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Integration of copper subcellular distribution and chemical forms to understand copper toxicity in apple trees

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ABSTRACT

Little information is known about the tolerance mechanisms of apple trees growing in soils with excess Cu. This study investigated not only the accumulation and translocation of Cu in the organs of two apple tree cultivars (Fuji and Ralls), but also the detoxification mechanisms of their roots at the cell level. Most accumulated Cu was within apple tree root, especially in the fibrous root, for both cultivars. Compared with Fuji, Ralls had significantly higher Cu concentrations in roots, especially for fibrous roots, while lower concentrations in the aboveground parts. Combining the results from subcellular distribution and chemical forms in the fibrous roots of two apple tree cultivars, more Cu amounts were sequestrated in cell wall and vacuole for Ralls and Cu accumulated in the fibrous root of Fuji had higher in vivo mobility. The results of this study implied that different apple tree cultivars differ in the accumulation, transportation and tolerance to toxic levels of soil Cu, and the chemical forms and subcellular partitioning of Cu in the fibrous roots can elucidate some of the variation of apple trees in Cu sensitivity.

1. Introduction

The gradual accumulations of copper (Cu) in soils of fruit tree areas resulting from the intensive and long-term usage of Cu-based fungicides have been reported all over the world (Komárek et al., 2010). For example, Cu maximum concentration value as much as 3200 mg kg^{-1} has been found in the vineyards of southern Brazil (Mirlean et al., 2007). Results from apple orchards of Shandong Province—a well famous fruit producing area of China, have shown that soil Cu concentration increased at a rate of 1.37 mg kg^{-1} to 1.45 mg kg^{-1} per year during the years from 2008 to 2014 (Wang et al., 2009b, 2015b). Consequently, Cu contaminations in orchard and vineyard soils have become a growing public concern worldwide. In an environmental perspective, one of the major issues is to quantify the bioavailability and toxicity of soil Cu to a range of living plants (Lai et al., 2010; Wang et al., 2015a). The elevated soil Cu can affect the phenology, growth and reproduction of ruderal plant species or crops growing on vineyard soils (Brun et al., 2003; Michaud et al., 2007). A number of studies have also been carried out to characterize Cu phytotoxicity in fruit trees (Chopin et al., 2008; Lai et al., 2010). Excessive soil Cu concentrations not only can result in the increase of Cu concentrations in fruit tree organs, but also can exhibit toxicity symptoms to the plant tissues (Brun et al., 1998; Fernández-Calviño et al., 2009). Thus, the annual increasing soil Cu content and its potential environmental risk have prompted European Union to restrict the application rates of Cu to fruit trees (Commission Regulation (EC) No 473/2002). However, in most developing countries, such as China, no guideline is established for the sustainable management of Cu-based fungicides during fruit producing process (Wang et al., 2009a,b).

In heavy metal-contaminated soils, plants cope with the potential metal stress in different ways. Some plant species adopt an exclusion strategy to avoid the excessive uptake and transport of metal ions. In contrast, some plant roots can take up large amounts of metals and transport them to the shoots (Clemens et al., 2002; Xin and Huang, 2014). The accumulation abilities of heavy metal in plants vary greatly not only among plant species but also among cultivars or genotypes within the same species (Wang et al., 2007). Plants have evolved several strategies for metal detoxification (He et al., 2013a,b). In order to avoid heavy metal toxicity, metal in plant can be compartmentalized into cell wall, vacuole or other subcellular fractions (Fu et al., 2011; Zhao et al., 2015). He et al. (2013a) have proved that subcellular compartmentation played a critical role in the Cd accumulation and detoxification in woody plants. Moreover, it has been reported that the chemical forms of heavy metal in plant tissues can explain some of the differences in uptake and translocation of heavy metal among cultivars or

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genotypes (Fu et al., 2011; Xin and Huang, 2014). Chemical form approach quantifies the metal fates within cells by sequentially extracting metals with different chemical solutions (Wu et al., 2005). Although these chemical phases are operationally defined, studies have provided clear evidence that heavy metal in inorganic form and water–soluble form migrate more readily than metals binding to pectates, phosphates and oxalates, and these forms have stronger negative effects on plant cells (Fu et al., 2011; Xu et al., 2013). Taking the Cd-resistant and Cd-sensitive barley genotypes for example, Wu et al. (2005) have found that the former had higher concentrations of pectate and protein integrated Cd. Additionally, studies have also convinced that the biological activity of heavy metal in plant is associated with its subcellular distribution and chemical forms (Fu et al., 2011; Zhao et al., 2015).

Apple tree is one of the most important fruit crops worldwide, and a long history has been reported about the application of Cu-based fungicides to protect apple trees from serious fungal attacks (Wang et al., 2009a,b). Although a few studies have been carried out to investigate the uptake and transportation of Cu in apple tree organs (Wang et al., 2015b), the tolerance mechanisms of apple tree in response to Cu stress remain unclear, especially for different apple tree cultivars. Moreover, the selection of low-Cu cultivars could be a useful tool in reducing the Cu amount entering human food chain. The main objective of this study was to evaluate the differences in Cu uptake and accumulation between two apple tree cultivars through the analyses of Cu concentrations in plant organs. A further aim was to investigate the possible tolerance and detoxification mechanisms of apple trees under soil Cu stress by examining Cu subcellular distributions and chemical forms in apple tree roots.

