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# Arbuscular mycorrhizal colonisation increases copper binding capacity of root cell walls of *Oryza sativa* L. and reduces copper uptake

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ABSTRACT

There is evidence that colonisation by mycorrhizal fungi can protect host plants from toxic concentrations of heavy metals. The mechanism by which protection is provided by the fungus for any particular metal is poorly understood. Rice (*Oryza sativa* L.) plants were inoculated with *Glomus mosseae* and grown for 4 weeks to ensure strong colonisation. The plants were then exposed to low to toxic concentrations of copper (Cu) and the uptake and distribution were examined. The effect of mycorrhizal colonisation on the cell wall composition and Cu binding capacity of roots was also investigated. Mycorrhizal plants showed moderate reductions in Cu concentrations in roots but large reductions in shoots. In roots, mycorrhizal plants accumulated more Cu in cell walls but much less in the symplasm compared to non-mycorrhizal plants. The differences in cell wall binding of Cu could be partly explained by changes in the composition of the cell wall. The mechanistic basis for the reduced Cu accumulation and the potential beneficial consequences of mycorrhizal associations on plant growth in Cu toxic soil are discussed.


## 1. Introduction

Plant tolerance to heavy metals can be achieved through a range of physiological and morphological changes (Sanitá di Toppi and Gabbrielli, 1999; Hall, 2002). Metal retention by roots is often cited as a beneficial strategy (Wagner, 1993), although in few cases has the site of increased metal storage in roots been identified. Similarly, some mycorrhizal fungi have been shown to reduce accumulation of certain heavy metals in shoots and it is presumed that this is due to increased retention within the root/mycorrhizal structures (Brunner and Frey, 2000; Chen et al., 2004; Zhang et al., 2005). There are a number of possible explanations for the observed reductions in heavy metal concentrations in shoots of mycorrhizal plants. These include (1) a greater selectivity of metal transporters in the cell membranes of mycorrhizae, (2) compartmentation of metals in fungal vacuoles, thereby reducing transfer of the metals to the plant, and (3) increased capacity of mycorrhizal roots to immobilise metals within their cell walls. The binding of heavy metal ions to plant root most probably involves charged groups on cell wall polysaccharides binding metal efficiently (Cohen-Shoel et al., 2002). Of these the acidic side groups of galacturonic acid in pectin are likely to be most important. (Eticha et al., 2005). Pectins have been implicated as the main cell wall binding site for $Al^{3+}$ (Eticha et al., 2005).

Evidence for intracellular sequestration of metals in ectomycorrhizal fungi is largely indirect and metal-specific (Blaudez et al., 2000; Frey et al., 2000). However, there appear to be no data on relative metal selectivity between plants and endomycorrhizal fungi; the obligate nature of the endomycorrhizal association makes it difficult to separate binding to plant root from binding to the fungus. Adsorption on or precipitation of heavy metals in plant cell walls has been reasonably well described (Lignell et al., 1982; Pellegrini et al., 1993; Amado Filho et al., 1999; Andrade et al., 2002), but there is limited information on metal binding by mycorrhizal cell walls. Joner and Leyval (1997) reported that the arbuscular mycorrhizal fungus *Glomus mosseae* had a high capacity for Cd uptake but they did not distinguish between immobilization on cell walls or in vacuoles. Tam (1995) demonstrated that in the ectomycorrhizal fungus *Pisolithus tinctorius*, Cu and Zn were retained in extrahyphal slime, while Bradley et al. (1981, 1982) showed that in the dwarf shrub *Calluna vulgaris* (L.), Cu accumulated in the associated ericoid mycorrhizal fungus *Pezizella ericae*. Despite the apparent versatility of different types of mycorrhizae to accumulate heavy metals, how this is achieved largely remains a mystery.

Rice is an important crop in Asia, especially in China. However, contamination with a mixture of heavy metals of paddy soils is not

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uncommon (Chen et al., 2005; Lin et al., 2007). Based on evidence with other heavy metals (Brunner and Frey, 2000) mycorrhizal associations with rice could potentially reduce the uptake of Cu. In this study we have focused on the role of cell walls in binding of Cu in the association between rice and the arbuscular mycorrhizal fungus *G. mosseae*. The principal aim was to try to establish whether reduced uptake of copper could be explained by compositional changes in cell walls of roots as a result of mycorrhizal colonisation.

## 2. Materials and methods

### 2.1. Cu toxicity trial

Seeds of rice (*Oryza sativa* L.) cultivars, Jiahua 1, were surface-sterilized with 10% (v/v) $H_2O_2$ for 10 min and immersed in deionized water for 24 h. They were then germinated on moist filter paper until the radicles appeared. For each treatment, 20 uniform seedlings were placed in 20 ml of treatment solution ($KNO_3$ 0.2 mM, Ca $(NO_3)_2$ 0.2 mM, $MgSO_4$ 0.1 mM) with varying concentrations of Cu supplied as Cu $(NO_3)_2$. The solutions were changed every 12 h for 7 days then the root lengths were measured.

### 2.2. Production of mycorrhizal and non-mycorrhizal plants

Since the presence of different concentrations of Cu in the medium during the inoculation and colonisation of plants by the mycorrhiza could potentially lead to variations in the degree of colonisation that would complicate interpretation of the results, plants were pre-cultured without Cu for 4 weeks until strong and uniform colonisation was established. Plants were then exposed to Cu over the following 2 weeks to examine the uptake and distribution of Cu.

