In Situ Distribution and Speciation of Toxic Copper, Nickel, and Zinc in Hydrated Roots of Cowpea1[W][OA]

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The phytotoxicity of trace metals is of global concern due to contamination of the landscape by human activities. Using synchrotron-based x-ray fluorescence microscopy and x-ray absorption spectroscopy, the distribution and speciation of copper (Cu), nickel (Ni), and zinc (Zn) was examined in situ using hydrated roots of cowpea (Vigna unguiculata) exposed to 1.5 μM Cu, 5 μM Ni, or 40 μM Zn for 1 to 24 h. After 24 h of exposure, most Cu was bound to polygalacturonic acid of the rhizodermis and outer cortex, suggesting that binding of Cu to walls of cells in the rhizodermis possibly contributes to the toxic effects of Cu. When exposed to Zn, cortical concentrations remained comparatively low with much of the Zn accumulating in the meristematic region and moving into the stele; approximately 60% to 85% of the total Zn stored as Zn phytate within 3 h of exposure. While Ni concentrations were high in both the cortex and meristem, concentrations in the stele were comparatively low. To our knowledge, this is the first report of the in situ distribution and speciation of Cu, Ni, and Zn in hydrated (and fresh) plant tissues, providing valuable information on the potential mechanisms by which they are toxic.

Trace metals are natural components of the environment, but elevated and potentially toxic levels sometimes occur. The toxicity of trace metals to plants is an important environmental and economic problem. The release of copper (Cu), nickel (Ni), or zinc (Zn) from anthropogenic sources (such as mining and refining, fungicide and manure use, and the disposal of biosolids) is of concern due to their potentially detrimental effects on the environment. It is estimated that hundreds of thousands of sites are contaminated globally (Canadian Environmental Industries, 2005). The mechanisms by which trace metals are toxic to plants remain unclear, despite their toxic effects having been researched for >100 years in the case of aluminum. Similarly, the mechanisms used by plants to tolerate excess trace metals continue to be debated. To answer these questions, it is important to determine (1) the distribution of trace metals within the root tissue, and (2) what ligands the trace metals bind to within the root. For this purpose, we contend that it is necessary to investigate the distribution and speciation in situ using hydrated (and preferably, fresh) plant tissues following comparatively short periods of exposure to environmentally relevant concentrations of the metal. To our knowledge, no studies meeting all of these criteria have investigated the speciation of Cu, Ni, or Zn in plant roots. For example, many studies use high and environmentally irrelevant concentrations of metals, particularly for Cu where the higher concentrations are most likely used to increase plant uptake and hence improve the signal-noise ratio within analyses (Mijovilovich et al., 2009); the extended x-ray absorption fine structure (EXAFS) analyses of Sahi et al. (2007) used 630 μM Cu while those of Shi et al. (2008) used 300 μM Cu. In contrast, studies have shown Cu to be toxic at concentrations <10 μM (Kopittke et al., 2010). Further, the use of freeze- or oven-dried plant materials, such as by Sahi et al. (2007), Balkanov et al. (2009), Mesjasz-Przybyłowicz et al. (2007), Serret et al. (2002), Terzano et al. (2008), and Straccek et al.
(2008), is not ideal given the potential influence of drying on sample integrity directly affecting metal speciation and distribution. Finally, although metals are known to induce toxicities within minutes or hours of exposure (Blamey et al., 2004, 2011), most studies determine metal speciation after comparatively long periods of exposure (often days or weeks). For example, although Mijovilovich et al. (2009), van Steveninck et al. (1994), and Küpper et al. (2009) utilized frozen, hydrated samples grown at relevant concentrations, the plants were exposed to metals for comparatively long periods of time (7 d to 4 months). While these studies provide useful data about long-term toxicity, the speciation after these extended periods may not be related to the initial toxic effects of the metals (or the initial response of the plants to metal toxicity).

It is also interesting to note that although many studies have examined the distribution and speciation of trace metals in plants, most have used hyperaccumulators (for example, see the review of Callahan et al., 2006). Somewhat surprisingly, despite their agronomic and environmental importance, data examining the in situ distribution and speciation of metals in nonhyperaccumulating plants are comparatively scarce. One likely reason is that analyses in nonhyperaccumulating plants are more challenging due to their lower concentrations (Lombi and Susini, 2009).

