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Subcellular distribution and chemical forms of cadmium in *Morus alba* L.

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ABSTRACT

Morus alba L. (mulberry) is a perennial woody tree and a species with great potential for Cd phyremediation owing to its large biomass and extensive root system. The mechanisms involved in Cd detoxification were investigated by analyzing the subcellular distribution and chemical forms of Cd in mulberry in the present study. These results indicated that 53.27–70.17% of Cd mulberry accumulated was stored in the root and only about 10% were in the leaves. Lots of the Cd was located in the cell wall of the mulberry root and in soluble fraction of the mulberry leaf. Moreover, in roots, the largest amount of Cd was in the form of undissolved Cd-phosphate. While in mulberry leaves and stems, most of the Cd was extracted by 2% Acetic acid and 0.6 M HCl, representing Cd-phosphate and Cd-oxalate. It could be concluded that the Cd combination with peptides and organo-ligands in vacuole of leaf or complexed with proteins or cellulose in the cell wall of root might be contributed to the tolerance of mulberry to Cd stress. The mulberry could be used to remediate the Cd polluted farmland soils.

KEYWORDS

mulberry; cadmium; subcellular distribution; chemical form; phytoremediation

1. Introduction

Soil contamination by heavy metals has become a global environmental problem, giving rise to a concern in developing innovative and sound remediation technologies (Zeng et al. 2011; Zhong et al. 2015). Cadmium (Cd) is a non-essential and highly toxic heavy metal element, which can be released into the environment by a series of different ways, such as metallurgical alloying, ceramics, metal plating, photograph development, pigment works, textile printing industries, lead–zinc mining, alkaline batteries and electroplating (Mohapatra and Anand, 2007; Wang et al. 2009). Phytoremediation is one of the most effective methods to remediate the heavy metals in soils. A number of studies have proposed that the use of some woody plants could be an alternative method for the removal or stabilization of metals from contaminated soils (Pulford and Watson, 2003; French et al. 2006; Peng et al. 2012; Delplanque et al. 2013; Zhou et al. 2015). Mulberry also exhibits the potential for remediating heavy metals-polluted soils (Katayama et al. 2013; Ranđelović et al. 2014; Zhou et al. 2015). Mulberry is a perennial woody tree with the characteristics of extensive root systems, deep and rapid growth, and high biomass production (Zhou et al. 2015). Although the concentration of heavy metal in mulberry is lower than that of the hyperaccumulator, the total amount of heavy metal migration is still considerable because of the large amount of biomass (Jiang et al. 2017). Recently, many papers have proven that there are a few differences in the growth and development of silkworms, the production and quality of cocoons when the silkworm was fed on leaves from the mulberry cultivated in heavy metal polluted or not field areas (Wang et al. 2004; Jiang et al. 2017). Developing sericulture may be a safe, economic, eco-efficient utilization of heavy metals-contaminated areas (Wang et al. 1998; Yan et al. 2014; Jiang et al. 2015; Zhou et al. 2015). Not only does it take advantage of the heavy metals-contaminated land, increases the income of farmers, but also reduces the harm of heavy metal to the human body through the food chain. Wang (Wang 2002) conducted a 7-year micro-plot experiment to evaluate the Cd tolerance of mulberry, and found that mulberry trees are physiologically tolerant to soil Cd pollution. When the Cd concentration was 8.49 mg/kg, there was no harmful effect on the growth of mulberry. A yield reduction in the mulberry leaves was obviously at the soil Cd content of 75.8 mg/kg and the lethal concentration of Cd in the soil for mulberry was 145 mg/kg (Wang et al. 2004). However, excess Cd in mulberry could deeply interfere with a series of physiological processes such as plant growth, nutritional quality, enzyme activity and photosynthesis (Prince et al. 2001; Wang 2002; Wang et al. 2004; Tewari et al. 2013). For reducing Cd toxicity, some plants have induced intra and extra cellular mechanisms for metal detoxification, such as binding and precipitation in the cell wall, complexation and compartmentalization in vacuoles (Wang et al. 2008; Xin et al. 2014).

