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REGULAR ARTICLE

Identifying the species of copper that are toxic to plant roots in alkaline nutrient solutions

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Abstract
Background and aims The pH of the growth medium influences Cu speciation in solution, the negativity of plasma membrane (PM) surface potential, and hence the rhizotoxicity of Cu.
Methods Solution culture experiments were conducted with wheat (*Triticum aestivum* L.) seedlings to examine the toxicity of various Cu species at pH values ranging from 4.50 to 8.25. The toxic species of Cu was identified, giving particular consideration to the electrical properties at the plant cell membrane and ion activities at the PM surface.
Results The solution culture studies showed that at pH<6.60 (i.e., free Cu^{2+} >95 % of total Cu), the addition of cations (Ca^{2+} or H^+) decreased the toxic effects of Cu by decreasing the negativity of the PM surface potential (and hence decreasing the activity of Cu^{2+} at the PM surface). For solutions with pH values from 7.50 to 8.25 ($CuCO_3^0$ >50 % of total Cu), an increase in pH significantly enhanced the toxicity of Cu, whilst the addition of Ca had negligible influence on toxicity.
Conclusions Root growth in solution cultures was influenced primarily by the surface activities of free Cu^{2+} and $CuCO_3^0$. Across all experiments, the data indicate that it was $CuCO_3^0$, rather than $CuOH^+$, that contributed Cu toxicity over pH>7.00. Although our data do not explore the mechanism of toxicity, we propose that $CuCO_3^0$ has an important role in Cu rhizotoxicity in alkaline growth media.

Keywords Cu speciation · Rhizotoxicity · Copper carbonate · Plasma membrane surface potential · Alkalinity

Introduction

Copper is an essential micronutrient required for normal plant growth and development. However, at elevated concentrations, it is also highly toxic to plant growth potentially resulting in complete inhibition of growth. Some contaminated soils have been reported

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to contain more than 1,000 mg Cu kg^{-1} (Kabata-Pendias and Pendias 2001; Luo et al. 2006). Most Cu in soil is present in strongly bound forms with the soil solids, with relatively low levels in the soil solution (and potentially bioavailable forms) (Luo et al. 2006). Of the Cu in the soil solution, Cu is present as the free Cu^{2+} ion and other species bound with inorganic (e.g., CO_3^{2-}, OH^-) or organic ligands (Sauvé et al. 1997). Solution pH has an important influence on the distribution of Cu species. For example, in the Cu^{2+}-H^+-CO_3^{2-} system in equilibrium with atmospheric CO_2, the free Cu^{2+} ion is the dominant species (>95 %) at pH<6.60, whilst at pH>7.50 $CuCO_3^0$ is the dominant Cu species (Powell et al. 2007; Millero et al. 2010).

It is well-established that the free Cu^{2+} ion (the activity of which is denoted as $\{Cu^{2+}\}$) is the form available to organisms, as described by the free ion activity model (FIAM) (Campbell 1996). The effect of pH on the rhizotoxicity of Cu has been studied extensively in nutrient solutions at pH values <7.0. For these solutions (i.e. <7.0), an increase in pH (decrease in $\{H^+\}$) has been reported to increase the rhizotoxicity of Cu to wheat (*Triticum aestivum*) (Parker et al. 1998; Luo et al. 2008; Wang et al. 2008), cowpea (*Vigna unguiculata*) (Kopittke et al. 2011a), and barley (*Hordeum vulgare*) (Lock et al. 2007). Recent analyses have demonstrated that this increased toxicity can be attributable to an increased activity of Cu^{2+} at the plasma membrane (PM) surface. Hereafter, the activity of Cu species M in the bulk solution is designated as $\{M^Z\}_b$ while its activity at the outer PM surface is $\{M^Z\}_0$, where Z is the charge of the ion. Increasing pH can polarize the PM and increase the negativity of electrical potential at the outer surface of the PM (ψ_0), and thereby increase $\{Cu^{2+}\}_0$ and increase toxicity (Kinraide 2006; Wang et al. 2008; Kinraide and Wang 2010; Kopittke et al. 2011a).

Much less information is available regarding Cu toxicity in alkaline solutions (pH>7.0) (which is important, for example, in the alkaline soils contaminated by Cu-containing fungicides (Poschenrieder et al. 2001; Komárek et al. 2010)). Interestingly, studying root elongation of barley (*H. vulgare*), Wang et al. (2009) reported that the EC50 for total Cu decreased three-fold as pH increased from 6.5 to 8.0. Similarly, Lock et al. (2007) reported that Cu-containing solutions were substantially more toxic to roots of barley at pH 7.7 than at pH 6.9 (and this increased toxicity was not associated with increased uptake of Cu). Over this pH range (ca. 7 to 8), changes in ψ_0 due to changes in H$^+$ activity are too small (<1 mV) to account for increases in toxicity. However, it is noteworthy that the speciation of Cu changes substantially across these pH values and species other than Cu^{2+} dominate. This raises the possibility that Cu-species other than Cu^{2+} are toxic. Wang et al. (2009) attributed this enhanced toxicity to $CuOH^+$. In another study with *Daphnia*, De Schamphelaere and Janssen (2002) also proposed intoxication from $CuOH^+$. However, the study of the toxicity of pH-dependent, ligand-bound metals (e.g., $CuOH^+$, $CuHCO_3^+$, or $CuCO_3^0$) is difficult due to problems of inter-correlation among variables (including pH) and the added complication of changes in PM surface potentials (Kinraide 2006).