On the basis of the above discussion, this study aims to investigate the in situ distribution and speciation of Cu, Ni, and Zn in roots of a nonhyperaccumulator (cowpea [Vigna unguiculata]) following short periods of exposure (1–24 h) to toxic, but environmentally relevant, levels of trace metals. For this purpose, synchrotron-based x-ray fluorescence microscopy (μ-XRF) and x-ray absorption spectroscopy (XAS) are ideally suited due to their high sensitivity and ability to analyze hydrated (and in the case of μ-XRF, fresh) samples.

RESULTS

Root Growth

Although not measured in this experiment, it was apparent that the addition of Cu, Ni, or Zn reduced root growth substantially compared to that in the control. For Cu (but not Ni or Zn), light microscopy revealed that the reduction in growth was associated with the rupturing and tearing of the root epidermis and outer cortex (Supplemental Fig. S1; see also Kopittke et al., 2008). After 1 to 24 h of exposure, metal concentrations in root tissues ranged between 5.1 and 71 μg g⁻¹ on a fresh mass basis (see Supplemental Table S1); concentrations that are low compared to those in root tissues at the time of analysis (commonly freeze dried) in most previous studies. For example, Sahi et al. (2007) reported that the freeze-dried roots of Sesbania drummondii contained a Cu concentration of 27,440 μg g⁻¹ when analyzed, Shi et al. (2008) reported that freeze-dried roots of Elsholtzia splendens contained a Cu concentration of 11,755 μg g⁻¹ when analyzed, and Sarret et al. (2002) reported Zn concentrations of 1,100 to 14,400 μg g⁻¹ (17–218 μmol g⁻¹) in freeze-dried roots of the hyperaccumulator Arabidopsis halleri. The low tissue concentrations of this study result from the use of hydrated roots, short exposure periods, the use of a nonhyperaccumulating species, and comparatively low concentrations of metals within the nutrient solution.

μ-XRF

There was no evidence that the μ-XRF analyses damaged the fresh, hydrated roots (see Lombi et al., 2011 for more details regarding sample damage for the current experimental system). Indeed, light microscopy revealed that the roots remained healthy and hydrated after analysis (Supplemental Fig. S2).

The two-dimensional (2D) elemental maps presented here are a projection of the three-dimensional volume of the roots. This is due to the ability of the x-ray beam to penetrate and generate signal from the whole thickness of the sample. However, since the morphology of the root, with the exception of the root tip, is cyndrical, the images can be quite easily interpreted. In the case of the root tip it should be considered that its diameter becomes progressively smaller. Therefore, apparently lower concentrations in the 2D elemental maps in the root tips may simply be the result of the progressively smaller volume probed closer to the apex.

For the 24-h roots, Cu was located largely within the rhizodermis (and possibly the outer cortex), with only low concentrations observed in the cortex and stele (Fig. 1). Comparatively high concentrations of Cu were also observed at the root apex, although close examination tends to suggest that the Cu at the root apex was likely bound to the outer layers of the root cap rather than being internalized (as observed for Zn; Fig. 1). A similar pattern was observed after 6 h of exposure, with most Cu within approximately 1 mm of the root apex (Fig. 1). For roots exposed to Cu for 24 h, rupturing and tearing was observed in the rhizodermis and outer cortex (Supplemental Fig. S1). In these ruptured roots, Cu tended to accumulate in the rhizodermal cells on either side of the rupture, but not in the cortical cells at the base of the rupture even though these cells were exposed directly to the bulk solution (Fig. 1). However, it should also be considered that this direct exposure only started upon rupture of the rhizodermis.

As expected, and as observed for Cu, concentrations of Ni were higher in the distal portions of the root (Fig. 1). Interestingly, although Ni accumulated in the cortex (with the highest concentrations generally in the inner cortex), concentrations in the stele were substantially lower along the entire length of root analyzed (apparently including the procambium near the root apex). It was also noteworthy that although Ni accumulated within the meristem, concentrations were lower in the adjacent root cap (Fig. 1). However, as
In particular, it was noted that the distribution of Zn was discrete, with some cells having high concentrations and other cells having comparatively low concentrations (Fig. 2). For the cells in the stele/procambium, it is possible that these high concentrations of Zn correspond to the xylem. A 2D scan collected after the tomographic series revealed that the tomographic analysis did not cause damage to the fresh, hydrated root (Fig. 2).

Although the distribution of calcium and other nutrients was also examined, no differences could be observed between treatments (data not presented).