Subcellular distribution and chemical forms of heavy metal in plants may be related to metal tolerance and detoxification (Wang et al. 2008; Wang et al. 2009; Fu et al. 2011; Xin et al. 2013b; Xin et al. 2014; Chen and Lai, 2016; Lu et al. 2017; Shi et al. 2017). However, to our knowledge, little information is available on Cd distribution pattern in response to Cd stress in the mulberry species concerned. Therefore, this study was to investigate the characteristics of Cd subcellular distribution and chemical forms in mulberry, for the purpose of providing

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theoretical basis for further utilization of mulberry trees in the restoration of cultivated land contaminated by heavy metals and resources utilization of mulberry leaves. We hypothesized that the Cd content in leaves was less, the subsellular distribution of Cd in mulberry is mainly located in the cell wall of the root
\and leaf and the forming of precipitates between Cd and phosphate may be the key method to tolerance the Cd toxicity in mulberry.

2. Materials and methods

2.1 Soil preparation, plant materials and growth conditions

Soil for the pot experiment was replaced with a seedling substrate, which was purchased from the commercial market. Its basic properties were as follows: bulk density 0.2–0.4/g/cm^3, total porosity≥60%, relative water content<35%, particle size<20 mm, pH 5.5–7.0, organic matter≥40%. Three kilograms (dry weight) of the prepared substrate were added to each pot (15 cm in upper diameter and 35 cm in height). Three pots were replicated for all the Cd treatments.

The one-year-old mulberry plant stocks (var. Yu71–1), which were raised from cuttings obtained from a single plant by taking hardwood cuttings, were used as experimental plant materials. One plant was transplanted in each pot in August and irrigated with tap water once a week. All the pots were placed randomly in an outdoor pavilion, and the plants grew under natural conditions. The $CdCl_2$ was added into the Hoagland nutrient solution at the concentration of 0, 8, 45 and 90 mg/L. In consideration of the higher porosity and organic matter of the seedling substrate, better Cd tolerance of the mulberry tree, one liter Hoagland nutrient solution with different $CdCl_2$ concentrations was added to each pot every month. Though the $CdCl_2$ was added in the solution form in this study, different from hydroponic experiment, the Cd added would be adsorbed by the substrate and interacted with organic matter in substrate, reducing the content of Cd^{2+}, alleviating the toxicity to mulberry. After three months (from August, 2015 to October, 2015), the experimental plants were harvested and separated into roots, stems and leaves, and immediately used in the following analysis and test.

2.2 Tissue fractionation

Plant materials (2 g) were pretreated according to the methods described by Wang et al. (Wang et al. 2008) with a few modifications. In brief, plant tissues (root and leaf) were homogenized in the extraction buffer (50 mM Tris-HCl (pH 7.5), 1.0 mM $C_4H_{10}O_2S_2$, 250 mM sucrose). The homogenate was centrifuged (Himac CF 15RX, Japan) at 1000 × g for 15 min and the precipitation was designated as 'cell wall fraction' mainly consisting of cell walls and cell wall debris. The resulting supernatant solution was further centrifuged at 20000 × g for 30 min. The deposition and supernatant solution were referred to as 'organelle containing fraction' and 'soluble fraction', respectively. All steps were performed at 4 °C. The fractions were dried at 70 °C and wet digested with HNO_3–$HClO_4$ (3:1, v/v) by electric heating plate digestion separately, and then Cd concentrations in the digests were determined by atomic absorption spectrometry (AAS)(Thermo Fisher ICE-3400, America).