The aim of the present study was to identify the toxic ionic species of Cu, giving particular consideration to the electrical properties at the plant cell membrane and ion activities at the PM surface. Treatments were designed in order to minimise inter-correlation among the activities of the various species in the bulk culture media or at the PM surface. Furthermore, Ca was added to solutions to reduce the negativity of ψ_0 and thereby allow differentiation between Cu species with different valencies (through changing their activities at the PM surface).

Materials and methods

Solution culture design

Experiments were conducted in solution culture to study the effects of Cu speciation on toxicity over a wide range of pH values (4.50–8.25). Experiment 1 was designed to investigate the effect of pH on Cu toxicity when the total Cu concentrations were maintained constant. Solutions were arranged in a factorial design with eleven pH values (4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.25, 7.50, 7.75, 8.00, and 8.25), three $Ca(NO_3)_2$ concentrations (0.25, 1.00, and 4.00 mM), and four $Cu(NO_3)_2$ concentrations (0–1.50 µM).

Experiment 2 was designed to investigate Cu toxicity in alkaline solutions by holding the activity of Cu^{2+} constant whilst varying the activity of other Cu-species. Toxicity was examined at four pH values (7.50, 7.75, 8.00, and 8.25), with $\{Cu^{2+}\}_b$ being held constant at 0.064 µM by varying the total $Cu(NO_3)_2$

concentration at two $Ca(NO_3)_2$ concentrations (0.50 and 8.00 mM). With these treatments, the $\{Cu^{2+}\}_0$ was also constant (ca. 0.18 or 2.39 μM) given that the negativity of ψ_0 changed by <0.5 mV as pH increased from 7.5 to 8.25. Therefore, this experiment held $\{Cu^{2+}\}_0$ constant whilst activities of the other Cu species (i.e., $CuCO_3^0$, $CuOH^+$, $CuHCO_3^+$) varied in both the bulk solution and at the PM surface. For instance, $\{CuCO_3^0\}_0$ (which is equal to $\{CuCO_3^0\}_b$ since it has a valency of zero) ranged from 0 to 2.53 μM.

Experiment 3 examined whether Ca alleviates Cu toxicity at pH 8.00 (as is observed in acidic solutions). Seven Ca concentrations (0.25 to 10.0 mM) were examined at pH 8.00 with the bulk Cu^{2+} activity being held constant at either 0.48 or 0.96 μM (with the corresponding $CuCO_3^0$ activity constant at 0.60 or 1.20 μM). An increase in Ca reduced the ψ_0 negativity, resulting in a decrease in the activities of Cu species with a positive charge (i.e., Cu^{2+}, $CuHCO_3^+$ and $CuOH^+$) at the PM surface, whilst changes in the activity of $CuCO_3^0$ were negligible as it is an uncharged ion-pair.

Solution pH values were maintained constant by the use of buffers. At pH≤6.00, MES (2-2-[N-morpholino] ethane sulfonic acid, 2.0 mM) was used as a buffer, whilst at pH>6.00, pH was maintained using MOPS (3-[N-morpholino] propane sulfonic acid, 3.6 mM) and addition of NaOH. For solutions with a pH<5.50, HNO_3 was used for pH adjustment. Both buffers do not form complexes with Cu (Kandegedara and Rorabacher 1999; Lock et al. 2007) and did not affect Cu toxicity to plants at the concentrations used in this study. Solution pH was measured both at the start and upon completion of each experiment, and the concentrations of Ca, Cu and Na were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; POEMS-II, TJA, USA).

For all three experiments, 2 d old wheat (*T. aestivum* L. cv. Yangmai 14) seedlings with uniform root length (1–2 cm) were cultured in darkness for 48 h at 25 °C in acid-washed polyethylene beakers containing 500 mL of continuously aerated test medium. For each treatment series (i.e. different pH or Ca concentration), a control without Cu was included. The treatments were replicated twice with seven seedlings per replicate. At the completion of the experiment, the two longest roots of each seedling were measured and the mean of the 14 measured values per replicate were recorded. Further details of the growth experiments and precautions for the preparation of the test solutions have been presented previously (Wang et al. 2008, 2011a).

Cu speciation

The accuracy of the equilibrium constants used to compute the activities of the Cu species may influence the conclusions regarding the identification of the toxic species. Table 1 presents ten sets of equilibrium constants compiled from various sources. It is noteworthy that there is no consensus regarding the stability constants for Cu complexes with OH^-, which appear to depend largely upon the ionic strength (Paulson and Kester 1980; Santana-Casiano et al. 2008). For example, Paulson and Kester (1980) reported a value of −7.99 at an ionic strength of 0.05 M but −7.20 at 3.0 M. The modelling for the present study used the recommended and provisional stability constants from the International Union of Pure and Applied Chemistry (IUPAC) technical report (Powell et al. 2007), which are very close to two new measurements (Santana-Casiano et al. 2008; Millero et al. 2010). All thermodynamic modeling in the nutrient solutions was conducted using WHAM 6.5 with the stability constants of Cu complexes of IUPAC (Table 1). For the solution cultures, the equilibrium phases included atmospheric CO_2 ($P_{CO2}=10^{-3.5}$ atm).