**XAS**

Roots exposed to Cu or Zn were examined using XAS. These two metals were chosen as they differed markedly in their distribution within cowpea roots (there was insufficient beamtime to permit examination of Ni also). The Zn EXAFS spectra for the model compounds reported in Figure 3 are similar to previously reported data (for example, Sarret et al., 2009). The spectra of aqueous Zn and Zn complexed by polygalacturonic acid and citric acid have a similar oscillation that is consistent with octahedrally coordinated Zn. The x-ray absorption near edge structure (XANES) spectra of these compounds also shows strong similarities (Fig. 4). Zn-His, previously reported by Sarret et al. (2009), has a distinctive second shell feature. Zn bound to Cys shows a frequency consistent with longer Zn-sulfur (S) bond distance and a lower energy edge position in comparison with other Zn compounds.
compounds. The frequency in the EXAFS spectra of Zn bound to phosphate groups and the XANES spectra provide several features that can be used to differentiate these compounds. For instance, the XANES of Zn phytate has a characteristic broader, almost double-peaked, main feature (Fig. 4). Linear combination fitting (LCF) of the EXAFS root data revealed that the majority of Zn was in a form resembling Zn bound to phytic acid; good correspondence was observed between the EXAFS spectra for phytic acid and those from the 3- and 24-h roots (Fig. 3). Indeed, using LCF of the EXAFS data, it was estimated that approximately 60% to 85% of the Zn was associated with phytic acid for both the 3- and 24-h exposure periods (Table I; Fig. 5, A and B). These results regarding the importance of phytic acid were reinforced by the examination of the XANES data. The XANES spectra for the root samples are visually similar to the spectrum of Zn phytate, and this was confirmed by LCF (Table I; Fig. 5, C and D). These results regarding the importance of phytic acid were reinforced by the examination of the XANES data. The XANES spectra for the root samples are visually similar to the spectrum of Zn phytate, and this was confirmed by LCF (Table I; Fig. 5, C and D). However, the speciation of the remaining 15% to 40% of the Zn was not clear, with LCF of the EXAFS and XANES data suggesting that it may be bound to Cys, His, or precipitated as Zn₃(PO₄)₂ (Table I). It should be noted here that the LCF analysis is not free of uncertainties and limitations. For instance, the fitting combinations obtained are necessarily the result of the choice of the standards used in the fitting procedure (Lombi and Susini, 2009). Thus, additional confirmation of the XANES and EXAFS LCF data were conducted via shell-by-shell fitting of the radial distribution functions (RDFs) to decipher the coordination environment of Zn and Cu. The Fourier transform Zn EXAFS signal supports the results of LCF, demonstrating the presence of Zn-oxygen (O) and Zn-phosphorus shells for Zn phytate and Zn-nitrogen, Zn-S, and Zn-carbon (C) shells from the Zn-Cys model (Supplemental Fig. S4; Supplemental Table S2). However, we have endeavored to include representative spectra of the main compounds and functional groups likely to coordinate metals in plants. The convergence of XANES and EXAFS LCF analyses with regards to the likely dominance of Zn phytate provides a reinforcing argument in terms of the reliability of the LCF approach.

The tetrahedrally coordinated Cu-Cys complex produced characteristic EXAFS and XANES spectra easily distinguished from the other Cu standards analyzed. The spectra of Cu-Cys complex reported in Figure 3 and Figure 4 are similar to those shown recently by Dokken et al. (2009) who suggested that Cu is mainly bound by S bonds on the thiol groups of this organic ligand. For roots exposed to Cu for 3 h, the low levels of Cu in the root tissue (Supplemental Table S1) reduced the accuracy of the analysis due to a reduced signal-noise ratio. Regardless, visual observation of the root spectra clearly shows that the
speciation at 3 h was markedly different from that at 24-h exposure (compare the EXAFS and XANES curves for 3 and 24 h, Figs. 3 and 4). For both EXAFS and XANES, LCF suggested that approximately 45% to 60% of the Cu at 3 h was associated with Cys, with the remainder associated with either citric acid (EXAFS) or His (XANES; Table I; Fig. 6). These results are supported by fitting of the RDFs for Cu-identified Cu-O and Cu-C shells for Cu citrate and Cu-nitrogen, Cu-S, and Cu-C shells for Cu Cys (Supplemental Fig. S4; Supplemental Table S2).