2.3 Chemical forms assay

In order to determine the chemical forms of Cd in the root, stem and leaf of mulberry, the extraction experiment was conducted by the specific solutions in the following order (Wang et al. 2008; Fu et al. 2011):

(1) F1(Fraction1), 80% ethanol, extracting inorganic Cd such as nitrate/nitrite, chloride, and aminophenol cadmium.
(2) F2, deionized water, extracting water soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$.
(3) F3, 1 M NaCl, extracting pectate and protein integrated Cd.
(4) F4, 2% Acetic acid (HAc), extracting insoluble $CdHPO_4$ and $Cd_3(PO_4)_2$ and other Cd–phosphate complexes.
(5) F5, 0.6 M HCl, extracting cadmium oxalate.
(6) F6, Cd in residues.

The fresh tissues (3 g) were cut into small pieces of 1–2 mm^2 and put into a 50-mL centrifuge tube with 15 ml extraction solution. The mixture was incubated at 30°C for 18 h, and then the extraction solution was pooled. The residues were extracted twice in the next 4 h with the same extraction solution (15 mL) for another 2 h, and the solution was also collected. A total of 45 mL extraction solution was aggregated and evaporated to constant weight and digested with HNO_3–$HClO_4$ (3:1, v/v). After collection of the former extraction solution, the plant materials retained in the tubes were subjected to the next extraction solution with the same extraction procedures. For determination of Cd in residues, plant material was digested with HNO_3–$HClO_4$ (3:1, v/v) at the end of the sequential extraction. Cd concentrations associated with different chemical forms were determined by AAS (Thermo Fisher ICE-3400, America).

2.4 Cd concentrations in different organs of mulberry

After harvest, the mulberry roots were washed twice with tap water and finally with de-ionized water. The plant samples (root, stem, leaf) were pre-dried at 105 °C in an oven for 30 min, and then the root and stem were cut into slices, respectively. The whole plant samples were dried to a constant weight at 70 °C, and then the mulberry samples were ground to powder and sieved. The mulberry materials were digested with HNO_3–$HClO_4$ (3:1, v/v) and Cd concentrations were determined by AAS (Thermo Fisher ICE-3400, America).

2.5 Statistical analysis

Analysis of variance (ANOVA) was used for statistically significant differences by SPSS software (version 17.0). All data are the means±SD of three replicates. Duncan′s multiple range test ($P<0.05$) was utilized to compare any significant differences between means of different treatments.

Table 1. Cd (mg/kg dw) distribution in subcellular fractions from leaves and roots of mulberry after Cd treatment (mg/L).

Organs	Cd treatments	Cell wall	Soluble fraction	Organelle	Total
Root	0	0.36±0.03a (0.69)	0.12±0.12b (0.19)	2.92±0.05b (0.12)	0.77±0.03
	8	16.29±1.96a (0.68)	7.95±2.72a (0.29)	31.66±5.21a (0.03)	15.60±0.86
	45	18.75±3.98a (0.77)	2.91±0.71b (0.16)	18.91±4.90a (0.07)	27.55±4.84
	90	38.44±5.88a (0.65)	3.07±0.57b (0.22)	23.10±4.99a (0.13)	31.60±6.49
Leaf	0	0.059±0.06b (0.36)	0.06±0.04b (0.56)	0.64±0.09b (0.08)	0.63±0.13
	8	0.62±0.31a (0.31)	0.49±0.06a (0.66)	1.40±0.16ab (0.04)	2.63±0.51
	45	0.86±0.18a (0.34)	0.45±0.02a (0.62)	1.58±0.36ab (0.04)	3.21±0.84
	90	1.04±0.43a (0.41)	0.46±0.02a (0.56)	1.75±0.45a (0.03)	8.57±0.69

Cd concentrations present mean±S.D (n = 3). The data in bracket indicate the percentages of Cd accumulation in the different fractions of mulberry. Treatments with the same letter were not significantly different (Duncan test, $p < 0.05$).

