fisher Technology Ltd).

After 5 extraction, some deionized water was added to the sediments into a triangle bottle. The mixture was dried on an electric heating plate, and added 2 mL nitric acid and a few drops of perchloric acid until clarified. The solution was determined the residue formation (FR) with a flame atomic absorption spectrophotometer (Thermo ICETM 3300AAS, Thermo Fisher Technology Ltd) (Yu et al., 2018).

**Data statistical analysis**







The data were sorted out by Excel 2010 software and plotted by Origin Pro 9.1. The statistical analysis software SPSS Statistics 19 was used to test the difference(*P*<0.05).

**Results**

**Cadmium content in Panax notoginseng**

The Cd content in shoots and roots decreased significantly with increase of lime application rates under Cd stress(Fig. 1). The contents of Cd in shoot and root decreased by 67.78% and 70.07%, respectively, with 3 750 kg·hm-2 lime application under Cd stress. Cd contents in roots were higher than in shoots.

**Fig.1** Effect of lime application on contents of Cd in shoots and roots of *Panax notoginseng* under cadmium stress

Notes: The figure legend showed different lime treatments. Lowercase letters indicate significant difference at 5% level between lime application treatments. The same as below.

**Effects of different lime application rates on organic acids contents in Panax notoginseng under Cd stress**

The oxalic acid and tartaric acid were the main organic acids in the roots. The acetic acid contents were the lowest in shoots or roots. The oxalic acid contents in shoots was higher than in roots. The tartaric acid contents in shoots were less than in roots (Fig. 2).

Under Cd stress and no lime application, the contents of organic acids except succinic acid increased slightly, which indicated that the synthesis of oxalic acid, citric acid, acetic acid, malic acid and tartaric acid in plant shoots were promoted. With the increase of lime application rates, the contents of oxalic acid, citric acid and malic acid gradually increased, which increased by 49.90%, 64.71% and 85.61%, respectively, under 3 750 kg·h m-2 lime application compared with no lime application. The contents of succinic acid decreased first and then increased with lime application rates. The oxalic acid contents increased significantly with the increase of lime application rates, which increased by 33.59% with 3 750 kg·h m-2 lime application compared with no lime application. There was no significant difference between malic acid and acetic acid contents.

The contents of oxalic acid and tartaric acid in the roots of *P. notoginseng* increased first and then decreased with the increase of lime application, while succinic acid contents decreased first and then increased. The oxalic acid contents increased first and then decreased with the increase of lime application rates, which increased by 76.90% with 3 750 kg·h m-2 lime application compared with no lime application.

**Fig.2** Effect of lime application on contents of the organic acid in *Panax notoginseng* under cadmium stress

**Effects of different lime application rates on organic acids contents of root exudates of Panax notoginseng under Cd stress**

Under Cd stress, the contents of organic acid secreted by *P. notoginseng* roots were changed by applying lime (Fig.3). Under 0 mg·kg-1 Cd treatment, the contents of citric acid did not change significantly with four different rates of lime application. Under 6 mg·kg-1Cd treatment, citric acid contents with 750, 2 250 and 3 750 kg·hm-2 lime application, which the difference between lime treatments was not significant, were significantly lower than that of 0 kg·hm-2 lime treatment.

Under 0 mg·kg-1 Cd treatment, the contents of malic acid changed with different rates of lime application. With the increase of lime application rates, the contents of malic acid increased significantly. Compared with 0 kg·hm-2 lime treatment, the contents of malic acid increased by 80.91% with 2 250 kg·hm-2 lime application. The secretion of malic acid was inhibited with the 3 750 kg·hm-2 lime application. Under 6 mg·kg-1Cd treatment, the secretion of malic acid was inhibited with the increase of lime application rates. The contents of malic acid secreted was reduced by 76.09% with 2 250 kg·hm-2 lime application compared with 0 kg·hm-2lime treatment. The contents of malic acid increased with the application of 3 750 kg·hm-2lime, which was still significantly lower than that with the treatment of 0 kg·hm-2 lime. The change trend of acetic acid contents was consistent with the change of malic acid with lime application under Cd stress. The contents of acetic acid with 2 250 kg·hm-2 lime application decreased by 46.72% compared with 0 kg·hm-2 lime treatment under 6mg·kg-1 Cd stress.