Ion surface activity at the PM surface

Values for ψ_0 were calculated using the Gouy-Chapman-Stern (GCS) model (computer program available from the authors) with the average of the measured pH values and other cation concentrations in each treatment. The GCS model combines electrostatic theory (Gouy-Chapman theory) with ion binding (Stern model) so that ψ_0 can be computed (Kinraide et al. 1998; Tatulian 1999; Yermiyahu and Kinraide 2005; Kinraide and Wang 2010). This model incorporates the intrinsic surface charge density (σ_0) of a membrane, the ion composition of the bulk medium (BM), and ion binding to the membrane (see Yermiyahu and Kinraide (2005) and Kinraide and Wang (2010)). Knowledge of ψ_0 enables the calculation of ion activities at the PM surface. The activity of ion I^Z at the PM surface ($\{I^Z\}_0$) is computed from the activity of I^Z in the bulk-phase medium ($\{I^Z\}_b$) according to the Nernst equation:

$$\{I^Z\}_0 = \{I^Z\}_b \exp[-ZF\psi_0/(RT)] \quad (1)$$

where Z, F, R, and T are the charge on the ion, the Faraday constant, the gas constant, and temperature,

Table 1 Equilibrium constants (log *K*) for Cu species complied from different sources

Equilibrium reactions	Baes and Mesmer (1981)	Lindsay (1979)	Paulson and Kester (1980)[a]	Santana-Casiano et al. (2008)[b]	Millero et al. (2010)	IUPAC[c]	WHAM 6.5[d]	NIST[e]	Visual MINTEQ3.0[f]	Phreeqc[g]
$Cu^{2+}+H_2O \leftrightarrow CuOH^{+}+H^{+}$	−8.00	−7.70	−7.20 to −8.10	−7.33 to −8.12		−7.95	−7.52	−7.52	−7.50	−8.00
$Cu^{2+}+2H_2O \leftrightarrow Cu(OH)_2+2H^{+}$	−17.30	−13.78	−14.6 to −16.7	−16.40 to −16.68		−16.20	−16.22	−16.22	−16.23	−13.68
$Cu^{2+}+CO_3^{2-} \leftrightarrow CuCO_3$	6.73	6.72			6.74	6.75	6.75	6.77	6.77	6.73
$Cu^{2+}+H^{+}+CO_3^{2-} \leftrightarrow CuHCO_3^{+}$		12.42			12.20	12.17	14.62	12.13	12.13	12.13

[a] Ion strength was from 0.001 to 3.0 M

[b] Ion strength from 0.050 to 3.5 M

[c] Taken from International Union of Pure and Applied Chemistry (IUPAC) technical report (Powell et al. 2007)

[d] Taken from default database of Windermere Humic Aqueous Model (WHAM 6.5) (Tipping et al. 2003)

[e] Taken from National Institute of Standards and Technology (NIST) standard reference database 46 (Martell et al. 1997)

[f] Taken from default database of the equilibrium speciation model Visual MINTEQ 3.0 <www2.lwr.kth.se/english/OurSoftware/Vminteq/>

[g] Taken from the database of Phereeqc <http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqc/>

respectively ($F/(RT)=1/25.7$ at 25 °C when ψ_0 is expressed in mV).

Analysis of root elongation

Root elongation was evaluated as relative root elongation (RRE, %). The RRE was calculated using the formula:

$$\mathrm{RRE,\%} = 100(\mathrm{RL_T} - \mathrm{RL_S})/(\mathrm{RL_C} - \mathrm{RL_S}) \quad (2)$$

where RL_T represents the mean root length (RL) in the presence of Cu, RL_C represents RL in the corresponding Cu-free control, and RL_S represents RL in toxicant sufficient to saturate growth-inhibitory processes. The RL_S is nearly equal to RL at the time of seedling transfer to the test media. When growth responds to measures of toxicant intensity (T, such as the activity (μM) of free Cu^{2+} activity or other species in the bulk or at the PM surface), the resulting curves can be expressed by the following equation:

$$\mathrm{RRE} = 100/\exp\left[(a_1T)^{\beta 1}\right] \quad (3)$$

where a_1 (μM^{-1}) is a strength coefficient that increases with the strength of the metal toxicity, and β_1 (dimensionless) is a shape coefficient (Taylor et al. 1991; Wang et al. 2008, 2011a; Kopittke et al. 2011a). It is noteworthy that sometimes large differences in tolerance are observed among plant species. The differences in the a_1 and β_1 coefficients for Eq. 3 may denote differences in sensitivity (Wang et al. 2011b).

Statistics

All coefficients in equations were evaluated by multiple, non-linear regression analysis using SYSTAT 13 (Cranes Software International Ltd, India). Significance levels are $P<0.05$ for all reported regression and coefficients. Root mean square error (RMSE) is also given to estimate how close the predictions are to the observations.

Results

Effect of pH on Cu speciation in the absence of DOM

Figure 1 shows a speciation diagram for the Cu^{2+}-H^{+}-CO_3^{2-} system from pH 4.00 to 8.50, based on

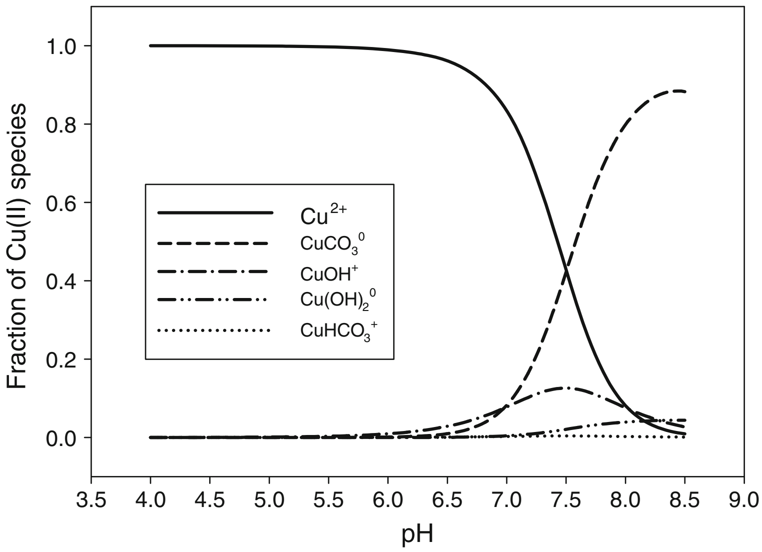

Fig. 1 Speciation of Cu as function of pH in solution containing a total of 1.0 μM $Cu(NO_3)_2$ and 1.0 mM $Ca(NO_3)_2$. The speciation was modelled using the equilibrium constants of IUPAC (Powell et al. 2007, Table 1) in equilibrium with atmospheric CO_2 (p_{CO2}= $10^{-3.5}$ atm). Only the five most abundant species are presented