3. Results and discussion

3.1 Uptake and subcellular distribution of Cd

The accumulation characteristics and subcellular distribution of Cd in mulberry were showed in Table 1. The concentrations of Cd in different tissues (root, stem and leaf) of mulberry increased with the increase of Cd treatment concentrations and these results were consistent with those of *Brassica parachinensis* L. (Qiu et al. 2011), *Capsicum annuum* L. (Xin et al. 2014), *Brassica napus* (Mwamba et al. 2016) and *Salix matsudana*. (Wu et al. 2016). And for each treatment, the Cd content in different organs of mulberry was in the order of roots>-stems>leaves (Fig. 1). The concentrations of Cd in the root, stem and leave of mulberry indicated that a lot of Cd (53.27–70.17%) was stored in the root and only about 10% was in the mulberry leaves (Fig. 2). Less Cd in the mulberry leaves, less injury to the silkworms. In this study, the maximum Cd concentration in substrate was 90 mg/kg, the Cd content in mulberry leaf was 8.57 mg/kg, correspondingly (Table 1). Wang et al. (Wang et al. 2004) conducted a 3-year micro-plot

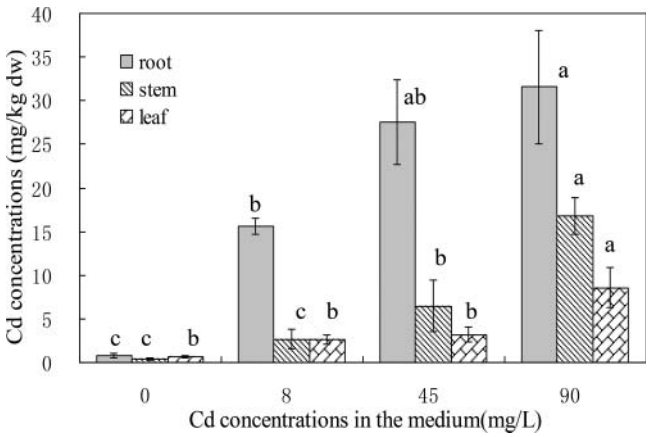

Figure 1. Cd concentration in different parts of mulberry. Bars indicate the standard error of the mean. Treatments with the same letter were not significantly different (Duncan test, $p < 0.05$).

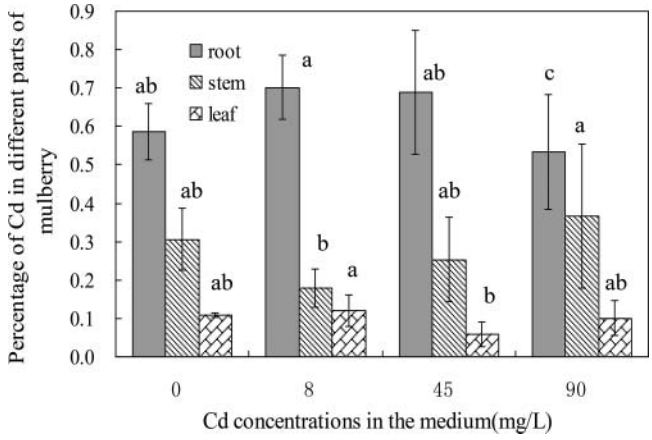

Figure 2. Percentages of Cd accumulated in different parts of mulberry. Bars indicate the standard error of the mean. Treatments with the same letter were not significantly different (Duncan test, $p < 0.05$).

experiment of mulberry cultivation with Cd-polluted soil and silkworm breeding experiments to evaluate the toxic effects of Cd on mulberry and silkworms. They concluded that the leaves yield was obviously reduced when the soil Cd content was 75.8 mg/kg. When the Cd concentration in soil was 145 mg/kg, the Cd in the mulberry leaf was 3.27 mg/kg (dry basis), the mulberry plants exhibited marginal growth, and the ingestive and digestive rates were significantly affected, but the growth of silkworms and the quality of cocoons was not significantly affected (Wang et al. 2004). These differences may come from the various soils, the addition forms of Cd, and the duration of the experiment.