2. Materials and methods

2.1. Soil sample collection and preservation

Experimental soil was collected from farmland which was planted with corn since cultivation. The soil is classified as Mollisols according to the USDA soil taxonomy system, with a 0–50 cm mollic epipedon. Soil sample was collected at a depth of 0–20 cm (after removing the litter layer). Soil pH (1:5 soil to water), soil organic carbon content and total soil Cu content of the sampling site were 5.78, 18.80 g kg^{-1} and 24.93 mg kg^{-1}, respectively. The soil sample was first air dried and then passed through an 8 mm sieve to remove litter and stones.

To homogenize the Cu concentrations in soils, each 15 kg soil sample was spread onto a table and was sprayed with 100 mL of $CuSO_4$ solutions. Three soil Cu treatments were with target Cu concentrations of 105 mg kg^{-1} (T1), 161 mg kg^{-1} (T2) and 533 mg kg^{-1} (T3), respectively. Then, soil sample was replaced into a pot. Six replicates were used for each Cu treatment. Pots were watered lightly during the "stabilization period" for one year. It has been reported that aging can influence the mobility and bioavailability of metals added to soil, and this long period allowed for the equilibration of various sorption mechanisms in soils (Ma et al., 2005). Then, about 10 g of fertilizer containing 15% nitrogen (N), 15% phosphorus (P) and 15% potassium (K) was applied to each pot at the start of the pot experiment to ensure adequate nutrition for the growth of apple tree.

2.2. Pot experiments

In this study, two apple tree cultivars (Fuji and Ralls) extensively grown in China, were selected as plant materials. The same apple rootstock (*Malus hupehensis* Rehd.), which is widely used as an apple rootstock in the many fruit producing areas of China, such as Liaodong Peninsula and Shandong Peninsula, was used for Fuji and Ralls. Studies have found that *M. hupehensis* is capable of limiting the accumulation of Cu in aerial tissues (Liu et al., 2011). One-year-old apple trees were transplanted into the pretreated pots (one plant per pot). Then, pots were buried into soil to simulate the actual soil environment. Plants were watered with distilled water without heavy metals and fertilized one to two times per week. The pot experiments were conducted in a greenhouse with an air temperature of 18–35 °C. Growth of plants continued for 10 months (from March to December in the year of 2014).

2.3. Sample sampling and preparation

In December, apple trees were harvested, and were divided into roots, trunk, branches and leaves. Moreover, the root samples were divided into fibrous roots, lateral roots and tap roots. Plant samples of aerial organs, lateral root, tap root and part of the fibrous root samples were washed in the following order: tap water, 2 mol L^{-1} HCl, and deionized water. Then, the samples were dried in an oven at 70 °C. While the other part of the fresh fibrous root samples were immediately frozen in liquid N_2 and stored in a freezer.

2.4. Determination of copper in soil and plant samples

Soil samples were first ground to pass through a 100 mesh screen (diameter of the sieve is 0.149 mm). Then, samples were digested with an acid mixture of HNO_3–$HClO_4$–HF (2:1:1, v/v/v) for the determination of total soil Cu concentration using flame atomic absorption spectrometer (AA-6300C, Shimadzu).

About 0.50 g of the plant samples were digested with 2 mL of 30% (w/v) H_2O_2 and 5 mL of ultrapure HNO_3. After digestion treatment, samples were filtrated through Whatman No. 42 filter papers. Finally, the filtrate was made to 25 mL with highly purified deionized water.

By definition, bioaccumulation factor (BAF) is the ratio between Cu concentrations in soil and plant root (Lai et al., 2010):

$$\mathrm{BAF} = \frac{C_{\mathrm{root}}}{C_{\mathrm{soil}}}$$

where C_{root} is the Cu concentration in root (mg kg^{-1}), and C_{soil} is the Cu concentration in soil (mg kg^{-1}).

Translocation factor (T_f) indicates the ability of plants to translocate metal from roots to aerial tissues of a plant (Shi et al., 2010).

$$T_{\mathrm{f}} = \frac{C_{\mathrm{trunk}}}{C_{\mathrm{root}}}$$

where C_{trunk} is the Cu concentration in the trunk of apple tree (mg kg^{-1}), and C_{root} is the Cu concentration in root (mg kg^{-1}).

Accuracy and precision of the methods used for the determinations of Cu concentrations in soil and plant samples were confirmed by the analyses of related certified standard reference materials (soil reference, GBW-07403; apple reference, GBW-10019) provided by the National Research Center for Certified Reference Material (CRM), China. Digestions of these standard reference materials were performed with the same digestion procedures used for soil and plant samples. In view of data quality assurance, each sample batch was analyzed in a triplicate under standard conditions within the confidence limit of 95%.

2.5. Separation of tissue fractions

The subcellular distribution of Cu within the fibrous root was determined according to the procedure used by Zeng et al. (2011). Frozen fibrous roots (0.20 g) were weighed and homogenized using a mortar and a pestle in 10 mL buffer solution containing