The octahedrally coordinated Cu model complexes showed a characteristic preedge feature (visible in the first derivative of the spectra) that is characteristic of a 1s → 3d transition and a more pronounced shoulder due to 1s → 4p transition. Both these features are indicative of tetragonal distortion (Kosugi et al., 1984). For the roots exposed to Cu for 24 h, much of the Cu

| Table 1. Results of LCF of K-edge EXAFS and XANES data to cowpea roots exposed to 40 μM Zn or 1.5 μM Cu for 3 or 24 h |
|-----------------------------------------------|
| **Data are rounded to two significant figures, with means ± the SD.** |
| **Standard** | **EXAFS** | **XANES** | **EXAFS** | **XANES** |
|---|---|---|---|---|
| **Zn** | | | | |
| Phytic acid (%) | 62 ± 3.1 | 74 ± 0.6 | 85 ± 4.1 | 71 ± 0.8 |
| Cys (%) | 26 ± 0.6 | 26 ± 0.6 | 26 ± 0.6 | 26 ± 0.6 |
| His (%) | 38 ± 4.3 | 15 ± 4.6 | 16 ± 0.7 | 16 ± 0.7 |
| Phosphate (%) | 0.030 | 0.00033 | 0.047 | 8.1 × 10⁻⁵ |
| R factor | | | | |
| **Cu** | | | | |
| Polygalacturonic acid (%) | 43 ± 2.9 | 60 ± 5.8 | 29 ± 2.2 | 29 ± 2.2 |
| Aqueous (%) | 18 ± 5.5 | 35 ± 0.7 | 35 ± 0.7 | 35 ± 0.7 |
| Citric acid (%) | 57 ± 2.5 | 45 ± 1.1 | 22 ± 1.8 | 22 ± 1.8 |
| Cys (%) | 55 ± 2.3 | 55 ± 2.3 | 55 ± 2.3 | 55 ± 2.3 |
| His (%) | 0.091 | 0.00058 | 0.068 | 8.9 × 10⁻⁵ |
| R factor | | | | |

*R factor = Σ(experimental-fit)²/Σ(experimental)², where the sums are over the data points in the fitting region.*
appeared to be bound to polygalacturonic acid (Figs. 3 and 4). The LCF of the EXAFS data confirmed this observation, with an estimated 60% of the Cu associated with polygalacturonic acid at 24 h (Table I; Fig. 6, A and B). Once again, the importance of polygalacturonic acid was confirmed using LCF of the XANES data (Table I; Fig. 6, C and D), although the similarity of the XANES spectra of these compounds may translate some uncertainty in regards to their relative contribution. As noted for the Zn, the speciation of the remaining Cu in the 24-h roots was not certain, although it appeared to be associated with Cys or citric acid, and as soluble Cu (EXAFS; Table I; Fig. 6).

Overall, the LCF results were generally similar from both the EXAFS and XANES spectra for all four treatments (Table I) and confirmed by EXAFS shell fitting of the RDFs (Supplemental Fig. S4; Supplemental Table S2). However, some discrepancies were evident, particularly for the roots exposed to Cu for 3 h and in the other treatments for ligands with compar-
DISCUSSION

Determining the distribution and speciation of metals in roots is crucial in elucidating the mechanisms by which they are toxic to plants; this study provided data for fresh, hydrated roots (distribution) or frozen, hydrated roots (speciation) of cowpea. For these roots, most Cu accumulated within the rhizodermis (and possibly the outer cortex) and root cap, with substantially lower concentrations within the inner cortex and stele (Fig. 1). These results for Cu are in line with the known affinity of Cu for the cell wall. Indeed, these results regarding Cu distribution are also consistent with the findings from our XAS analyses that, after 24 h of exposure, substantial quantities of Cu were bound to polygalacturonic acid (the main component of pectin; Table I; Fig. 6). Similarly, Nishizono et al. (1987) reported that 70% to 90% of the Cu in roots of Athyrium yokoscense was located in the cell wall. Interestingly, it was noted that although Cu accumulated in the rhizodermis, concentrations within the root tissues at the base of the ruptures were low even though these cells were exposed directly to the Cu-containing bulk solution (Fig. 1; Supplemental Fig. S1). These observations are consistent with the proposal of Kopittke et al. (2008) who suggested that Cu inhibits root growth, at least in part, by binding to the cell wall and preventing the wall from loosening as part of the elongation process. Ruptures thereby form due to the presence of rigid (slowly expanding) outer cells overlying cells of the stele and inner cortex that are expanding at a faster rate (given that these inner cells have less Cu bound to the cell wall and hence are less rigid). Certainly, the observation that Cu was bound to polygalacturonic acid in the rhizodermis (but not the cortex and stele) is consistent with this hypothesis regarding the mechanism of Cu toxicity. The reason as to why only comparatively low concentrations of Cu are located in the tissues at the base of the ruptures is unclear. Presumably this occurs because the cell walls of these cortical cells have a lower negative charge than those in the rhizodermis (for examples, see Boudjeko et al., 2006 or Douchiche et al., 2010). Further work is required in this regard.

