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Full Length Research Paper

Subcellular localization of cadmium in hyperaccumulator *Populus* × *canescens*

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In this study, subcellular localization of cadmium in hyperaccumulator grey poplar (*Populus* × *canescens*) was investigated by the transmission electron microscopy (TEM) method. Young *Populus* × *canescens* were grown and hydroponic experiments were conducted under four Cd^{2+} concentrations (10, 30, 50, and 70 µM) in contrast to the control (non-Cd) for 28 day(s). Results indicate that Cd stress could induce serious damage in root cells of grey poplar. Cd induced plasmolysis, concentrated cytoplasm, disappearance of the cristae in mitochondria, ambiguity of framework and destruction of mitochondria envelope in root tip, stem and leaves cells. The hollowing cells were also found in Cd treatment, and there existed some different granule in these hollowing cells. Plasmolysis was found in all treatments in cells of stem, but no damage was detected in cells of leaves. The degree of damage to organs of grey poplar was as follows: root > stem> leaves. It was suggested that the *Populus* × *canescens* as a renewable resource has the potential to decontaminate cadmium stress development, accumulation and distribution.

Key words: Cadmium, phytoremediation, hyperaccumulator, grey poplar, organ.

INTRODUCTION

Cadmium (Cd) is a widespread pollutant with a long biological half-life; it enters into the food chain and constitutes a potential risk for both animals and humans (Wojas et al., 2008; Dai et al., 2011). Excess Cd typically causes direct or indirect inhibition of physiological processes, such as respiration, transpiration, photosynthesis, oxidative stress, cell elongation, nitrogen metabolism and mineral nutrition, resulting in growth retardation, leaf chlorosis and low biomass in plants (Sanità di Toppi and Gabbrielli, 1999). Phytoremediation technique is less costly, less invasive, less risky and environmental benefits to remove contaminants from environments. Thus, the technology has many advantages over traditional engineering based method (Alkorta and Garbisu, 2001; Roosens et al., 2005; Solís-Domínguez et al., 2007; Harfouche et al., 2010). Plants used for phytoremediation display a wide range of mechanisms at the cellular level including the detoxification processes (Laureysens et al., 2004, 2005; Mertens et al., 2004; Tuskan et al., 2006; Ghosh and Singh, 2005; Ebbs et al., 2009; Xu et al., 2009), and thus the plants usually have a high tolerance to heavy metals and metalloid stress (Pilon-Smits and LeDuc, 2009).

It is well known that the roots are the main route through which Cd enters plants (Klang-westin and Eriksson, 2003), and about 80% of Cd is accumulated in roots of some plants. Most Cd in roots is localized in the insoluble fraction of cell walls and nuclei, which is connected with the detoxification mechanism of Cd (Boominathan and Doran, 2003; Cobbett and Goldsbrough, 2002; Malá et al.,

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2010). With increasing Cd concentration in cells, a series of alterations at ultrastructural level appear. Electron microscopy (EM) techniques are very useful in localizing Cd in plant tissues (Jiang et al., 2009; Liu et al., 2009). They make it possible to identify the main accumulations of Cd in cells and cellular organelles and observe alterations in cell ultrastructure (Samardakiewicz and Woźny, 2000; Broadley et al., 2001, 2004; Ni and Wei, 2003; Jiang et al., 2009). Plants have a range of potential mechanisms at different levels that might be involved in the detoxification and thus tolerance to heavy metal stress (Hall, 2002; Clemens, 2006; White and Brown, 2010; Dai et al., 2011). The main detoxifying strategy of plants contaminated by heavy metals is the production of phytochelatins (PCs) (Hall, 2002; Nishikawa et al., 2006). PCs, a family of metal-induced peptides, are produced in plants upon exposure to excess heavy metals, such as Cu, Cd or Zn (Clemens, 2001; Cobbett, 2000), and can be detected in plant tissues and cell cultures (Keltjens and van Beusichem, 1998). Several studies have reported that PCs can form complexes with Pb, Ag and Hg ***in vitro*** (Rauser, 1999).

Although there are extensive literature relating to cellular levels and physiological studies on the influence of heavy metals on plants, Cd tolerance strategies of plants have not been fully explained yet (Castiglione et al., 2007; Borghi et al., 2008). Poplar is easy to propagate and establish (Castiglione et al., 2007; Dai et al., 2011), and has the advantages of rapid growth, high biomass production, and the ability to accumulate high concentrations of nutrients and heavy metals after long-term exposure. Thus, it is a potential plant for absorption and accumulation of heavy metals (Giachetti and Sebastiani, 2006). In a previous investigation, the effects of different concentrations (10, 30, 50 and 70 µM) of Cd on growth for 28 days were investigated in hydroponically grown ***Populus*** × ***canescens***. Cd had significant inhibitory effects on stem and leaves growth at high concentrations (70 µM) on roots at 50 and 70 µM Cd during the entire experiment. In the present study, we used EM and cytochemistry to investigate ultrastructural alterations in plasma membrane, dictyosomes, endoplasm reticulum (ER) and mitochondria, to identify the synthesis and distribution of cysteine-rich proteins induced by Cd and to explain the possible mechanisms of the Cd-induced cellular defense system in the root and (stem and leaves) meristematic cells of ***Populus*** × ***canescens***.