Under 0 mg·kg-1 Cd treatment, the secretion of succinic acid was inhibited with the increase of lime application rates. Under 6 mg·kg-1 Cd treatment, the contents of succinic acid increased first and then decreases with increase of the rates of lime application. Compared with 0 kg·hm-2 lime treatment, the contents of succinic acid increased by 100.00% with 750 kg·hm-2 lime application. Compared with 750 kg·hm-2 lime application, the contents of succinic acid were significantly inhibited with 3 750 kg·hm-2 lime application.

Under 0 mg·kg-1 Cd treatment, the content of oxalic acid decreased by 17.66% with 750 k g·hm-2 lime compared with 0 kg·hm-2 lime, which there were no significant differences between other lime treatments. Under 6 mg·kg-1 Cd stress, oxalic acid contents decreased significantly with the increase of lime application rates. The oxalic acid contents with 2 250 kg·hm-2 lime application reduced by 52.05% compared with 0 kg·hm-2 lime application. The contents of tartaric acid with 7 50 kg·hm-2 lime application were significantly increased compared with 0kg·hm-2 lime application and 0 mg·kg-1 Cd treatment. There was no significant difference in the secretion of tartaric acid under 6 mg·kg-1 Cd treatment.

**Fig.3** Effect of lime application on contents of organic acids of root exudates of *Panax notoginseng* under cadmium stress

**Effects of foliar spraying oxalic acid on biomass of Panax notoginseng under Cd stress with different lime application**

The root biomass increased first and then decreased with the increase of oxalic acid spraying concentrations (Table 1). when oxalic acid was not applied, the root biomass increased with the increase of lime application rates. With 0.1 and 0.2 mol·L-1 oxalic acid treatments, the root biomass increased first and then decreased with the increase of lime application rates. Compared with the control, the root biomass of *P. notoginseng* increased by 65.36% with 750 kg·hm-2 lime and 0.1 mol·L-1 oxalic acid treatment. However, the root biomass decreased by 4.15% with 3 750 kg·hm-2 lime and 0.2 mol·L-1 oxalic acid treatment compared with the control. Without lime application, the root biomass decreased significantly by 32.97% with 0.2 mol·L-1 oxalic acid treatment comparing with 0.1mol·L-1 oxalic acid treatment. Under 750kg·hm-2 lime treatment, the root biomass of oxalic acid increased significantly by 57.59% and 35.58%, respectively, with 0.1 mol·L-1 and 0.2 mol·L-1 oxalic acid spraying. Under 2 250 kg·hm-2 lime treatment, the root biomass decreased significantly by 23.51% with 0.2 mol·L-1 oxalic acid treatment comparing with 0.1mol·L-1 oxalic acid treatment.

When lime was not applied, the biomass of stem increases first and then decreases with the increase of oxalic acid concentrations. With 750 kg·hm-2 lime application, the biomass of stem decreased first and then increased with with the increase of oxalic acid concentrations. However, with 2 250 kg·h m-2 and 3 750 kg·hm-2 lime application, the stem biomass decreased with the increase of oxalic acid concentrations. With 0 and 0.2 mol·L-1 oxalic acid spraying, the biomass of stem increased first and then decreased with increase of lime application rates. Compared with the control, except 0.2 mol·L-1 oxalic acid treatment, the biomass of *P. notoginseng* stem was higher than that of control. The biomass of *P. notoginseng* stem increased by 30.73% with 750 kg·hm-2 lime application + 0.2 mol·L-1 oxalic acid spraying. Without lime application, the stem biomass decreased significantly by 32.08% with 0.2 mol·L-1oxalic acid treatment comparing with 0.1mol·L-1 oxalic acid treatment. With 2 250 kg·h m-2 lime application, the biomass of stem decreased by 24.38% with 0.2 mol·L-1 oxalic acid treatment compared with non oxalic acid spraying.

Under 0 and 750 kg·hm-2 lime application, the leaf biomass increased first and then decreased with the increase of oxalic acid concentrations. Under 2 250 kg·hm-2 and 3 750 kg·h m-2 lime application, leaf biomass decreased with the increase of oxalic acid concentrations.The leaf biomass increased first and then decreased with the increase of lime application rates. Compared with the control, the biomass of *P. notoginseng* leaves increased by 36.39% with 750 kg·hm-2 lime and 0.1 mol·L-1 oxalic acid treatment. Without lime application, the leaf biomass under the treatment of 0.2 mol·L-1 oxalic acid reduced by 40.60% comparing with 0.1mol·L-1oxalic acid treatment. Under 750kg·hm-2 lime application, leaf biomass reduced by 33.40% with 0.2 mol·L-1oxalic acid treatment compared with 0.1mol·L-1 oxalic acid treatment.