Concentrations of Zn were highest in the meristemic region and lowest in the cortex, with intermediate concentrations observed along the stele (Fig. 1). Tomographic analysis 0.75 mm from the root apex confirmed the presence of Zn in the procambium/stele, with the Zn largely present in discrete locations (perhaps in the xylem; Fig. 2). Some accumulation was also evident in the rhizodermis and outer cortex at this location (Fig. 2). Although the speciation data from the XAS was not spatially resolved, approximately 60% to 85% of Zn within the apical 5 mm was found to be bound to phytic acid (Table I; Fig. 5; Supplemental Fig. S4; Supplemental Table S2). Thus, accumulation as Zn phytate would appear to be a rapid response, accounting for the majority of the Zn in cowpea roots. Indeed, van Steveninck et al. (1994) suggested that the sequestration of Zn as Zn phytate within vacuoles of root cells may provide a strategy for limiting transport to the shoot in several agronomic species. This is consistent with the observations from the tomographic analysis, where the size of the Zn accumulations in the rhizodermis and outer cortex would tend to suggest that the Zn was largely intracellular (rather than in the cell wall; Fig. 2). Regardless, the observed distribution of Zn (Fig. 1) would tend to suggest that much of the Zn is taken up close to the root apex (for example, near the meristem, where the Caspian strip is not fully formed), stored as Zn phytate, with some Zn moving into the stele and presumably into the shoot. Using a cadmium (Cd)-sensitive microelectrode, a similar pattern of uptake has been reported in roots of Thlaspi caerulescens exposed to 5 mM Cd (Piñeros et al., 1998).

The distribution of Ni was different to that of both Cu and Zn (speciation of Ni was not examined). While Ni concentrations were high in both the cortex and meristem, there was reduced movement into the stele. Also, there tended to be accumulation at the inner cortex, apparently at the Caspian strip (in particular, note the more mature portions of the root; Fig. 1). These findings are in accordance with the results of Lombi et al. (2011) who conducted tomography on a fresh, hydrated cowpea root exposed to 5 mM Ni for 24 h. Similarly, Seregín et al. (2007) studied several plant species exposed to 10 to 400 μM Ni and reported that Ni moves readily into the root meristem, often accumulating at the endodermis in nonhyperaccumulators.

These differences in the distribution of these trace metals suggest differences in the transport mechanisms. For example, the uptake and transport mechanisms for Zn are comparatively well understood (see Kramer, 2010), and the data here demonstrate the movement and uptake of Zn into the root tissue. In contrast, for Ni there was reduced movement into the stele; the mechanisms of Ni transport and delivery are largely unknown (Mizuno et al., 2005). Clearly, further work is warranted, and use of the current experimental technique in combination with genetic tools will provide substantial advancements.

As already noted, the speciation of Cu differed markedly from Zn; approximately 60% to 85% of Zn was most likely bound to phytic acid while much of the Cu was likely associated with Cys (3 h) or polygalacturonic acid (24 h; Table I; Figs. 5 and 6). Interestingly, both Cu and Zn have similar stability constants for phytic acid (Crea et al., 2008). Phytate,
myoinositol hexakisphosphate, contains a high density of negatively charged phosphate groups and hence forms stable complexes with many ions, including Cu and Zn. However, one notable difference between Cu and Zn phytate is that the Zn form is less soluble, resulting in its precipitation from solution (Champagne and Fisher, 1990). It is therefore possible that the precipitation of Zn phytate results in the increased production of phytic acid, leading to the accumulation of Zn in this form. Whether the accumulation, and presumed precipitation, of Zn phytate is a specific mechanism of tolerance, or whether the formation of Zn phytate occurs simply because of the high concentrations of these elements in biological tissues, is unknown. Regardless, the presence of this compound in tissues exposed to excess Zn for \( \leq 3 \) h suggests that it may be important in regulating the toxicity of Zn to plants (and their tolerance to Zn).