MATERIALS AND METHODS

Cultivation of plants and Cd exposure

The experiments were performed in the orchard of Northwest A and F University, Yangling (34°20′N, 108°24′E), China. Plantlets of ***Populus*** × ***canescens*** (***P. tremula*** × ***P. alba***) were produced by micropropagation (Leplé et al., 1992) and cultivated in a climate chamber (day/night temperature, 25/18°C; relative air humidity, 50 to 60%; light per day, 16 h and photosynthetic photon flux, 150 µmol m^{-2} s^{-1}). After 4 weeks, the rooted plantlets were transferred to an aerated Hoagland nutrient solution in a growth room with the same environmental condition as in the climate chamber. The nutrient solution was exchanged every 3 days. After a 12-week cultivation, the plants were treated with five $CdSO_4 \cdot 7\ H_2O$ concentrations of 0 (control), 10, 30, 50 and 70 µM $CdSO_4$ by adding $CdSO_4$ into the nutrient solution.

After Cd exposure for 28 day(s), each $CdSO_4$ exposure had six plants and six control plants were harvested per date. The plants were separated into root, stem and leaves, and were then separately weighed and oven-dried at 70°C until a constant weight was achieved. The root apparatus was carefully rinsed with a deionized water solution soon after harvesting took place to remove Cd ions adsorbed at the root surface. Based on leaves, stem and root dry weights metal resistance was calculated according to the tolerance index developed by Landberg and Greger (2002). The effects of Cd exposure on leaves, stem, and root dry weight were assessed using two-way ANOVA.

Transmission electron microscopy (TEM)

The subcellular localization of Cd in leaves, stem and roots tip cells was evaluated according to the method reported previously (Sahi et al., 2002), with minor modification. After 12-week cultivation, the plants were treated with nutrient solution of control (0) and 10, 30, 50 and 70 µM. Subsequently, the leaves, stem and root samples were taken and washed.

Determination of phytochelatins (PCs) contents

Plant material frozen in liquid nitrogen was extracted in 5% sulfosalicylic acid, 6 mM diethylenetriaminepentaacetic acid as described by Zhang et al. (2005). The homogenate was centrifuged at 10,000 × g, 4°C for 10 min to remove cellular debris and precipitated proteins. Total non-protein thiols in the supernatant were then quantitated spectrophotometrically with Elman's reagent at 412 nm by the method of Zhang et al. (2005).

Metal analysis

Samples of leaves, stem and roots were separately oven-dried at 70°C and ground to a powder in a laboratory mill. Then 0.5 g of plant material was mineralized in $HCl–HNO_3$ (dilution factor 3:1, v: v) (Cho and Seo, 2005; Ma et al., 2010) clarified with ultra pure water and used for measurements of total Cd in an atomic absorption spectrophotometer (Atomic Absorption Spectrometer 373, Perkin-Elmer, Norwalk, CT, USA).

Statistical analysis

A completely randomized design incorporating six replicates was used for each time point. Data were subjected to analysis of variance (ANOVA) to examine the effects of time, treatment on different organs. Statistical analysis was conducted using STATISTICA 5.1 software (Statsoft Inc., United States of America). Separation of means was carried out using Fisher's LSD test at $P<0.05$ and $P<0.01$ significance level.