the recommended stability constants of IUPAC (Martell et al. 1997) (Table 1). For solutions at pH<6.60, the free Cu^{2+} ion accounts for >95 % of the total Cu. However, for solutions at pH>6.60, the contribution of Cu^{2+} to the total Cu species decreases sharply. At pH>7.50, $CuCO_3{}^0$ is the dominant species with $CuOH^+$ also accounting for approximately 12.6 % of the total Cu at pH 7.50. $CuHCO_3{}^+$ formation constants are sufficiently small that $CuHCO_3{}^+$ is unlikely to be a significantly environmental species ($CuHCO_3{}^+$ for <0.37 % of the total Cu, Fig. 1) under normal environmental conditions. It can, however, become a significant species in condition with high levels of bicarbonate (Powell et al. 2007). It should be noted that $CuHCO_3{}^+$ maybe possibly contributes to Cu toxicity in some calcareous soils.

Effects of pH and Ca on Cu toxicity

In the range of pH from 4.50 to 6.50, increases in Ca^{2+} or H^+ (i.e. decreases in pH) alleviated Cu toxicity, but there was no significant relationship between RRE and $\{Cu^{2+}\}_b$ ($P>0.05$) (Fig. 2). The Ca- or H-induced alleviation of Cu toxicity resulted not from specific competition (also see Wang et al. 2012 for detail), but from a non-specific reduction in the negativity of ψ_0 which decreased $\{Cu^{2+}\}_0$ (and hence decreased inhibition of root growth). Indeed, RRE was significantly related to $\{Cu^{2+}\}_0$ (R^2=0.823; $P<0.001$, Fig. 2) irrespective of solution pH and Ca concentration, indicating that the effects of Ca^{2+} and H^+ in this pH range on Cu toxicity were attributed to the electrostatic effect of ψ_0. Using the fitted curves, an EA50$\{Cu^{2+}\}_0$ value (i.e., the effective PM activity of Cu^{2+} causing a 50 % inhibition in growth) can be derived as 15.6 μM. This value is similar to the value of 14.1 μM for wheat (Wang et al. 2008), and higher than 4.4 μM $\{Cu^{2+}\}_0$ for cowpea (Kopittke et al. 2011a). Thus, ion activities at the PM surface are more appropriate for examining plant-ion interactions than are bulk activities.

The data indicate that in the pH range of 6.50 to 8.25 (where species such as $CuCO_3{}^0$ become increasingly important, see Fig. 1 and discussion above), species other than Cu^{2+} probably also contribute to toxicity. For example, for solutions in which the activity of Cu^{2+} was held constant (but total Cu was increased due to the formation of other species), toxicity increased as pH increased; an increase in pH from 7.50 to 8.25 decreased RRE by 60 %, despite a constant $\{Cu^{2+}\}_b$ of 0.064 μM (Fig. 3).

So which of the other Cu-species formed in alkaline solutions is toxic? Interestingly, in Experiment 2 when solution pH was varied (and free Cu^{2+} ion was maintained constant by varying the total Cu), root elongation correlated with $\{CuCO_3{}^0\}_0$ (R^2=0.980, $P<0.001$) more closely than to $\{CuOH^+\}_0$ (R^2=0.884, $P<0.001$), or $\{Cu^{2+}\}_0$ ($P>0.05$) (Fig. 4). These results regarding the toxicity of $CuCO_3{}^0$ were extended by Experiment 3, which investigated the effect of Ca on Cu toxicity in solutions at pH 8.00. In this experiment, the addition of

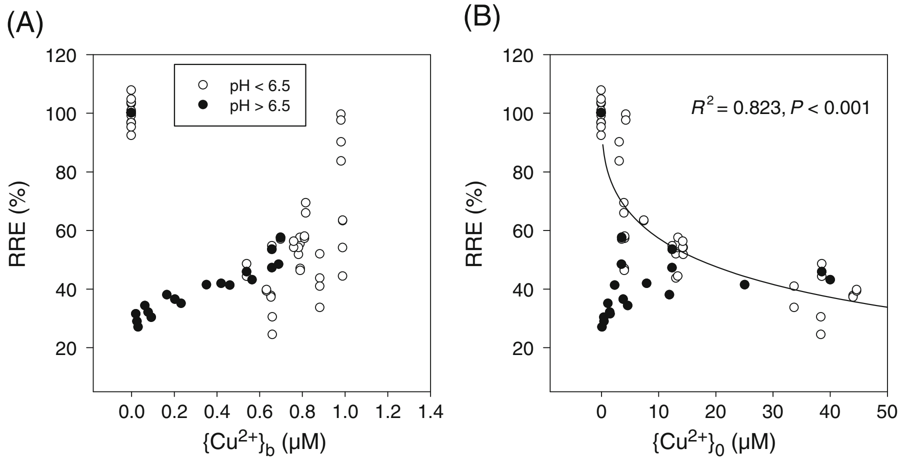

Fig. 2 Root elongation of wheat roots exposed to Cu at pH from 4.50 to 8.25 in response to either the activity of Cu^{2+} in the bulk solution (**a**) or its activity at the PM surface (**b**) (Experiment 1). The solid line is the regression line for the data with pH<6.5. The legend applies to the two panels