The finding that only comparatively small amounts of Zn were bound to S-containing ligands (such as Cys) is perhaps not unexpected, given that Zn prefers O and N as ligands. Indeed, Zn binds relatively strongly to hard ligands (O and N) but only weakly to soft ligands (S; Kinraide, 2009). As a result, S-rich compounds tend not to be involved in Zn accumulation. In contrast, Cu binds very strongly to soft ligands (Kinraide, 2009), which is reflected in this study by the LCF results indicating binding of Cu to a ligand, Cys, which contains thiol groups. It should be noted here that Cys is a key component of metal-scavenging proteins such as metallothioneins and phytochelatins. Further, the finding that substantial quantities of Cu after 24 h of exposure are bound to polygalacturonic acid is also not unexpected. Polygalacturonic acid is the main component of pectin, and Cu is known to bind strongly to cell walls. The reason for the apparent shift in Cu speciation between 3 (Cys, and perhaps citric acid or His) and 24 h (polygalacturonic acid) is not known. One possibility is that Cu initially accumulates with Cys, or ligands possessing thiol groups, due to its strong binding to S. However, once these sites are saturated, the Cu then accumulates within the cell wall with the polygalacturonic acid.

The importance of Zn phytate has been reported previously in other nonhyperaccumulating species. For example, Terzano et al. (2008) reported that freeze-dried roots of Eruca vesicaria grown for 7 d in Zn-toxic solutions were able to partly block Zn immediately outside the endodermis in the form of Zn phytate. Similarly, using scanning electron microscopy with energy-dispersive x-ray spectroscopy on cryo-preserved samples, van Steveninck et al. (1994) reported that various agronomic species, such as lucerne (Medicago sativa), soybean (Glycine max Merr.), and tomato (Solanum lycopersicum), formed Zn phytate in their roots after 7 to 14 d of exposure to solutions containing 80 to 300 \( \mu \)M Zn. Other ligands have also been reported to be of importance in roots, particularly in hyperaccumulating plants (see Broadley et al., 2007 for more information). For example, Zn has been reported to be associated with His in roots of T. caerulescens, but with organic acids in the xylem (Salt et al., 1999). In freeze-dried roots of A. halieri grown on contaminated soil, Zn was reported to be associated with malic acid, citric acid, and phosphorus possibly either \( \text{Zn}_3(\text{PO}_4)_2 \) or Zn phytate; Sarret et al., 2002. Finally, Straczek et al. (2008) reported that Zn was bound to oxalic acid and other carboxyl groups (likely associated with the cell wall) in freeze-dried roots of Nicotiana tabacum.

The findings regarding the speciation of Cu are also in agreement with previous findings. For example, in the roots, stems, and leaves of E. splendens (a Cu-tolerant plant) grown in 300 \( \mu \)C Cu for 10 to 60 d, most Cu was bound to O-containing ligands in the cell wall (Shi et al., 2008). Similarly, Küpper et al. (2009) reported that, in frozen, hydrated shoots of Crassula helmsii grown for 8 d at 10 \( \mu \)M Cu, Cu was bound by O ligands (such as organic acids). However, other studies have reported Cu to be bound to S-containing ligands. Similarly, Cu has been reported to bind to phytochelatins in stems (but not roots) of soil-grown Larrea tridentata (Polette et al., 2000), while Cu in leaf samples of T. caerulescens (a Zn/Cd hyperaccumulator) grown in solutions containing 10 \( \mu \)M Cu for 4 months was reported to be bound by S ligands such as in metallothioneins (Mijoviclovich et al., 2009).

Although previous studies have investigated the distribution and speciation of Cu and Zn (as discussed above), to our knowledge this is the first study to provide in situ data for these metals in hydrated tissues following exposure to environmentally relevant concentrations for comparatively short periods of time. We contend that these factors are important in establishing the underlying mechanisms of toxicity and tolerance for these metals.