RESULTS

Cd accumulation and biomass accumulation

Generally, Cd causes cell damage by acting as an

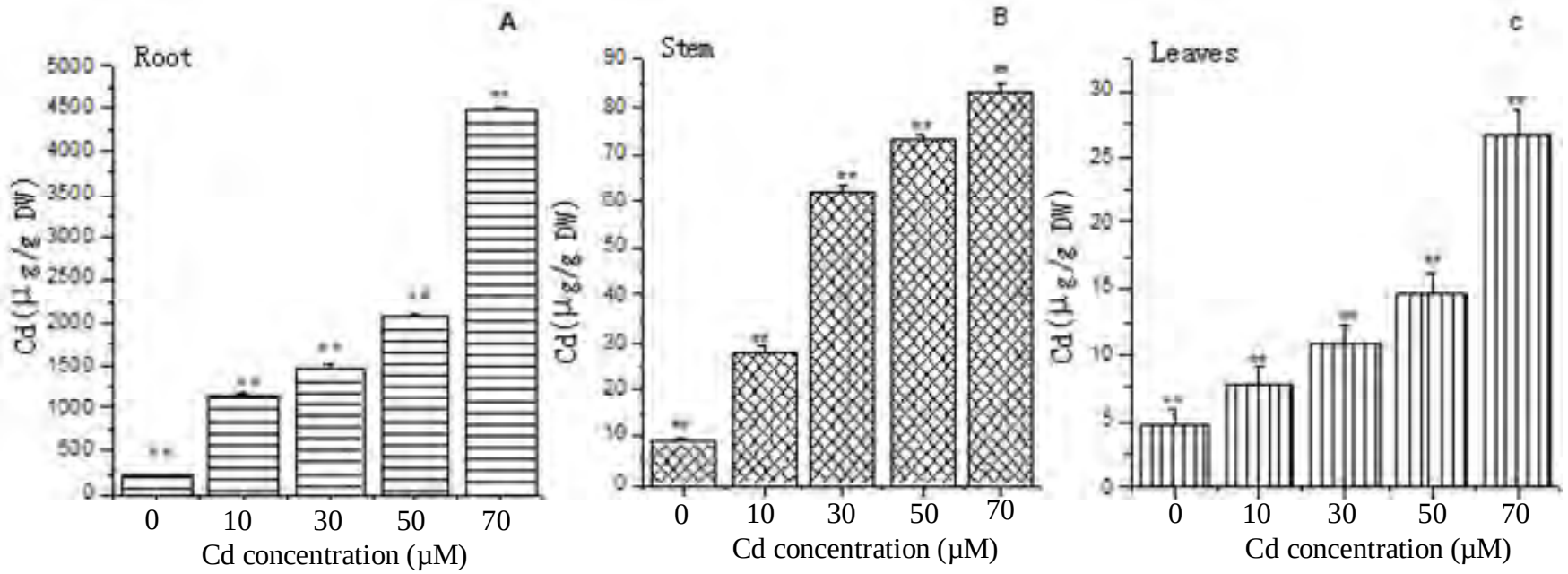

Figure 1. Cd content (μg . g^{-1} DW) of ***Populus* × *canescens*** grown with 0 (control), 10, 30, 50 and 70 μM L^{-1} $CdSO_4$ for 28 days. Data were presented as mean ± standard error (n = 6). *Represents the significance level at ***p***<0.05; **represents the significance level at ***p***<0.01 when compared with control.

enzyme inhibitor, precipitating essential elements and/or metabolites (Schützendübel and Polle, 2002; Ghosh and Singh, 2005; Garnier et al., 2006; Solís-Domínguez et al., 2007) as observed in plant tissues exposed to high concentration metals. Exposure of ***Populus* × *canescens*** to 10, 30, 50 and 70 μM $CdSO_4$ for 28 day(s) led to significant increases in Cd concentrations in the following order: roots > stem >leaves, P< 0.001(Figure 1). Furthermore, the Cd influx into roots of ***Populus* × *canescens*** was more than 100 times higher than that observed in wheat exposed to the same Cd concentration. This finding suggests that poplar roots still absorb large quantities of Cd after Cd exposure for 28 days and underlines that the sink for Cd is much larger in ***Populus* × *canescens*** than that in wheat.

In this study, to analyze the toxic effects of Cd on plant growth, biomass of root, stem and leaves was recorded (Figure 2). The plant growth decreased following the increased Cd concentrations. The ***Populus* × *canescens*** after different $CdSO_4$ exposure for 28 days, increasing external Cd concentrations may not affect biomass decline in leaves, stem and root.

Changes in levels of phytochelatins

To compare the different levels of PCs formation in Cd-stressed ***Populus* × *canescens*** (Figure 3), ***P.* × *canescens*** were exposed to 10, 30, 50 and 70 μM Cd and harvested at 28 days of Cd treatment. PCs contents of roots were highest, followed by stem and leaves under different levels Cd exposed.

Subcellular localization of Cd in leaves cells

The TEM revealed that Cd content was below (although sometimes at) the detection limit in mesophyll cells of both the palisade and spongy parenchyma of the leaves tissue. The detectable Cd content was located in the cytoplasm of plants after they were exposed to 10, 30, 50 and 70 μM Cd for 28 days. Different concentration Cd