up to 10 mM Ca (which decreased ψ_0 negativity from −55.2 to −10.9 mV) had no significant impact on root growth in Cu-containing solutions at pH 8.00 ($P>0.05$) (Fig. 5), which was in contrast to the alleviatory-effect of Ca observed in acid solutions where Cu^{2+} dominates (Fig. 2). This observation supports the proposed toxicity of the $CuCO_3{}^0$ species as being uncharged its activity at the PM surface is not influenced by the addition of depolarizing cations and the associated changes in the negativity of ψ_0. Furthermore, it is noteworthy that RREs were related to $\{CuCO_3{}^0\}_0$ ($R^2=0.986$, $P<0.001$), but not to $\{Cu^{2+}\}_0$ or $\{CuOH^+\}_0$ ($P>0.05$) in these solutions at pH 8.00 (Fig. 6). These results suggest that the increased Cu rhizotoxicity caused by an increase in pH of that range resulted from increases in $\{CuCO_3{}^0\}_0$, rather than $\{CuOH^+\}_0$.

Modelling RRE in solution culture

Across all three experiments, no relationships were found between RRE and any single Cu species using Eq. 3 ($P>0.05$ for regression or coefficients). Equation 3, therefore, was modified to incorporate the toxicity of multiple Cu species (e.g., Cu^{2+} and $CuCO_3{}^0$) (see Kinraide 1999):

$$\mathrm{RRE}=100/\exp\left\{\left[a_1\left(\left\{\mathrm{Cu}^{2+}\right\}_0+a_{12}\left\{\mathrm{CuCO}_3^0\right\}_0\right)\right]^{\beta 1}\right\} \quad (4)$$

In this equation, the value of the coefficient a_{12} represents the relative rhizotoxicity of the Cu species $\{CuCO_3{}^0\}_0$ relative to the rhizotoxicity of the Cu species $\{Cu^{2+}\}_0$. For example in Eq. 4, an a_{12} value of 10 means

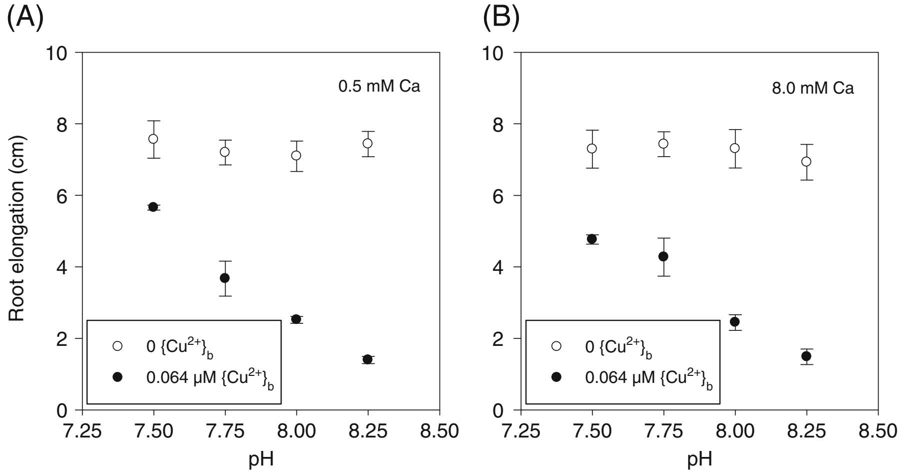

Fig. 3 Root elongation in response to pH in media with varying pH (7.5 to 8.25) and $Cu(NO_3)_2$ at 0.50 or 8.0 mM $Ca(NO_3)_2$ to hold the bulk Cu^{2+} activity at 0.064 µM (Experiment 2)

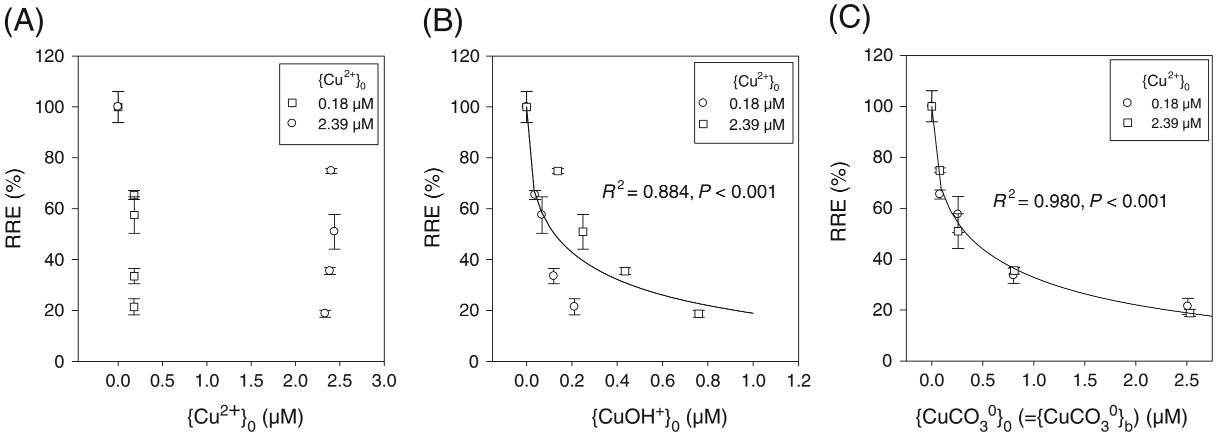

Fig. 4 Relative root elongation (RRE) of wheat roots as functions of surface activities of Cu^{2+} (**a**), $CuOH^+$ (**b**), or $CuCO_3^0$ (**c**) in solution with varying pH (7.5 to 8.25) and $Cu(NO_3)_2$ at 0.50 or 8.0 mM $Ca(NO_3)_2$ to hold the Cu^{2+} surface activity at 0.18 µM or 2.39 µM (Experiment 2)