**Table 1:** Effects of oxalic acid and lime on the biomass (dry weight) of *Panax notoginseng* under Cd stress

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Oxalic acid concentration/mol·L-1 | | Lime application/kg·hm-2 | | | |
| 0 | 750 | 2 250 | 3 750 |
| Root | 0 | 8.92±1.19ab | 9.36±1.45b | 10.75±1.50ab | 12.12±2.87a |
| 0.1 | 12.80±2.41a | 14.75±1.19a | 13.57±1.72a | 13.11±1.53a |
| 0.2 | 8.58±0.80b | 12.69±1.00a | 10.38±1.02b | 8.55±2.38a |
| Stem | 0 | 1.79±0.14ab | 1.81±0.20a | 2.42±0.28a | 2.21±0.13a |
| 0.1 | 2.12±0.34a | 1.68±0.18a | 2.23±0.27ab | 1.84±0.32a |
| 0.2 | 1.44±0.13b | 2.34±0.37a | 1.83±0.20b | 1.81±0.18a |
| Leaves | 0 | 3.49±0.34a | 3.52±0.51ab | 4.22±0.80a | 4.03±0.94a |
| 0.1 | 4.63±0.38a | 4.76±0.65a | 3.16±0.55b | 3.07±0.58a |
| 0.2 | 2.75±0.58b | 3.17±0.27b | 2.90±0.41b | 2.99±0.58a |

Notes: Date were means ±SD. Different lowercase letters in the same column of the same part indicate significant differences at the level of *P* <0.05.

**Cd Effects of foliar spraying oxalic acid on Cd content and chemical formation of Panax notoginseng under different lime applications**

The Cd contents of *P. notoginseng* were between 0.15-0.70mg·kg-1. With the same oxalic acid concentration spraying, the Cd content of each part was greatly reduced with increased of lime application from 0 to 2 250 kg·hm-2. The Cd content of the root decreased more significantly. Compared with the control, Cd content decreased 68.57% with 2 250 kg·h m-2 lime application and 0.1 mol·L-1 oxalic acid spraying. With 0 and 750 kg·hm-2 lime application, the Cd contents of root, stem and leaf of *P. notoginseng* decreased with the increase of oxalic acid concentrations. With lime and oxalic acid treatments, the cadmium content in the root met the standard value (GB19086-2008, Cd ≤0.5 mg·kg-1).

The two-factor ANOVA showed that the Cd contents of *P. notoginseng* root, stem and leaf were significantly affected by the rate of lime application. The Cd contents of stem and leaf were significantly affected by the interaction between lime and oxalic acid (*P* <0.05).

**Table 2** Effects of oxalic acid and lime on the cadmium contents and accumulation characteristics of *Panax notoginseng* under Cd stress

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Lime application/kg·hm-2 | Oxalic acid concentration/mol·L-1 | Cd content/ mg·kg-1 | | | | | EC | TF | BTF |
| Root | Stem | | Leaves | |
| 0 | 0 | 0.70±0.09a | 0.55±0.06a | | 0.59±0.09a | | 0.10 | 0.84 | 0.51 |
| 0.1 | 0.61±0.07ab | 0.50±0.01ab | | 0.43±0.02b | | 0.09 | 0.75 | 0.41 |
| 0.2 | 0.56±0.07bc | 0.42±0.03b | | 0.39±0.08bc | | 0.08 | 0.73 | 0.36 |
| 750 | 0 | 0.47±0.04c | 0.45±0.07b | | 0.41±0.07bc | | 0.07 | 0.90 | 0.51 |
| 0.1 | 0.33±0.04d | 0.31±0.04c | | 0.30±0.04cd | | 0.05 | 0.92 | 0.40 |
| 0.2 | 0.28±0.01d | 0.25±0.01cd | | 0.24±0.02de | | 0.04 | 0.89 | 0.39 |
| 2 250 | 0 | 0.31±0.03d | 0.25±0.02cd | | 0.22±0.01de | | 0.04 | 0.75 | 0.49 |
| 0.1 | 0.22±0.05d | 0.21±0.03d | | 0.15±0.02e | | 0.03 | 0.84 | 0.34 |
| 0.2 | 0.23±0.03d | 0.19±0.02d | | 0.17±0.02e | | 0.03 | 0.80 | 0.36 |
| 3 750 | 0 | 0.23±0.04d | 0.21±0.02d | | 0.19±0.05de | | 0.03 | 0.86 | 0.49 |
| 0.1 | 0.25±0.01d | 0.24±0.02cd | | 0.22±0.04de | | 0.04 | 0.96 | 0.36 |
| 0.2 | 0.27±0.02d | 0.22±0.05d | | 0.23±0.06de | | 0.04 | 0.84 | 0.53 |
| ANOVA | |  | |  | |  |  |  |  |
| Lime | | 74.99\*\* | | 67.89\*\* | | 40.91\*\* |  |  |  |
| Oxalic acid | | 7.72\* | | 12.50\* | | 8.25\*\* |  |  |  |
| lime × oxalic acid | | 1.97 | | 3.46\* | | 2.60\* |  |  |  |