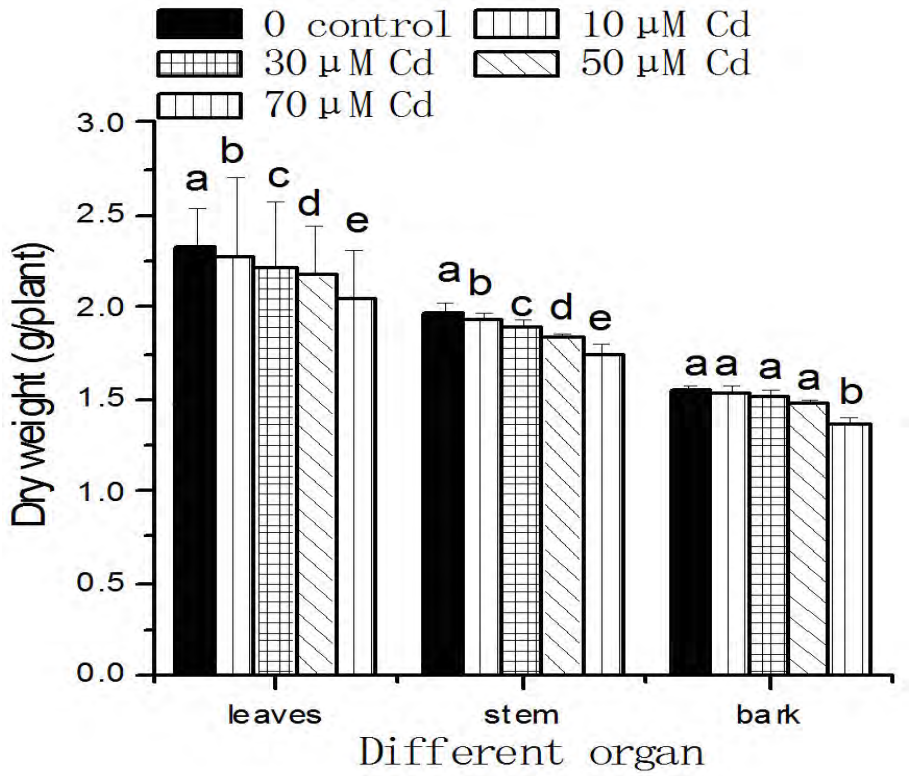

Figure 2. Dry weight (mg · g^{-1}) of ***Populus*** × ***canescens*** grown with 0 (control), 10, 30, 50 and 70 μM L^{-1} $CdSO_4$ for 28 days. Values are means of six replicates ± standard deviation. Different letters represent the significant difference at $P \leq 0.01$.

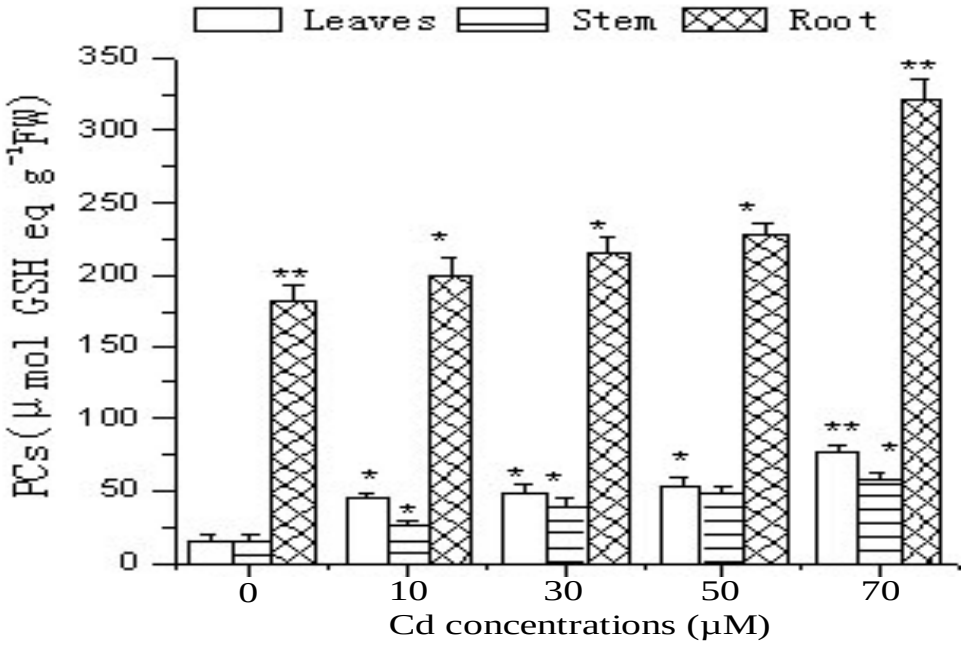

Figure 3. PCs levels in roots, stem and leaves of P. ×canescens in response to Cd exposure. ***Populus* × *canescens*** treated with 0 (control), 10, 30, 50 and 70 μM L^{-1} $CdSO_4$ for 28 days. Each value of PCs represents the average of six experiments. Data were presented as mean ± standard error (n = 6). *Represents the significance level at $p<0.05$; **represents the significance level at $p<0.01$ when compared with the control.

exposures are illustrated in Figure 4b to e vs. Figure 4a (control). Compared to the control tissue (Figure 4a), from 10 to 70 μM Cd exposure (Figure 4b to e), the cell walls increased approximately one- to three-fold in thickness (arrows), condensation of the nuclei, and occasional accumulation of vacuolar phenolics. The most dramatic structural change occurred in the chloroplasts: probably unaffected size, accumulated lipids, and increased size and frequency of starch grains (sg) under 50 and 70 μM Cd exposure (Figure 4d and e). Cd is mainly uploaded in the free ionic form from roots via the xylem to the leaves.