that $\{CuCO_3^0\}_0$ is 10-times more toxic than $\{Cu^{2+}\}_0$. Fitting all data (Experiments 1–3) to this equation yielded an R^2 value 0.912 (cf. R^2 of 0.858 when considering $\{Cu^{2+}\}_b$ and $\{CuCO_3^0\}_b$), with a_1= 0.021, a_{12}=56.1, and β_1=0.419 (Table 2, Fig. 7). The value of a_{12} indicates that the same level of growth inhibition as defined by $\{CuCO_3^0\}_0$ required 56.1 times of $\{Cu^{2+}\}_0$. Interestingly, when species $\{CuCO_3^0\}_0$ was replaced with $\{CuOH^+\}_0$, no significant coefficient (P>0.05) was obtained for a_{12}.

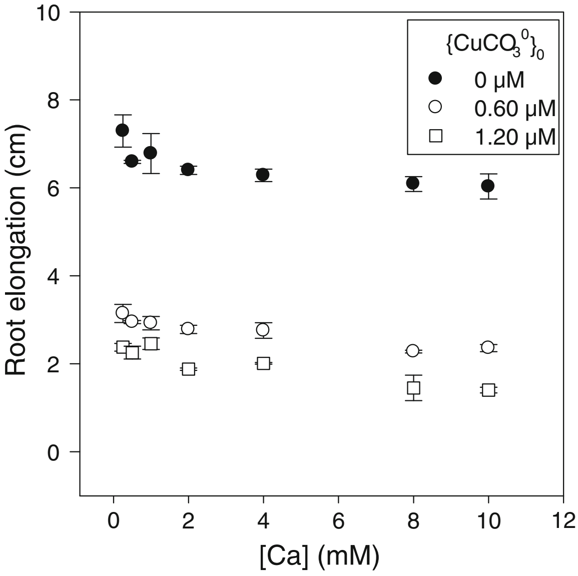

Fig. 5 Root elongation in response to Ca concentration in media at constant pH 8.00, with the surface activity of $CuCO_3^0$ being hold at 0.60 and 1.20 µM (Experiment 3)

Discussion

At pH values <6.50, the addition of cations (Ca^{2+} or H^+) to the bathing solution tended to alleviate the rhizotoxicity of Cu^{2+}. Similar results have been reported previously (Parker et al. 1998; Kinraide 2006; Lock et al. 2007; Wang et al. 2008, 2012; Kopittke et al. 2011a). This alleviatory effect can be attributed to non-specific changes in $\{Cu^{2+}\}_0$ (Kinraide 2006; Wang et al. 2008; Kopittke et al. 2011b). The addition of these cations reduces the negativity of ψ_0 and thereby decreases $\{Cu^{2+}\}_0$.

In the pH range from 6.50 to 8.25, an increase in pH significantly enhanced Cu toxicity (due to changes in Cu species), whilst the addition of Ca, (contributing substantially to changes in the negativity of ψ_0) did not influence root growth in these Cu-containing solutions (Figs. 3 and 5). Based on the following evidence, we propose that it is $CuCO_3^0$, rather than $CuOH^+$, that contributes substantially to Cu toxicity at pH>6.50:

(i) RRE in Experiment 2 decreased sharply across the entire pH range from 7.50 to 8.25 (Fig. 3), in accordance with the changes in speciation for $CuCO_3^0$, (rather than for $CuOH^+$, for which there is a maximum in formation at pH 7.5, see Fig. 1).

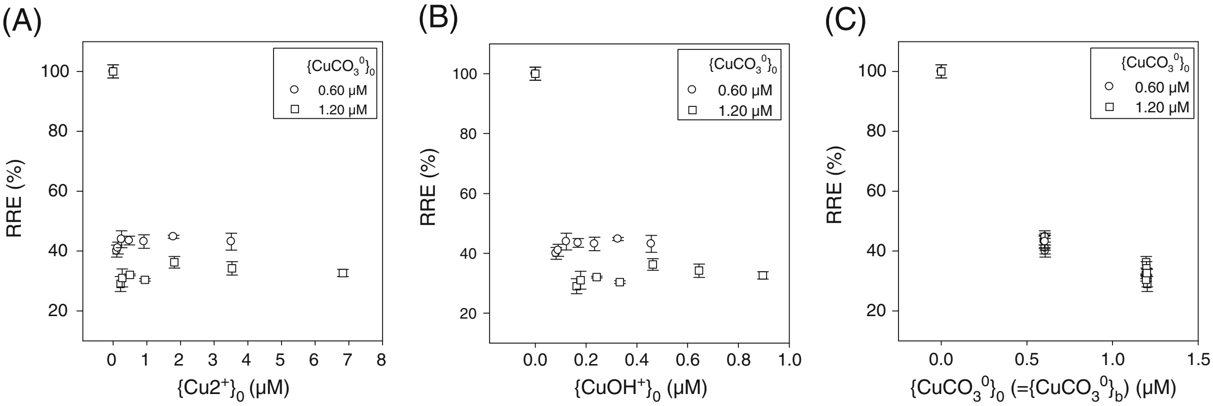

Fig. 6 Effects of Cu main species on the relative root elongation (RRE) of wheat roots exposed to solution at constant pH 8.00, with varying $Ca(NO_3)_2$ and $Cu(NO_3)_2$ to keep the surface activity of $CuCO_3^0$ constant at 0.60 μM or 1.20 μM (Experiment 3)

(ii) RRE in Experiment 3 was dependent only on $CuCO_3^0$ at pH 8.00, not on $\{Cu^{2+}\}$ or $\{CuOH^+\}$ (Fig. 6).