Notes：Date were means ±SD. Different lowercase letters in the same column indicate significant differences at the level of *P* <0.05.

The percentage of sodium chloride extraction and acetic acid extraction formation in roots, stems and leaves were the highest, followed by deionized water extraction, ethanol extraction and residue state (Fig .4). The percentage of sodium chloride extraction formation without lime application was generally higher than that with lime application. The percentage of sodium chloride extraction formation in roots, stems and leaves with control was 80.5%, 73.8% and 65.8%, respectively. With increase of the rates of lime application, the percentage of sodium chloride extraction formation in roots, stems and leaves decreased gradually, while the extraction state of acetic acid and deionized water formation increased gradually. Different from the roots, the percentage of Cd ethanol extraction formation in leaves increased greatly with spraying of oxalic acid concentrations (Fig.4).



**Fig.4** Effects of foliar spraying of oxalic acid on the chemical formation percentage of cadmium in *Panax notoginseng* under cadmium stress

Notes: The legend shows the percentage of six cadmium extraction states, FF:ethanol extraction state, FW : deionized water extraction state, FNaCl :sodium chloride extraction state, FHAc: acetic acid extraction state, FHCl: hydrochloric acid extraction state, FR: residue state.A,B,C,and D respectively represent the lime application rate of 0,750,2 250,and 3 750kg·hm-2.The same below.

The analysis of variance showed that the Cd ethanol extraction formation contents of *P. notoginseng* roots were significantly affected by the rates of lime application. The Cd ethanol extraction formation contents of stems and leaves were greatly affected by lime and oxalic acid. The Cd deionized water extraction formation contents of roots, stems and leaves were significantly affected by lime and oxalic acid. The sodium chloride extraction formation contents in roots, stems and leaves were significantly affected by lime application. The contents of Cd acetic acid extraction formation of *P. notoginseng* roots, stems and leaves were significantly affected by lime, lime and oxalic acid. The contents of Cd acetic acid extraction formation in leaves were also regulated by oxalic acid, and the contents of Cd hydrochloric acid extraction formation in stems and leaves were significantly affected by lime.

**Table 3:** Variance analysis of the effects of oxalic acid and lime on the chemical formation of Cd in various parts of *Panax notoginseng* (F value)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Root | | | | | |
| Treatments | FF | FW | FNaCl | FHAc | FHCl | FR |
| Lime | 3.78\* | 0.68 | 27.49\*\* | 10.12\*\* | 2.11 | 1.22 |
| Oxalic acid | 0.73 | 0.99 | 4.22\* | 1.51 | 0.79 | 7.00\*\* |
| lime × oxalic acid | 1.96 | 4.08\*\* | 1.76 | 6.34\*\* | 2.27 | 1.16 |
|  | Stem | | | | | |
|  | FF | FW | FNaCl | FHAc | FHCl | FR |
| Lime | 7.18\*\* | 4.57\* | 11.96\*\* | 6.32\*\* | 4.67\* | 5.71\*\* |
| Oxalic acid | 25.65\*\* | 0.32 | 0.49 | 2.56 | 2.51 | 1.94 |
| lime × oxalic acid | 8.81\*\* | 5.00\*\* | 0.33 | 5.84\*\* | 2.19 | 2.26 |
|  | Leaf | | | | | |
|  | FF | FW | FNaCl | FHAc | FHCl | FR |
| Lime | 3.70\* | 3.66\* | 18.96\*\* | 18.48\*\* | 3.49\* | 3.86\* |
| Oxalic acid | 4.92\* | 3.63\* | 1.48 | 5.53\* | 2.65 | 0.2 |
| lime × oxalic acid | 2.94\* | 2.51\* | 1.98 | 8.30\*\* | 2.03 | 1.25 |

Notes: In the two-way analysis of variance, \* indicates *P* <0.05, and \*\* indicates *P* <0.01.