Subcellular localization of Cd in stem and root tip cell

Ultrastructural studies of the stem and root tip cells of ***Populus* × *canescens*** grown in control solution and in solutions containing (10, 30, 50 and 70 μM) Cd for 28 days revealed extensive differences. Control cells had typical ultrastructure. Plasma membrane was unfolded with a uniform shape in all parts. Large amounts of rough ER, dictyosomes, mitochondria and ribosomes were immersed in dense cytoplasm. The nuclei with well-stained nucleoplasm and distinct nucleolus were located in the center of cells, whereas vesicles were distributed in stem and root tip cells (Figures 5a and 6). Under 10 μM Cd treatment, the observable effect of Cd at ultrastructural level was that the dictyosome vesicles increased, appearing as a compact mass of vesicles in the cytoplasm (Figures 5b and 6b). Under 30 μM Cd treatment, the ER with swollen cisternae appeared to be concentrically arranged along the cell wall, some flattened cisternae were broken up into small closed vesicles (Figure 5c). While under 50 and 70 μM Cd treatment, in some meristematic cells the nuclear envelope was generally more dilated compared with the control cells (Figure 5d). There were marked invaginations of plasmalemma (Figure 5e). In addition, there were some small vesicles containing electron-dense granules formed by the plasma membrane.

The morphological alterations above took place during 28 days of treatment with Cd, but no visible injury in other cellular components was seen. Under 10 μM Cd treatment, an interesting phenomenon was found at the 28^{th} day of Cd exposure in the root tip cell; many parallel arrays of ER with regularly extended cisternae were noticeable in cytoplasm (Figure 6b). Under 30 μM Cd treatment, there was high cytoplasmic vacuolization in root tip cells. Normally, several vesicles gradually fuse together to produce a large cytoplasmic vacuole, in which electron-dense granules can be seen (Figure 6c). Under 50 and 70 μM Cd treatment, the electron-dense granules were first found in cell walls and also deposited in spaces between the cell walls and plasma membrane (Figure 6d), and then there was a gradual accumulation of electron-dense granules in vacuoles, cytoplasm and mitochondrial membranes with increasing Cd treatment time (Figure 6e).

DISCUSSION

To our knowledge, this is the first report on the use of TEM technique in dicotyledonous plants. ***Populus* × *canescens***, a fast-growing plant with high biomass production that grows in soils or flooded areas. In this study, ***Populus* × *canescens*** tissues were able to tolerate and hyper accumulate high Cd concentrations (70 μM) for 28 days. ***Populus*** × canescens roots contribute to making metal ions more available to the uptake proteins as they not only acidify the rhizosphere through plasma membrane-localized proton pumps, but actively secrete low-molecular weight (LMW) compounds that can function as metal chelators (Cobbett, 2000; Cobbett and Goldsbrough, 2002; White and Brown, 2010; Lux et al., 2011). This finding suggests that in the tolerance mechanisms, there is a strong connection to the pathways for uptake, partitioning and accumulation of toxic metals. Chelation and sequestration processes result in removal of the toxic ions from sensitive sites and in accumulation.The uptake and accumulation of Cd in ***Allium cepa*** were investigated by inductively coupled plasma mass spectrometry (ICP-MS), and Wierzbicka et al. (2007) indicated that 56% of total Cd was located in cell walls. Our results indicated that Cd ions were localized and accumulated in cell walls and vacuoles in ***Populus* × *canescens***. Cd retention in the roots is based on binding of Cd to ion-exchange sites on the cell wall and extracellular precipitation, mainly in the form of Cd carbonate deposited in the cell wall (Kahle, 1993; Jiang et al., 2009; Lux et al., 2011). Once excessive Cd ions enter the cytoplasm, a defense mechanism is activated, protecting the cells against Cd toxicity at the cellular level. Endocytotic and exocytotic processes are well known in plant cells (Liu et al., 2003, 2007). The plasma membrane represents a 'living' barrier of the cell to free inward diffusion of Cd ions. The results here indicated some vesicles containing Cd deposits were found in cells and were obviously derived from the invaginations of plasmalemma and ER (Jiang et al., 2009). It was clearly shown that they could prevent the circulation of free Cd ions in the cytoplasm and could force them into a limited area.

Moreover, mobilization and transport of metal ions across the plasma membrane are only the first steps in metal uptake and accumulation (Clemens 2006). When Cd enters cells even in small amounts, it produces a wide range of adverse effects on physiological processes (Cobbett, 2000; Cobbett and Goldsbrough, 2002; Lunáčková et al., 2003; Clemens, 2006). The ultra-structural results in the present investigation showed some electron-dense granules in vacuoles, cell walls and cytoplasm in the meristematic cells after Cd treatment (Jiang et al., 2009). Plasma membrane function may be rapidly affected by heavy metals, as shown by increased leakage from cells in the presence of high concentrations of metals (Kolb et al., 2010). Our results showed that root tip cells had a rapid and effective defense system against