Rice seeds were surface-sterilized with 10% (v/v) $H_2O_2$ for 10 min and immersed in deionized water for 24 h. They were then germinated on moist filter paper until the radicles appeared. The seedlings were selected for uniformity before planting. *O. sativa* L. was inoculated with *G. mosseae* isolate (Gm) (BGC.XJ01, isolated from a non-contaminated soil, provided by YS Wang, Beijing Academy of Agriculture and Forestry) or left uninoculated (NM). The inoculum contained dried roots of sorghum, hyphae, spores and soil. Plants were grown in round plastic pots containing 200 g perlite plus 40 g inoculum for the mycorrhizal treatment, or 200 g perlite plus 40 g sterilized inoculum for the NM treatment. The inoculum was mixed with the perlite. Three germinated seedlings were sown per pot. Containers were watered and fertilized as needed with a 1/5 strength nutrient solution (Feigin et al., 1987). Pots were regularly weighed to maintain appropriate water content. After 4 weeks of preculture, some plants were removed gently from perlite, and were washed carefully to remove adhering particles on the root surface. Plants were then transferred to hydroponic treatment solutions containing varying concentrations of Cu for 2 weeks; other plants were grown for a further 4 weeks in perlite without Cu to provide material from which to extract cell walls for the Cu binding studies. For the hydroponic experiment, inoculated roots showing good colonisation, along with NM roots, were transferred into ¼ strength modified Hoagland's nutrient solution with or without Cu. Fe–EDTA was omitted from uptake solutions to avoid complexation of Cu. The pH of uptake solutions was maintained at 5.5. During the growth period, the containers were aerated with air pumps and arranged in an environment-controlled growth chamber with a 14-h photoperiod at a photon flux 280 $\mu mol\ m^{-2}\ s^{-1}$ in the range 400–700 nm.

### 2.3. Root colonisation by G. mosseae

To determine the degree of colonisation after 4-week and 8-week preculture, a portion of the roots (about 1 g fresh weight) was washed free of perlite with tap water, and then fully rinsed in deionized water. The clean roots were cut into segments around 1 cm long, cleared by soaking in 10% KOH and stained according to Phillips and Hayman (1970) but without using phenol. Percentage colonisation was determined by the grid intersect method (Giovannetti and Mosse, 1980).

### 2.4. Distribution of Cu between root apoplast and symplast

In order to distinguish between Cu bound to cell walls and Cu taken up into cells, roots were desorbed using a modified desorption procedure as described in Reid and Liu (2004). Briefly, after being treated with different concentrations of Cu in hydroponic solution, roots were excised. The entire root system from each treatment was then desorbed in 5 mM $CaCl_2$, which was changed every 5 min. After 20 min the roots were rapidly frozen in liquid nitrogen to disrupt cell membranes and desorption was continued for 40 min. The Cu released in the 20 min desorption plus the Cu remaining in the root after the freeze–thaw plus rinse was considered to be apoplastic. The Cu released following the freeze–thaw was considered symplastic. The Cu concentration in the desorbed solution and in the solution after freeze–thaw was analyzed directly by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

### 2.5. Measurement of tissue Cu content

Plants were harvested at the end of the treatments and the roots rinsed with Milli-Q water and blotted gently. The roots and shoots were oven-dried at 70 °C for 72 h and the dry weights determined. The dried samples were ground with a stainless steel mill and digested in concentrated nitric acid ($HNO_3$) at 160 °C. Cu in the extracts was measured by ICP-AES.

### 2.6. Preparation of cell walls

Root cell walls of 8 weeks mycorrhizal and non-mycorrhizal plants were extracted according to Zhong and Lauchli (1993). Roots were frozen in liquid $N_2$ and homogenized to a powder with a mortar and pestle. The root powder was washed into a 50-ml centrifuge tube with three aliquots of 15 ml ice-cold 75% ethanol, mixed well and left undisturbed for 20 min in an ice bath. The homogenates were then centrifuged for 10 min at 1000 g and the pellets were washed in three steps, each repeated three times, as follows: 1) ice-cold acetone (1:7, w/v), 2) ice-cold methanol–chloroform mixture (1:1, v/v) and 3) ice-cold methanol. The supernatant of each wash was discarded and the final pellet was freeze-dried overnight. The dried pellets were further ground to powder with a mortar and pestle and considered as crude cell walls. The powder was stored at 4 °C until required.

### 2.7. Fractionation of cell walls

Individual cell wall components were extracted according to Zhong and Lauchli (1993). The crude cell walls of mycorrhizal and non-mycorrhizal roots (0.1 g) were incubated in 4 ml 0.5% ammonium oxalate buffer (containing 0.1% $NaHB_4$), and washed twice in boiling water for 2 h. After centrifugation the supernatants were collected, considered as pectin, and the volume was recorded. The precipitate was washed with deionized water twice and freeze-dried. The dried precipitate was washed with 4 ml 4% NaOH (containing 0.1% $NaHB_4$) three times for 24 h at room temperature. After centrifugation the supernatants were collected and the volume was recorded. After being neutralized with cold acetic acid, the supernatant was freeze-dried and designated hemicellulose 1 (HC1). In the same way the precipitate was washed and freeze-