(iii) High Ca (up to 10.0 mM) alleviated the toxicity of Cu^{2+}, but had no influence on toxicity at pH 8.00 (therefore suggesting that the toxic ion was uncharged, $CuCO_3^0$).

(iv) RRE in Experiment 2 correlated better with $\{CuCO_3^0\}_0$ ($R^2=0.980$) than with $\{CuOH^+\}_0$ ($R^2=0.884$) or $\{Cu^{2+}\}_0$ ($P>0.05$) (Fig. 4).

(v) Across all three solution culture experiments, Eq. 4 yielded a significant coefficient ($P<0.05$) for $\{CuCO_3^0\}_0$ but not for $\{CuOH^+\}_0$ ($P>0.05$, Table 2). An equation incorporating both $\{Cu^{2+}\}_0$ and $\{CuCO_3^0\}_0$ explains 92.7 % of variation of RRE in these experiments.

(vi) Analysis of RRE vs. ion species with different equilibrium constants (Table 1) also confirmed that RRE depends upon the formation of both $\{Cu^{2+}\}_0$ and $\{CuCO_3^0\}_0$ (Table 2).

As noted in the Introduction, the toxicity of ionic species other Cu^{2+} has been reported previously. Interestingly, $CuCO_3^0$ has been reported previously to be

Table 2 Summary of statistics from regression analysis according to the Equations below

Selected equilibrium constants (see Table 1)	a_1	a_{12}	β_1	R^2
$\mathrm{RRE} = 100/\exp\left\{\left[a_1\left(\{Cu^{2+}\}_0 + a_{12}\{CuCO_3^0\}_0\right)\right]^{\beta 1}\right\}$				
IUPAC	0.021 (0.002) $P<0.001$	56.1 (7.3) $P<0.001$	0.419 (0.028) $P<0.001$	0.927
NIST	0.022 (0.002) $P<0.001$	60.3 (7.7) $P<0.001$	0.423 (0.027) $P<0.001$	0.929
WHAM	0.025 (0.003) $P<0.001$	85.1 (11.0) $P<0.001$	0.412 (0.026) $P<0.001$	0.929
$\mathrm{RRE} = 100/\exp\left\{\left[a_1\left(\{Cu^{2+}\}_0 + a_{12}\{CuOH^+\}_0\right)\right]^{\beta 1}\right\}$				
IUPAC	0.004 (0.003) $P>0.05$	610.2 (439.1) $P>0.05$	0.202 (0.035) $P<0.001$	0.872
NIST	0.004 (0.003) $P>0.05$	233.2 (159.8) $P>0.05$	0.210 (0.035) $P<0.001$	0.873
WHAM	0.005 (0.003) $P>0.05$	348.5 (215.8) $P>0.05$	0.234 (0.036) $P<0.001$	0.877

All ion activities are calculated with different equilibrium constants presented in Table 1 and the unit is expressed in μM. The values in parentheses are the standard errors

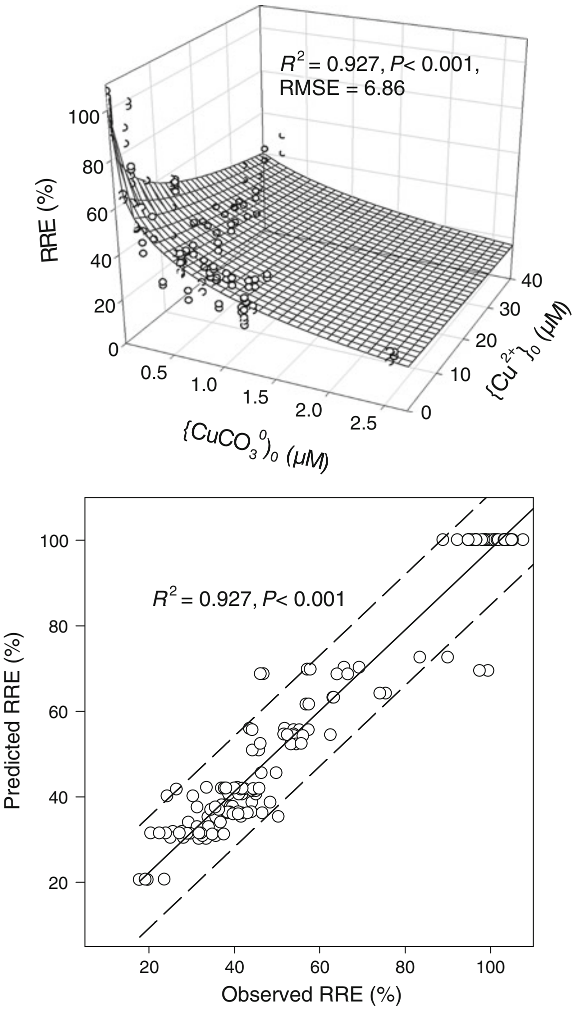

Fig. 7 The relative root elongation (RRE) as functions of the activity of $CuCO_3{}^0$ and Cu^{2+} at the plasma membrane surface ($\{CuCO_3{}^0\}_0$, $\{Cu^{2+}\}_0$). Regression coefficients are presented in Table 2