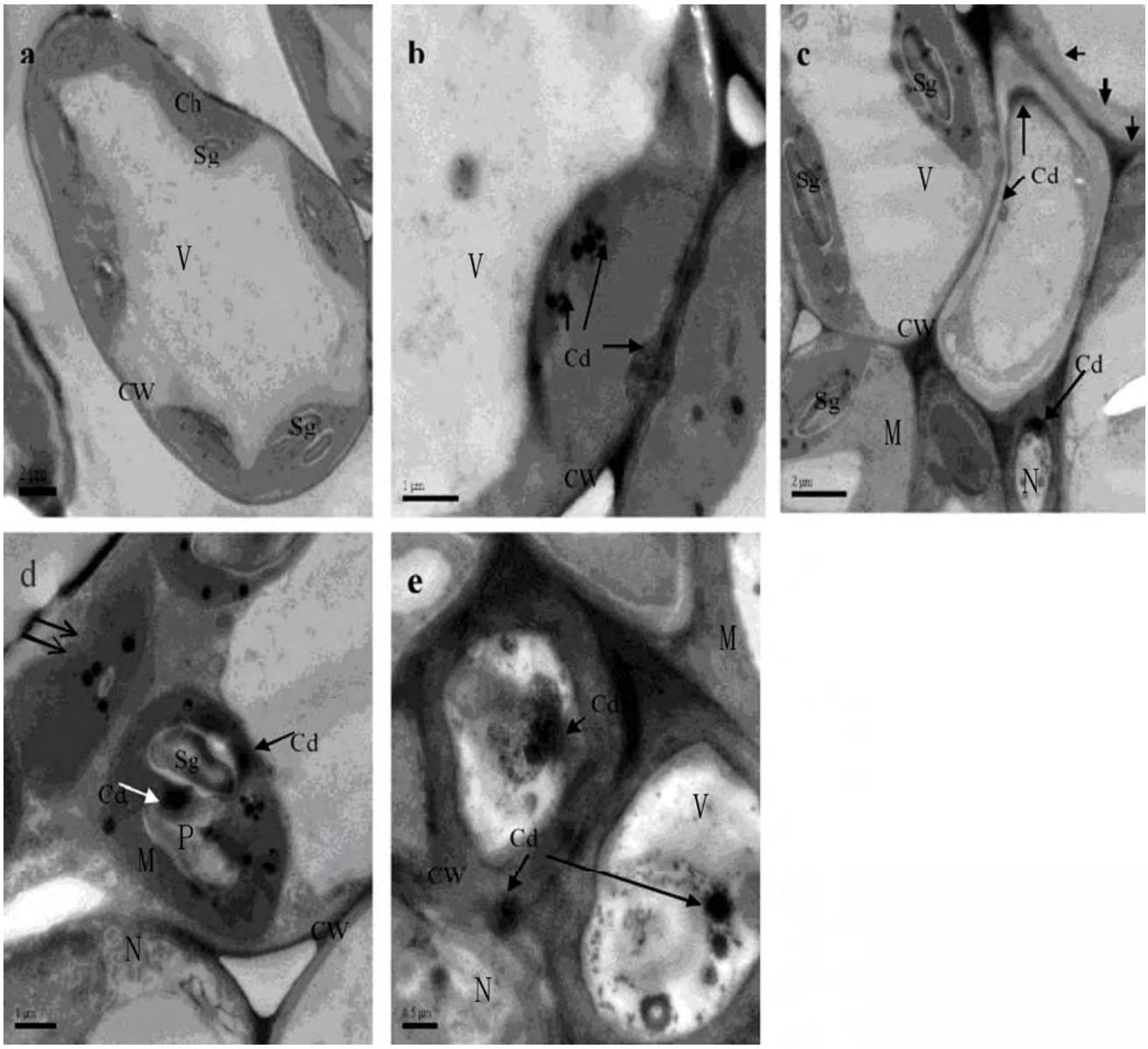

Figure 4. Cytological changes in the structure of assimilative cells (inside the leaves mesophyll of ***Populus* × *canescens***) caused by heavy metal exposure. Cd, Cadmium; CW, cell wall; ICS, intercellular space; Ch, chloroplasts; V, vacuole; Sg, starch grains; M, mitochondria; N, nuclei;

Cd toxicity involving ER and dictyosomes, which may be one mechanism accounting for lower toxicity of Cd. This phenomenon may be explained by the fact that once excessive Cd ions entered cytoplasm, the synthesis of new proteins of ER involved in heavy metal tolerance was stimulated. We assume that some vesicles from ER and dictyosomes may carry metal-complexing proteins or polysaccharide components, which participated in repair of membrane and cell wall following damage. Some vesicles may have carried the proteins, which bind Cd by formation of stable metal-PC complexes in cytoplasm, and in this way the free metal ions in the cytoplasm decreased. Cells can maintain sufficient PCs to bind with Cd. ER definitely plays a very important role in detoxification of Cd. The vacuole is the final destination for practically all toxic substances that plants can be exposed to, and the vacuoles of root (stem and leaves) cells are the major sites of metal sequestration (Clemens 2006).