**Discussion**

The soil pH in *P. notoginseng* planting area of experiment site was 5.4. Acidic soils are not conducive to crop growth due to the toxic effects of active aluminum, strong phosphorus-fixing ability, and other effective nutrient elements. The application of lime can alleviate the harm of acidic soil and promote the absorption of nutrients by plants. Studies have shown that the effects of lime application of 3 000 to 6 000 kg·hm-2 were significantly higher than that of 750 kg·hm-2 lime treatmentfor 5.0~5.5 pH acidic soil (Zeng et al., 2017). However, excessive use of lime was detrimental to plant growth (Yan et al., 2018). The application of lime reduced the effectiveness of soil Cd by changing soil pH, and the Cd contents in the shoots and root of *P. notoginseng* decreasing, thus alleviating the toxicity of Cd to plants. The limit value of Cd was Cd ≤0.5 mg·kg-1 according to Wenshan *Panax notoginseng* standard (GB19086-2008). Without lime treatment, Cd contents in all parts under Cd stress exceeded the standard value. The Cd content in the shoots and roots met the standard vaue with the 3 750 kg·hm-2 lime application, which was similar to that of Zeng’s reports (Zeng et al., 2017) .

Plants adapt to environmental stress by altering organic acid metabolism and rhizosphere organic acid secretion (Henry et al., 2007). Different lime application rates have different effects on organic acids in the shoots and roots of *P. notoginseng*. Contents of oxalic acid, citric acid and acetic acid were significantly changed in *P. notoginseng* with different lime application under Cd treatment. Oxalic acid contents was the highest organic acid in the shoots, and oxalic acid and tartaric acid were the main organic acids in the roots. Whether shoots or roots, oxalic acid contents showed a positive response to lime under Cd treatment. Both Cd2+ and Ca2+ promoted the increase of oxalic acid contents in *P. notoginseng*. Oxalic acid played an important role in regulating plant stress resistance. Qin et al studied organic acids in maize under Cd stress found that the increase of oxalic acid concentration in plants was helpful to improve the adaptability of plants to adversity (Qin, 2017). Under Cd stress, the slight increase of oxalic acid contents in *P. notoginseng* was the response to environmental stress. Under the application of lime, the contents of oxalic acid in the shoots of *P. notoginseng* increased significantly. The possible reason was that Ca2+ induced plants to adjust the contents of oxalic acid, which improved plant resistance and balances free Ca2+.

Meanwhile, the root secretion of organic acid under Cd stress changed with the application of increase of rates of lime. With the7 50kg·hm-2 lime application, the contents of citric acid, malic acid, acetic acid and oxalic acid were significantly lower than those without lime treatment, while the content of succinic acid increased significantly. The LMWOAs from root system secretion could bind to Cd ligands and participate in the process of Cd2+ absorption, accumulation and transport, which promotes the accumulation of Cd by plants (Zhang et al., 1999). Its effect on plant absorption Cd was not only related to organic acid species and contents, but also to plant species and environmental conditions. Liu et al found that exogenous application of oxalic acid to *Echinodorus osiris* increased the Cd accumulation in the shoots and roots of *E. osiris* (Liu et al., 2014). The possible mechanism was that the acid dissolution of oxalic acid (Wei et al., 2017; Zeng et al., 2008). The combination of ligand of oxalic acid and the Cd increased the absorption of root to Cd and further promoted the transport of Cd from shoots xylem, which leaded to the increase of Cd content in shoots. Therefore, the secretion of oxalic acid in *P. notoginseng* significantly reduced with less than 2 250 kg·hm-2 lime application, thus reduced Cd biological mobility, which may reduce the absorption of Cd by *P. notoginseng* through plant avoidance mechanism.

The contents of oxalic acid in plants increased and in root exudates decreased with application of lime, which oxalic acid regulated the growth and resistance of plants. Spraying oxalic acid on leaves without lime significantly reduced the Cd contents, enrichment coefficient and transport coefficient of *P. notoginseng* roots, stems and leaves. Therefore, it was suggested that the ability of Ca2+ to improve plant resistance may be related to the regulation of oxalic acid. Franceschi’s studies had also shown that changes in endogenous oxalic acid content may be regulated by the concentration of Ca2+ (Li et al., 2000). The root and leaf biomass of *P. notoginseng* significantly increased with 0.1 mol·L-1 oxalic acid spraying (*P* <0.05) due to oxalic acid improving the resistance metabolism of *P. notoginseng*, thereby enhancing the absorption of nutrients by *P. notoginseng*, which is beneficial to growth (Li et al., 2000). However, the high contents of oxalic acid inhibited the growth and decreased the biomass of *P. notoginseng*, which was consistent with the results of Luo et al (Luo et al., 2019).