The cell walls of plant, the shell of cells, as the first safeguard to keep the protoplast from Cd toxicity, mainly being comprised of cellulose, hemicellulose, pectin and protein, offer many adsorption sites such as carboxyl, phosphate, hydroxyl and amino groups. Those adsorption sites could bind Cd^{2+} in the cell wall and limit the migration of Cd across cell membrane (Fu et al. 2011; Qiao et al. 2015). In the present study, 65%−77% of Cd in roots was combined with cell wall, and only about 10% were stored in organelle. These results were consistent with those of Bechmeria nivea (L.) Gaud (Wang et al. 2008). Similarly, Wu et al. (Wu et al. 2016) found that 53% and 65% of Cd were located in the cell wall of *S. matsudana* roots at 10 μM and 100 μM Cd, respectively. In seaweed, 41.2%−79.2% of Cd was accumulated in the cell wall when the seaweed was exposed to various Cd concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 5.0 mg/L)(Zhao et al. 2015). Xin et al. (Xin et al. 2014) conducted a greenhouse experiment to study the subcellular distribution and chemical forms of Cd in two hot pepper cultivars differing in Cd accumulation. However, it was reported that most of Cd (77%−87%) in hot pepper roots was stored in the soluble fraction and only 8%−17% of Cd was located in the cell wall. In addition, 56%−64% of the total Cd in the hot pepper leaves was bounded in the cell wall. In mulberry leaves, however, a great deal of the Cd was existed in the soluble fraction, reaching 56%−66%. Also a small quantity of Cd was located in the organelle fraction. In both root and leaf of *Brassica napus*, the majority of Cd was accumulated in the solution fraction, less was stored in cell wall (Mwamba et al. 2016). These different results indicated that various plant

species or varieties could also have different uptake characteristics and compartmentalization mechanisms of Cd. The vacuole is an organelle which is dynamic and holds about 90% of the total cell volume (Pittman 2005; Xin et al. 2014). It could be speculated that the vacuole was the main accumulation site for Cd in mulberry leaf. The cell fluid in vacuoles containing carbohydrates, inorganic salts, pigments, proteins and other substances plays a regulatory role in the internal environment of the cell, so that the cell can keep a certain osmotic pressure to maintain the state of expansion. The heavy metals chelate with (in-) organo-ligands in the vacuoles could reduce the free heavy metals ions activity and thus reduce its damage to cell, and the organo-ligands in vacuoles for Cd complexation are primarily organic acids and sulfur-rich peptides (Fu et al. 2011). These results indicated that no matter Cd accumulation in mulberry root or leaf, less Cd stored in the organelle, fewer injuries in plant cells, less damage in the process of cell metabolism. Meanwhile, these results were in accord with those concluded by Wójcik et al. (Wójcik et al. 2005) and Qiu et al. (Qiu et al. 2011). Wójcik et al. investigated the tissue and cellular compartmentation of Cd in the hyperaccumulator *Thlaspi caerulescens* by energy-dispersive X-ray microanalysis. They concluded that in roots, the cell wall makes a great contribution to Cd retention. In leaves, however, the vacuoles are the major compartment of its storage and detoxification. Qiu et al. (Qiu et al. 2011) also reported that the majority of the Cd was located in the cell wall and the soluble fraction of two Chinese flowering cabbage cultivars (Lubao 70 and ChixinNo.4). So the organelles of cabbage were well protected from Cd toxicity.