Taken together, this study strongly suggest that: (1) cell walls, a first barrier against Cd stress, can immobilize some Cd ions, (2) the morphological alterations in plasma membrane, dictyosomes and ER reflect the features of detoxification and tolerance under Cd stress, and (3) vacuoles are ultimately one of the main storage sites of Cd. Thus, root and (stem and leaves) meristematic cells

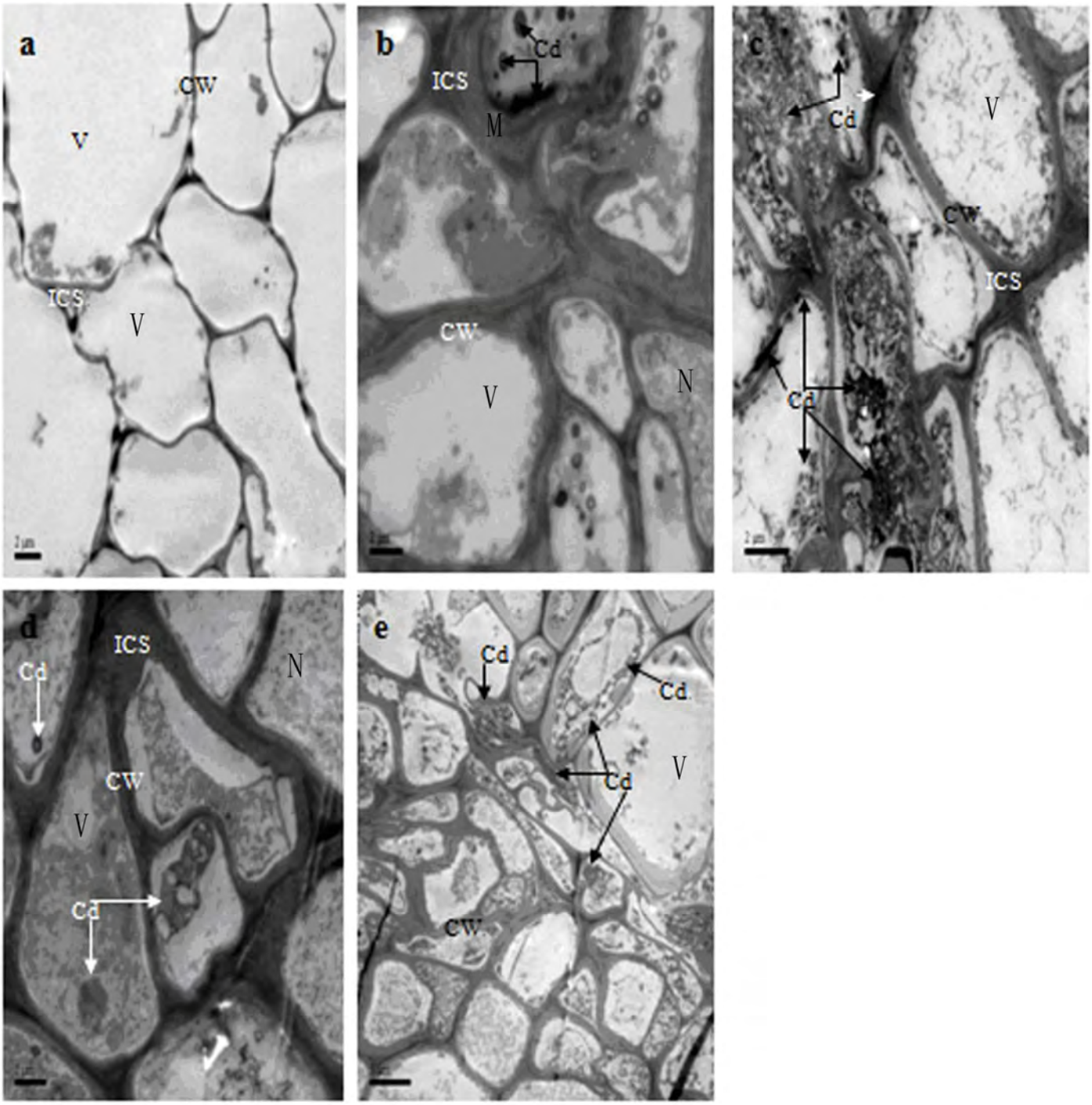

Figure 5. Transmission electron micrographs of stem cells of ***Populus* × *canescens*** grown in 1/2 Hoagland's nutrient solution, showing control (without addition of Cd), and with the addition of 10, 30, 50, and 70 µM Cd. (a) Cortical cells bordering intercellular space grown in 1/2 Hoagland nutrient solution composed of the control (without addition of Cd). (b) Cortical cells bordering intercellular space, showing Cd deposits along the plasma membrane of the stem cell treated with 10 µM Cd. (c) The plasmolysis of the cell, showing Cd deposits still along the plasma membrane of the cells (c, d, and e). Cd, Cadmium; CW, cell wall; ICS, intercellular space; Ch, chloroplasts; V, vacuole; Sg, starch grains; M, mitochondria; N, nuclei; P, plastids.

of ***Populus* × *canescens*** exposed to low Cd concentrations have a rapid and effective defense system, but at increased levels of Cd in the cytosol, cells are seriously injured. Moreover, it seems that ***Populus* × *canescens*** offer possibilities for phyto-stabilization and to a greater extent for phytoextraction.