Considering six different formation of continuous extraction Cd, the formation of ethanol extraction was inorganic Cd, deionized water extraction was water-soluble Cd and organic acid binding Cd (Su et al., 2014). These Cd formations were bioavailable Cd. The Cd formation of sodium chloride extraction was pectin and protein binding, acetic acid extraction Cd was insoluble phosphoric acid binding, hydrochloric acid extraction was oxalic acid binding, these three formations of Cd were inert Cd, generally low content in plants (Lu et al., 2019). The contents of sodium chloride extraction and acetic acid extraction Cd in roots, stems and leaves of *P. notoginseng*with were the highest, which indicated that *P. notoginseng* had certain tolerance to Cd stress. With the application of lime and oxalic acid, the Cd contents and the sodium chloride extraction contents of Cd decreased, which Cd were converted into the less mobile acetic acid extraction and hydrochloric acid extraction formations. Sodium chloride extracted formation Cd had a high affinity for protein, when Cd bound to protein to reduce enzyme activity, thus hindering crop metabolism (Wang et al., 2008). Therefore, the conversion of sodium chloride extraction formation Cd inert acetic acid extraction formation Cd reduced Cd biological toxicity. The percentage of ethanol-extracted and water-soluble Cd of *P. notoginseng* roots and stems increased slightly under the treatments of high concentration of oxalic acid and lime, which may be due to the influence of high concentration of oxalic acid and Ca2+ on the physiological metabolism of *P. notoginseng*. Cd2+ was transferred to store in the corresponding parts of *P. notoginseng* to form a detoxification defense mechanism, which was consistent with the research of Li et al (Li and Dong, 2015).

**Conclusions**

Field experiments were conducted to study effects of lime application on the LMWOAs contents in plants and root exudates of *P. notoginseng* and oxalic acid spraying on the accumulation and chemical formations of Cd in *P. notoginseng*. The results showed thatthe content of oxalic acid in shoots was the highest, while oxalic acid and tartaric acid contents in the root were the highest under Cd stress. The contents of oxalic acid in plants increase and in roots exudates decreased with lime application in a certain range. The biomass of *P. notoginseng* increased with the increase of lime application rates. The biomass of *P. notoginseng* increased first and then decreased with increase of oxalic acid spraying concentrations. The Cd contents of different parts of plants decreased with combined application of lime and foliar spraying oxalic acid, and the Cd formations in plants were mainly acetic acid extraction and hydrochloric acid extraction. Therefore, the combined application of lime and low concentration of oxalic acid spraying could be recommended on Cd contaminated soil to reduce the Cd contents and ensure the quality and safety of *P. notoginseng*.

**Acknowledgments**

The study was supported by the National Natural Science Foundation of China (Grant Nos. 41867055 and 31560163), The Yunnan Key Research and Development Program (2019BC001-04). We thank Professor Chenggang Ren from Yunnan Agricultural University, China for English writing improvement.

**References**

Anjum, N.A., M. Hasanuzzaman, M.A. Hossain, P. Thangavel, A. Roychoudhury, S.S. Gill, M.A. Rodrigo, V. Adam, M. Fujita, R. Kizek, A.C. Duarte, E. Pereira, I. Ahmad, 2015. Jacks of metal/metalloid chelation trade in plants-an overview. *Front Plant Sci*., 6: 1-16.

Choi, Y.E., E. Harada, M. Wada, H. Tsuboi, Y. Morita, T. Kusano, H. Sano, 2001. Detoxification of cadmium in tobacco plants: formation and active excretion of crystals containing cadmium and calcium through trichomes. *Planta*., 213: 45-50.

Choppala, G., Saifullah, N. Bolan, S. Bibi, M. Iqbal, Z. Rengel, A. Kunhikrishnan, N. Ashwath, Y.S. Ok, 2014. Cellular mechanisms in higher plants governing tolerance to cadmium toxicity. Crit. *Rev. Plant Sci*., 33: 374-391.

Fu, W.B., 2015. Effects of different calcium concentrations on the growth and photosynthetic physiological characteristics of typical karst plants. Guangxi Univ.

Henry, A., W. Doucette, J. Norton, B. Bugbee, 2007. Changes in Crested Wheatgrass Root Exudation Caused by Flood, Drought, and Nutrient Stress. *J. Environ. Qual*., 36: 904-912.