**CONCLUSION**

This study has provided data on the in situ analysis of hydrated (both fresh and frozen) roots of cowpea (an agronomic species) grown in Zn- and Cu-toxic solutions for comparatively short periods of time. After 24 h of exposure, most Cu was bound to polygalacturonic acid (in the cell wall) of the rhizodermis and outer cortex, which when considered together with the morphology of Cu-toxic roots, suggests that binding of Cu to walls of cells in the rhizodermis possibly contributes directly to the toxic effects of Cu. When exposed to excess Zn, cortical concentrations remained generally comparatively low, with much of the Zn accumulating in the meristematic region and moving into the stele; approximately 60% to 85% of the total Zn stored as Zn phytate within 3 h of exposure. While Ni concentrations were high in both the cortex and meristem, concentrations in the stele were comparatively low (in contrast to Zn). Also, there tended to be a slight accumulation at the inner cortex, apparently at the Casparian strip. These findings, together with knowledge of the underlying processes, are important in understanding both...
the toxicity of these metals and the response of plants to their toxicity.

MATERIALS AND METHODS

Plant Growth

For μ-XRF, seeds of cowpea (Vigna unguiculata ‘white Caloona’) were germinated in rolls of paper towel placed vertically in tap water for 3 d during which time they were transported to the Australian Synchrotron. Seven seedlings were placed in Perspex strips on top of 600-mL glass beakers filled to the brim (650 mL) with 1 mM CaCl$_2$ and 5 μM H$_2$BO$_3$. The beakers were placed in a water bath heated to 26°C, and the seedlings grown for approximately 12 to 18 h in this basal solution. The strips were then transferred to metal-containing solutions for the desired period of exposure. A total of three metals were investigated, being (μM) 1.5 Cu (6 and 24 h), 5 Ni (6 and 24 h), and 40 Zn (1 and 24 h). These concentrations have been shown to reduce growth by approximately 70% to 90% for a 48-h exposure period (Kopittke et al., 2011), and for Cu this concentration causes cowpea roots to rupture (Kopittke et al., 2008, 2009), but are briefly described here due to slight variations. Seeds of cowpea were placed in germination trays covered with paper towel moistened with tap water. Five plant growth containers were prepared, each with 22 L of continuously aerated nutrient solution consisting of 1 mM CaCl$_2$ and 5 μM H$_2$BO$_3$. After 2 d, approximately 230 seedlings were placed on shade cloth covering each of the five containers (giving a total of approximately 1,150 seedlings), and the seedlings were grown in this basal solution for a further 2 d. Five treatments were imposed, consisting of two metals (1.5 μM Cu or 40 μM Zn) both with two times of exposure (3 or 24 h), plus a single control (1 mM calcium). To add the metals, the shade cloth was lifted and the appropriate volume of a 6.5 mM stock solution (CuCl$_2$·2H$_2$O, NiCl$_2$·6H$_2$O, or ZnSO$_4$·7H$_2$O) added before the solution was stirred vigorously for 30 s and the shade cloth returned. The seedlings were removed from the metal-containing solutions at the appropriate times (3 or 24 h) and dipped in two separate solutions of 1 mM CaCl$_2$. While sitting in the second 1 mM CaCl$_2$ solution, the seedlings were pulled out of the shade cloth and the apical 5 mm of the root cut using a scalpel blade, blotted dry using whatman filter paper, immersed in liquid nitrogen, and immediately transferred to a dry shipper (cooled with liquid nitrogen). The sample was then transferred to the cryostat liquid helium) to hinder beam-induced artifacts and reduce thermal disorder. A tomographic analysis was conducted on a fresh, hydrated root exposed to 50 μM Zn for 24 h (see Lombi et al., 2011 for details). Briefly, a root was cut approximately 5 mm from the apex and placed in a polyimide capillary (internal diameter 860 μm) sealed with wax. The tube was partially filled with water to ensure the root did not dehydrate, however, the root was not immersed during imaging. The tomogram was generated by scanning the sample along a single transsect, 0.75 mm from the root tip, and repeating this scan 100 times (0°–360°). The sampling interval was 2 μm and the transit time was set at 3.9 ms (with corresponding acquisition times of about 9 min). The Zn and Compton sonograms were used to reconstruct the corresponding tomograms. Topography is possible on fresh, hydrated roots at the XFM beamline due to the greater data acquisition speed of the Maia detector compared to conventional detectors (Lombi et al., 2011).

Synchrotron Analysis (XAS)

The speciation of metals within root apices was examined using the XAS beamline at the Australian Synchrotron. Energy calibration of the spectra was obtained by simultaneous measurement, in transmission, of a metal foil reference (Cu or Zn). Both XANES and EXAFS spectra were collected in fluorescence mode using a 100-element solid-state Ge detector. The roots were mounted in a cryostat sample holder (maintained at approximately 12 K, liquid helium) to hinder beam-induced artifacts and reduce thermal disorder. The beam size was adjusted to approximately 1.2 × 0.7 mm.