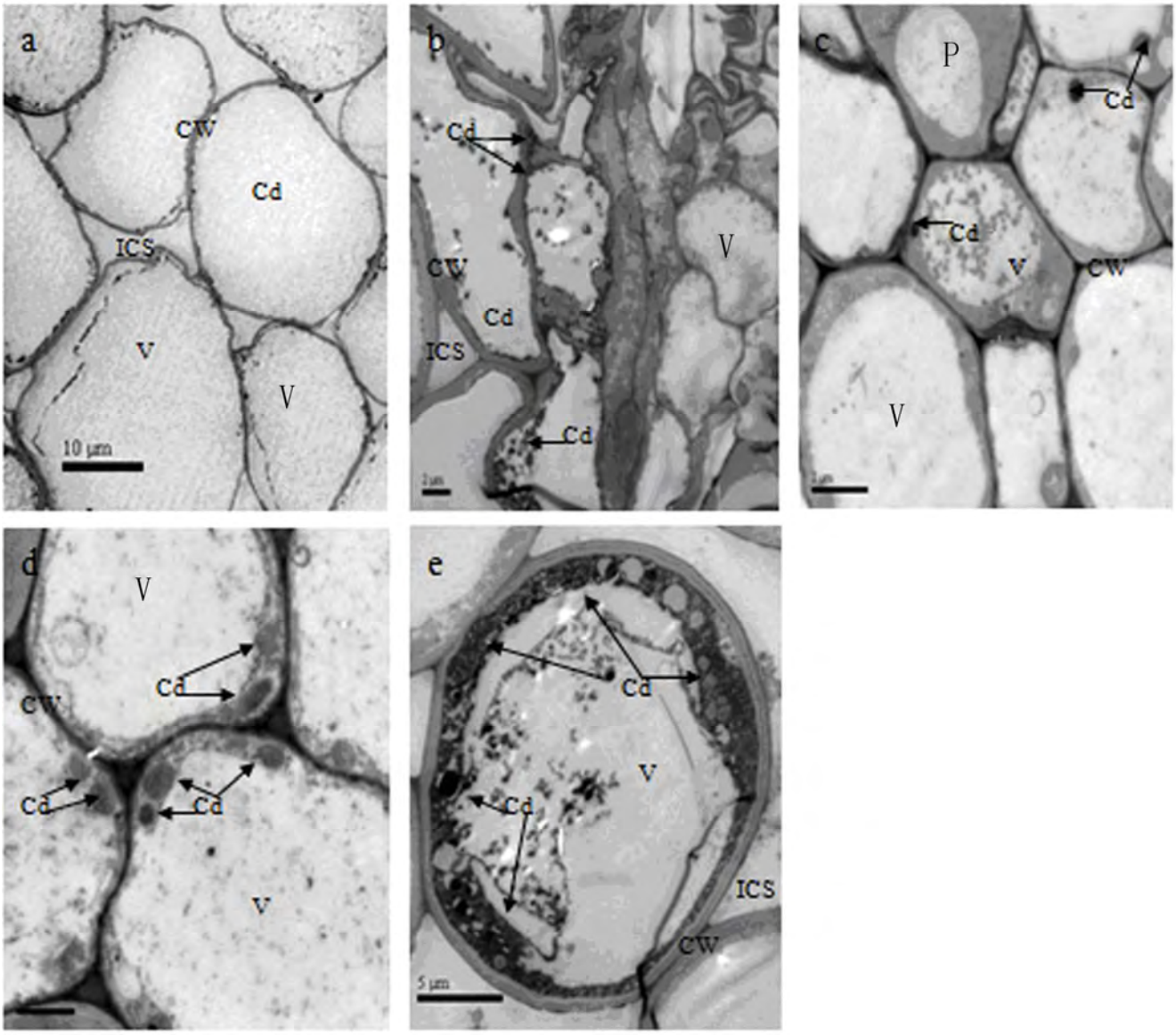

Figure 6. Transmission electron micrographs of root cells of ***Populus* × *canescens*** grown in 1/2 Hoagland's nutrient solution, including control (without addition of Cd) and with the addition of 0 (control), 10, 30, 50 and 70 μM Cd. (a) Cortical cells bordering intercellular space grown in 1/2 Hoagland nutrient solution composed of the control (without addition of Cd). (b) A section of a cell, showing Cd deposits along the plasma membrane of the root tip cell treated with10 μM. (c) One section of malformation cell, showing numerous Cd deposits in the cytoplasm of some malformed cells (c and d). (d) Magnified view of a section of the cell in Figure 6e, showing the Cd deposits in the cytoplasm of the some malformed cell. Cd, Cadmium; CW, cell wall; ICS, intercellular space; Ch, chloroplasts; V, vacuole; Sg, starch grains; M, mitochondria; N, nuclei; P, plastids.

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