Hu, Z.H., L. Jin, H.H. Zhu, 2017. The effect of lime on reducing cadmium content in rice and its influencing factors. *Hunan Agric. Sci*., 8: 20-23.

Kou, L.Y., K. Zhao, J.J. Cao, B.S. Jin, B.H. Zhou, 2019. Research on fitting model of low molecular weight organic acid for extracting some heavy metals from soil. *J. Environ. Sci*., 39: 2260-2268.

Li, B.S., X.X. Peng, M.Q. Li, 2000. The relationship between the accumulation of oxalic acid in plants and the metabolism of photorespiratory glycolic acid. *J. Plant Physiol*., 2: 148-152.

Li, F.F., S. Wang, S.C Gu, D.W. Cheng, H. Gu, M. Li, J.Y. Chen, Y.J. Yang, 2020. Effects of foliage spraying ABA and PDJ on fruit color and quality of 'Kyoho' grape. *J. Fruit Sci*., 37: 362-370.

Li, H.T., R. Dong, 2015. The absorption and accumulation of lead and cadmium by two Hemerocallis species and their subcellular distribution and chemical morphological characteristics. *J South China Agric. Univ*., 36: 59-64.

Li, S.S., G.C. Cao, P.C. Shi, G. Jiang, J. Yuan, 2015a. Spatial distribution of soil heavy metal elements in Qingdao urban area and evaluation of their status. *J. Ecol.* *Rural Environ*., 31: 112-117.

Li, Z.W., Y. Yang, X.M. Cui, P.R. Liao, J. Ge, C.X. Wang, X.Y. Yang, D.H. Liu, 2015b. Study on physiological response and enrichment characteristics of *Panax notoginseng* to cadmium stress. *Chinese J. of Chin. Mater. Medica*., 40: 2903-2908.

Li, X.F, 2019. Technical solutions for the safe utilization of heavy metal contaminated farmland in China: A critical review. *Land Degrad. Dev*., 30: 1773-1784.

Liu, W.R., L. Zhang, W.W. Yang, X.F. Li, L.P. Pan, Q. Li, C.L. Zhang, 2014. Effects of exogenous organic acids on the absorption and transport of cadmium by *Echinodorus osiris*. *Soil Bull*., 45: 205-209.

Lu, Q.H., Y.Q. Wang, H.B. Yang, 2020. Effects of exogenous calcium and abscisic acid treatments on the content of tartary buckwheat metabolites under salt stress. *J. Qingdao Agric. Univ (Nat. Sci. Ed.)*., 37: 95-101.

Lu, Y.M., Z.J. Nie, H.G. Liu, W. Gao, S.Y. Qin, C. Li, H.C. Fu, P. Zhao, 2019. Effects of zinc application on the distribution of cadmium in winter wheat subcellular cadmium and chemical forms of cadmium. *J. Henan Agric. Univ*., 53: 503-511.

Luo, L.F., J.X. Zhang, Y.C. Chuan, Y.W. Li, M.Z. Hao, H.R. Gu, S.S. Zhu, M. Yang, 2019. Study on the mitigation effect and mechanism of exogenous oxalic acid on the autotoxicity of notoginsenoside Rg1. *J. Northwest Sci-Tech Univ. Agri. Forestry (Nat. Sci. Ed.)*., 47: 101-108.

Qin, L., 2017. The accumulation characteristics of Cd and Pb and the mechanism of root secretion of low-molecular-weight organic acids in intercropping chrysanthemum and crops. Yunnan Agri. Uni.

Sallah-Ud-Din, R., M. Farid, R. Saeed, S. Ali, M. Rizwan, H.M. Tauqeer, S.A.H. Bukhari , 2017. Citric acid enhanced the antioxidant defense system and chromium uptake by *Lemna minor L.* grown in hydroponics under Cr stress. *Environ. Sci. Pollut. Res*., 24: 17669-17678.

Su, Y., J.L. Liu, Z.W. Lu, Z. Zhang, G.R. Shi, 2014. Effects of iron deficiency on subcellular distribution and chemical forms of cadmium in peanut root in relation to its translocation. *Environ. Exp. Botany*., 97: 40-48.

Subramanian, S., R. Viswanathan, 2007. Bulk density and friction coefficients of selected minor millet grains and flours. *J. Food Eng*., 81: 118-126.