toxic in aquatic species, including in rainbow trout (*Salmo gairdneri* Richardson) (Shaw and Brown 1974). Similarly, De Schamphelaere et al. (2002) included $CuCO_3{}^0$ (and $CuOH^+$) in a revised biotic ligand model for *Daphnia magna*. Furthermore, in studying Cu toxicity to plants, both Wang et al. (2009) and Lock et al. (2007) reported that the values of EC50$\{Cu^{2+}\}_b$ for barley root were increased significantly when the pH increased in high-pH range from about 7.0 to 8.0, and indicated that ionic species other than Cu^{2+} were toxic to roots of barley. Wang et al. (2009) attributed this toxicity to $CuOH^+$, and stated that $CuOH^+$ was more toxic than Cu^{2+} due to the stronger binding of $CuOH^+$ at the biotic ligand. The authors used the Cu stability constants from NIST (Table 1). By using these constants, it is estimated that concentrations of $CuOH^+$ are higher than those of $CuCO_3{}^0$ for solutions at pH<7.50, and that $CuOH^+$ accounts for approximately 28.5 % of the total Cu at pH 7.50 (Fig. S1). They included $CuOH^+$ in the BLM construct (see Eq. 7 of Wang et al. 2009) and found the linear relationship between $CuOH^+/Cu^{2+}$ and 1/EC50 $\{Cu^{2+}\}_b$ (Fig. 5 in that article). Actually, if $CuOH^+$ is replaced with $CuCO_3{}^0$ in Eq. 7 of that study, a linear relationship is also derived between $CuCO_3{}^0/Cu^{2+}$ and 1/EC50$\{Cu^{2+}\}_b$ (R^2=0.98). Similarly, a value of 0.336 is obtained for $K_{CuCO3BL}/K_{CuBL}$. The values for (Cu^{2+} + 2.92×$CuOH^+$) are significantly linear with the values for (Cu^{2+} + 0.336×$CuCO_3{}^0$) (R^2=0.83). Given this high correlation, RRE should be also highly correlated with the values for (Cu^{2+} + 0.336×$CuCO_3{}^0$), indicating that $CuCO_3{}^0$ could also interpret the enhanced toxicity observed in Wang et al. (2009). However, the attribution to $CuOH^+$ toxicity cannot explain the fact that RRE almost did not change with $\{CuOH^+\}_0$ varying from 0.09 to 0.89 μM in the present study, provided that $\{CuCO_3{}^0\}$ is held constant (Fig. 6; Experiment 3).

So how are these various Cu-species toxic? The free Cu^{2+} ion binds very strongly to soft ligands (Soft Ligand Scale of 0.88) but comparatively weakly to hard ligand (High Ligand Scale of −0.99) (Kinraide 2009). Recent in situ synchrotron-based experiments using hydrated root tissues of cowpea have demonstrated that the intoxication of Cu^{2+} results in accumulation of the Cu in the rhizodermis and outer cortex (Kopittke et al. 2011b), possibly inhibiting loosening of the cell wall as part of the elongation process (Kopittke et al. 2008, 2009). Additionally, a study examining Cu transport showed that more than 99.7 % of total Cu in tomato and chicory xylem sap was in a bound form (Liao 2000). However, the observation that $CuCO_3{}^0$ is also toxic (and, in fact, more toxic than Cu^{2+}) was unexpected given that $CuCO_3{}^0$ is an uncharged ion-pair which is considered to bind only weakly to physiologically active sites. According to the biotic ligand model, $CuCO_3{}^0$ is toxic by binding to the biotic ligand, although the binding constant of $CuCO_3{}^0$ for the biotic ligand was defined as being 10-fold lower than for Cu^{2+} (De Schamphelaere et al. 2002). We suggest, however, that $CuCO_3{}^0$ may be transported easily through the apoplast (in contrast to Cu^{2+}) and possibly be taken up as the uncharged ion-pair (as observed for the uptake of undissociated

H_3BO_3 by plant roots by mass flow). An alternative explanation would be that $CuCO_3^0$ complex is not strong, therefore may dissociate at the membrane surface where Cu^{2+} transporter proteins are located. Because of neutrality, $CuCO_3^0$ is much more easily transported through the apoplast to the membrane surface (followed by dissociation and uptake of Cu^{2+}), therefore appear to be more toxic. Exploring the mechanisms by which $CuCO_3^0$ is toxic requires further investigation.

Some of the foregoing discussions are based on the assumption that the equilibrium constants (from IUPAC, Table 1) used in this study are valid. The equilibrium constants used to compute the activities of the Cu species may influence the conclusions regarding the identification of the toxic species. Actually, the Cu species calculated using the three sets of constants are highly correlated (R^2>0.98). A sensitivity analysis was conducted which demonstrated that using the constants from the default database of WHAM (same as Visual MINTEQ 3.0) and NIST produced similar results (see Table 2), indicating $CuCO_3^0$ toxicity in alkaline solutions (i.e., significant values were obtained for coefficient 'a_{12}'), with the values for 'a_{12}' as large as those obtained based on the constants of IUPAC. This study focused on the root PM. A question of the study's validates may be raised if the sites of Cu intoxication lie within the cell wall rather than the PM? It should be noted, however, that the electrostatic characteristics of the cell wall are similar to the PM (Shomer et al. 2003; Kinraide 2004).

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