3.2 Chemical forms of Cd

When exposed to high concentration of Cd (90 mg/L), the mulberry trees showed no or few toxic symptoms, suggesting that mulberry tree (Var. Yu 71–1) has a high tolerance to the Cd contamination. Similarly, Xin et al. (Xin et al. 2013a) screened and identified the low Cd hot pepper among 30 hot pepper cultivars. It found that, compared with the control, the fruit biomass of 30 hot pepper cultivars did not significantly decrease in the Cd treatments (1.16 and 2.69 mg/kg), indicating that the hot pepper cultivars could bear the concentrations of Cd in agricultural soils. The chemical forms of Cd in plants are important to reflect the migration of Cd and toxicity degree. For example, Cd in inorganic form such as nitrate/nitrite, chloride, and aminophenol cadmium (extracted by 80% ethanol) and water soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$ (extracted by d-H_2O) exhibit better ability to transport and thus resulting in deleterious effects on protoplast compared with the pectate and protein integrated Cd (extracted by 1M NaCl), the insoluble $CdHPO_4$ and $Cd_3(PO_4)_2$ and other Cd–phosphate complexes (extracted by 2% HAc) and cadmium oxalate (extracted by 0.6 M HCl) (Wang et al. 2008; Mwamba et al. 2016). Cd concentrations of different chemical forms in the mulberry increased with the increase of Cd treatment concentrations (Table 2). In roots, the content of Cd extracted by 1M NaCl and 2% HAc was about 50% of the total amount, while the Cd forms extracted by other extracting solutions were relatively rather low. In stems, the main Cd forms were organic form and integrated with pectate and protein without Cd treatment. When mulberry was exposed to Cd solution, the cadmium oxalate and residual form were in the majority. In leaves, the extraction of 2% HAc and 0.6 M HCl occupied about half proportion of Cd accumulated by leaf. The percentages of other extraction forms were close (Table 2). In this paper, Cd was retained in each tissue of mulberry with various chemical forms. In roots, the most of the Cd was insoluble $CdHPO_4$ and $Cd_3(PO_4)_2$ and other Cd–phosphate complexes, indicating that less chan-

Table 2. Concentrations of different chemical forms of Cd in the roots, stems and leaves of mulberry (mg /kg dW) exposed to Cd treatments.

Organs	Cd treatments (mg/L)	F1	F2	F3	F4	F5	F6
Root	0	0.32±0.16b (0.06)	1.17±0.13b (0.10)	0.07±0.02c (0.12)	0.76±0.01b (0.39)	1.82±0.13b (0.17)	0.16±0.04b (0.16)
	8	3.56±1.6a (0.02)	19.13±0.01b (0.23)	2.92±0.88b (0.25)	7.92±0.17b (0.21)	21.15±12.15ab (0.12)	3.62±1.18ab (0.17)
	45	3.66±1.49a (0.03)	46.10±10.32a (0.17)	3.18±0.69b (0.23)	11.20±1.33b (0.28)	19.53±10.26ab (0.12)	3.37±1.12ab (0.17)
	90	4.88±2.37a (0.03)	61.65±21.97a (0.16)	4.98±1.21a (0.28)	25.88±11.42a (0.24)	24.88±15.43a (0.13)	5.56±0.33a (0.16)
Stem	0	0.68±0.59b (0.10)	4.49±1.70c (0.32)	0.14±0.03c (0.26)	0.28±0.06b (0.12)	2.49±0.31a (0.15)	0.02±0.01b (0.05)
	8	2.84±0.34a (0.18)	33.67±8.18b (0.10)	0.65±0.16ab (0.08)	2.51±0.08b (0.10)	17.51±2.15a (0.11)	1.35±0.64ab (0.43)
	45	2.54±0.31a (0.02)	43.33±11.94b (0.13)	1.88±0.84a (0.14)	4.57±0.04b (0.08)	102.33±33.32a (0.31)	8.31±1.71a (0.32)
	90	2.59±1.07a (0.01)	69.15±14.54a (0.13)	2.71±0.43ab (0.07)	25.41±11.28a (0.37)	117.16±17.24a (0.20)	9.25±0.80a (0.22)
Leaf	0	0.78±0.06b (0.09)	1.35±0.37a (0.10)	0.06±0.01b (0.22)	0.27±0.02a (0.14)	1.32±0.09b (0.27)	0.15±0.04c (0.18)
	8	1.18±0.12ab (0.14)	3.11±0.47a (0.19)	0.12±0.02ab (0.12)	0.93±0.05a (0.25)	3.63±0.90a (0.25)	0.16±0.08c (0.05)
	45	1.56±0.15a (0.21)	2.48±0.52a (0.17)	0.10±0.04ab (0.12)	0.60±0.04a (0.21)	1.37±0.45b (0.18)	0.29±0.06b (0.11)
	90	0.79±0.06b (0.10)	1.75±0.12a (0.18)	0.18±0.07a (0.19)	0.62±0.19a (0.21)	2.01±0.49b (0.20)	0.39±0.03a (0.12)