Sun, J.Y., Y.Q. Liu, B.L. Li, Y.W. Zhou, 2018. Research progress on plant tolerance to cadmium and remediation of cadmium contaminated soil. *Jiangsu Agric. Sci*., 46: 12-19.

Tian, F.X., X.H. Ji, Y.H. Xie, J.M. Wu, D. Guan, 2016. Effects of alkaline slow-release fertilizers on the absorption and accumulation of Cd in rice. *J. Agric. Environ. Sci*., 35: 2116-2122.

Verbruggen, N., C. Hermans, H. Schat, 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*., 181: 759-776.

Wang, S.T., Q. Dong, Z.L. Wang, 2017. Differential effects of citric acid on cadmium uptake and accumulation between tall fescue and *Kentucky bluegrass*. *Ecotoxicol. Environ. Saf*., 145: 200-206.

Wang, X., Y.G. Liu, G.M. Zeng, L.Y. Chai, X.C. Song, Z.Y. Min, X. Xiao, 2008. Subcellular distribution and chemical forms of cadmium in *Bechmeria nivea* (L.) *Gaud. Environ. Exp. Botany*., 62: 389-395.

Wei, J., Q.S. Li, Z.M. Xu, X.F. Zhou, Y.S. Yang, K.H. Chen, X. Lin, 2017. Activation effects of various organic acids on cadmium carbonate in soil. *J. Environ. Eng*., 11: 5298-5306.

White, P.J., Broadley, M.R., 2003. Calcium in Plants. *Ann. Botany*., 92(4),: 487-511.

Yan, J.P., X.D. Ding, L. Cui, L. Zhangf, 2018. Effects of different amendments and their combinations on the form and physical and chemical properties of soil cadmium. *J. Agric. Environ. Sci*., 37: 1842-1849.

Yu, B.G., L. Qin, F.D. Zhan, Y.Q. Zu, B. Li, J.X. Wang, Y. Li, 2018. The effect of intercropping on the distribution of lead, cadmium and zinc chemical forms in sedge and broad bean. *J. Agric. Environ. Sci*., 37: 621-631.

Yuan, F.Q., J.X. Yuan, J.F. Song, H.G. Zhang, 2019. Study on the secretion of organic acids from the root of *Larix olgensis* seedlings under Cd stress. *Soil Bull*., 50: 1218-1225.

Zeng, F.R., J.X. S. Chen, Y. Miao, F.B. Wu, G.P. Zhang, 2008. Changes of organic acid exudation and rhizosphere pH in rice plants under chromium stress. *Environ. Pollut*., 155: 284-289.

Zeng, T.T., Z.J. Cai, X.L. Wang, W.J. Liang, S.W. Zhou, M.G. Xu, 2017. Integrated analysis of increasing crop yields by applying lime to acid soils. *Chinese Agric. Sci*., 50(13): 2519-2527.

Zhang, J.S., H.F. Li, C.Z. Yi, F.S. Zhang, 1999. Effects of organic acids on activation of cadmium in soil and cadmium uptake by wheat. *Acta Pedologica Sin*. 1999: 61-66.

Zhang, Z.L., W.J. Zhai, X.F. Li, 2010. Experimental guidance of plant physiology[M]. Beijing: Higher Education Press.

Zhou, W., 2018. Deteriorative effects of cadmium stress on antioxidant system and cellular structure in germinating seeds of *Brassica napus* L. *J. Agric. Sci. Technol*., 17: 63-74.

Zhu, M.L., Z.J. Chen, Y. Jiang, F.G. Wei, B. Cui, Y.X. Jiang, H.B. Cao, W.S. Zhang, 2014. The effect of exogenous soil Cd stress on the accumulation of *Panax notoginseng* and its effective components. *Chin. patent med*., 36: 342-347.(in Chinese)

Zhuang, G.T., 2015. Current Status of Soil Pollution in my country and Strategies for Prevention and Control. Bull. *Chin. Acad. Sci*., 30: 477-483.

Zu, Y.Q., S.C. Cheng, H.L. Ke, X.H. Guo, J. Wu, Y. Li, 2017. Distribution characteristics and evaluation of Pb, Cd, Cu and Zn in *Panax notoginseng* planting area and soil. *J. Ecol. Rural Environ*., 33: 317-323.

Zu, Y.Q., X.Y. Mei, Q. Min, Y. Su, G.Q. Feng, Y. Li, 2016. Effects of arsenic stress on the content of notoginsenosides and flavonoids, key enzyme activities and their proteome analysis. *J. Appl. Ecol*., 27: 4013-4021.