To prepare roots for analysis, an agate mortar and pestle was cooled using liquid nitrogen. Approximately 50 frozen root apices were placed in the mortar and further liquid nitrogen added while the roots were homogenized. The roots were then placed into a sample holder with Kapton tape windows coated with liquid nitrogen. The sample was then transferred to the cryostat for analysis. At no stage between harvest and analysis were the roots allowed to thaw.

Standards, prepared by mixing the two metals with various ligands, were analyzed in hydrated (frozen) form using the cryostat. In all instances, the final concentration of the metal was 3 mM, together with 0.5% polygalacturonic acid, 15 mM Cys, 15 mM citric acid, 15 mM His, 15 mM phytic acid, or 15 mM oxalic acid. Similarly, 3 mM aqueous solutions of the metals (without additional ligand) were analyzed. In addition, solid Zn$_2$(PO$_4$)$_3$ (587583, Sigma Aldrich) was prepared for analysis by dissolving with boron nitride. All aqueous standards were mixed in 30% glycerol to avoid ice crystal formation. The Cys, citric acid, His, oxalic acid, and phytic acid standards were adjusted to approximately pH 6 using 0.5 M KOH (initial pH values were approximately 2–5), while Cu$_2$ or Zn$_2$ was not adjusted for the polygalacturonic acid (which formed a gel once mixed with the metal) or the 3 mM aqueous-metal standards (without organic ligands). Standards were prepared using 30 mM stock solutions of CuCl$_2$·2H$_2$O or ZnSO$_4$·7H$_2$O together with 1% polygalacturonic acid (P3850, Sigma Aldrich), or 150 mM solutions of i-Cys (W362305, Sigma Aldrich), citric acid (C9009, Sigma Aldrich), i-His (H8000, Sigma Aldrich), phytic acid (P8810, Sigma Aldrich), or oxalic acid (F58688, Sigma Aldrich). For the polygalacturonic acid, a 1% solution was prepared and mixed with Amberlite IR120 resin (Supelco) to convert the polygalacturonic acid from the sodium to hydrogen form. The free carboxyl groups for the 1% polygalacturonic acid were determined by titration with 0.02 M NaOH to neutrality (Walter, 1991), determined to be 30.4 μmol COO$^-$ mL$^{-1}$. The final 1% polygalacturonic acid
acid solution (hydrogen form) was prepared approximately 24 h before travel to the synchrotron and kept at 4°C until use. It was possible to model some of these standard solutions using GeoChem-EZ; the results indicating that >99% of both Zn and Cu were complexed with citric acid, >99% with oxalic acid, >98% for His, and >96% for Cys.

Both XANES and EXAFS data were collected at the Cu/Zn K-edge for the 24-h roots (three scans per metal treatment), 3-h roots (eight scans), and the standards (two scans). Thus, for each treatment there were three to eight scans, with approximately 50 homogenized root apices analyzed in each scan. Furthermore, as stated earlier, scans were conducted at two different times using independent sets of plants (with at least one scan done for each treatment on both occasions).

To examine the possibility of beam-induced damage, for a limited number of samples, spectra were collected twice from the same location and the spectra compared to determine if the beam had caused a change in speciation. In all other cases, to reduce the risk of beam damage and to obtain representative spectra, after each scan the sample position was moved by approximately 1.5 to 2.0 mm to sample different areas of each specimen. Spectra were energy normalized using the reference energy of the Cu and Zn foils, replicate spectra for each sample were then merged, and data were background and baseline corrected using Athena v.0.8.061 (Ravel and Newville, 2005). For both XANES (−20–30 E eV) and EXAFS (3–9 k A⁻³), LCF was performed using Athena. For the LCF, a maximum of three standards were permitted for each fit.

To further substantiate the LCF results of XANES and EXAFS, the EXAFS signal was extracted from the averaged spectra to identify the coordination environment of Zn and Cu. The data were converted from energy to photoelectron momentum (k-space) and weighted by 1/k. EXAFS spectra were calculated over a typical k-space range with a Bessel window and 1.0-width Gaussian wings. Fourier transforms were performed to obtain the RDF in R space. Plotted R-space (Å) data are not phase-shift corrected, the true Gaussian wings. Fourier transforms were performed to obtain the RDF in R space. Plotted R-space (Å) data are not phase-shift corrected, the true

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