Cd concentrations present mean±S.D (n = 3). The data in bracket indicate the percentages of Cd accumulation in the different fractions of mulberry. Treatments with the same letter were not significantly different (Duncan test, $p < 0.05$).

ces to be migrated to shoot. While in the mulberry leaves and stems, lots of Cd was combined with protein and pectate (extracted by 1M NaCl). The reasons may be that Cd was complexed by some certain chemical organic groups, such as carboxyl or hydroxyl, which could form a non-toxic complex with heavy metals. Furthermore, It could be speculated that larger percentages of 2% Acetic acid-extractable Cd (extracting insoluble $CdHPO_4$, $Cd_3(PO_4)_2$ and other Cd–phosphate complexes) in roots and NaCl-extractable Cd (extracting pectate and protein integrated Cd) in shoots contributed to the adaptation of mulberry to Cd tolerance and accumulation. These results along with the conclusions from Cd in subcellular distribution further indicated that complexation in vacuolar and sequestration in the cell wall may be important for mulberry to tolerance Cd stress.

Besides, the same kinds of plants with different genotypes may have different abilities to tolerance, detoxify and adapt to heavy metals ions. For example, compared with 3 Cd-resistant barley genotypes species, Wumaoliuling, a Cd-sensitive genotype, had higher Cd concentration in inorganic and water soluble forms, lower in pectate/protein integrated Cd (Wu et al. 2005). Xin et al. (Xin et al. 2014) investigated the characteristics of Cd uptake and distribution between a low-Cd and high-Cd hot peper cultivars by determining the subcellular locations and chemical forms of Cd in different tissues. They found that significant differences in the subcellular distribution and chemical forms of Cd between the low- and high-Cd hot pepper cultivars and these differences may be caused by the genotypic variation. So when in the study of the effects of other external factors on the accumulation of Cd in a mulberry, it is necessary to point out that the mulberry materials are from the same plant, to ensure the consistency of the gene. Though mulberry trees are widely distributed in China, which mulberry ecotypes could effectively be used to remediate the heavy metal polluted soil is still unknown. So, more laboratory or field studies are necessary to screen Cd sensitive mulberry ecotype to study the models of Cd tolerance and mechanisms of detoxification. A better understanding of Cd tolerance and detoxification in mulberry may eventually contribute to the phytoremediation and comprehensive utilization Cd contaminated soils by mulberry.

4. Conclusion

The concentrations of Cd in the subcellular part of mulberry tissues showed that most of Cd were accumulated in the soluble fraction of leaf and bounded to the cell wall fraction of root. In addition, the Cd in the root, stem and leaf of mulberry were with different chemical forms and Cd concentrations in various chemical forms in mulberry increased with Cd exposure concentrations. In roots, the majority of Cd was integrated with pectates and proteins, insoluble $CdHPO_4$ and $Cd_3(PO_4)_2$ and other Cd–phosphate complexes. While in stems and leaves, most of the Cd was in the form of Cd–phosphate complexes and cadmium oxalate. From this study, it could be deduced that the vacuoles and the cell walls contributed to the Cd tolerance and protected organelles from Cd toxicity.

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