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The rhizotoxicity of metal cations is related to their strength of binding to hard ligands

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ABSTRACT

Mechanisms whereby metal cations are toxic to plant roots remain largely unknown. Aluminum, for example, has been recognised as rhizotoxic for about 100 years but there is no consensus on its mode of action. We contend that the primary mechanism of rhizotoxicity of many metal cations is non-specific and that the magnitude of toxic effects is positively related to the strength with which they bind to hard ligands – especially carboxylate ligands of the cell wall pectic matrix. Specifically, we propose that metal cations have a common toxic mechanism through inhibiting the controlled relaxation of the cell wall as required for elongation. Metal cations, such as Al^{3+} and Hg^{2+} (amongst others), which bind strongly to hard ligands, are toxic at relatively low concentrations because they bind strongly to the walls of cells in the rhizodermis and outer cortex of the root elongation zone with little movement into the inner tissues. In contrast, metal cations, such as Ca^{2+}, Na^{+}, Mn^{2+}, and Zn^{2+}, which bind weakly to hard ligands bind only weakly to the cell wall and move farther into the root cylinder. Only at high concentrations is their weak binding sufficient to inhibit the relaxation of the cell wall. Finally, different mechanisms would explain why certain metal cations (for example, Tl^{+}, Ag^{+}, Cs^{+}, and Cu^{2+}) are sometimes more toxic than expected through binding to hard ligands. The data presented here demonstrate the importance of 'strength of binding to hard ligands' in influencing a range of important physiological processes within roots through non-specific mechanisms.

Keywords: binding; mechanism of toxicity; metals; root growth; symptoms.

INTRODUCTION

Ten elements, absorbed as cations, are essential for plant growth (Ca, Co, Cu, Fe, K, Mg, Mn, Na, Ni, and Zn) [1], but root growth is reduced due to toxicity when these and other, non-essential, cations are present at elevated concentrations. Despite the importance of rhizotoxicity, much remains to be learned regarding the mechanisms by which metal cations exert their toxic effects. For example, it remains unclear how soluble Al reduces root growth despite research on Al toxicity in acidic soils for > 100 years [2]. Aluminum has been reported to cause interference with DNA synthesis and mitosis [3], disrupt the function of the Golgi apparatus [4], interfere with signal transduction from the root cap [5], damage membrane integrity [6], disrupt cell expansion [7], and cause cell rupturing [8, 9]. Despite decades of concerted research, controversy remains as to the primary mechanism by which Al exerts its toxic effect and reduces root growth. This applies to many other metals also.

In pharmacology, quantitative structure-activity relationship (QSAR) models are used to predict the potency of compounds on the basis of their physicochemical properties. Environmental toxicologists have since adopted this approach to predict the toxicity of contaminants by using quantitative ion character-activity relationships (QICARs). For example, the QICAR model was used by Li et al. [10] to study metal toxicity in the freshwater ostracod, *Cypris subglobosa* Sowerby, relating the toxicity of metals to the physicochemical properties of the ions, such as atomic number, oxidation state, Pauling ionic radius, electronegativity, covalent index, and log of the first hydrolysis constant. Similarly, Wolterbeek and Verburg [11] related the toxic effects of cations to a range of physicochemical properties across a wide range of species (many of which were animals), and found that the toxicity of an ion could be predicted using

these general properties. However, there are surprisingly few studies using this approach in the study of plant-ion interactions (see Kinraide and Yermiyahu [12] as an example of such a study).

Typically, the primary purpose of developing QICAR models is to predict the toxicity of untested ions or of mixtures [10]. However, we have taken a slightly different approach in this study. Firstly, our aim is not to develop a model *per se* to relate the physicochemical properties of metal cations to their physiological effects, but rather, by examining whether such a relationship exists, to infer the mechanistic underpinnings that dictate how toxic metal cations behave within plant tissues. Secondly, we have not focussed narrowly on examining the concentrations of metal cations that have an effect on growth (root elongation, mass, etc), but have considered a range of observations relating to these toxic effects. Although focussing on plants, we have generally not included data from studies on hyperaccumulators due to large differences in the behaviour of metals within these plant species. Finally, although H^+ is not a metal cation, we refer to it accordingly for simplicity and brevity.

PHYSICOCHEMICAL PROPERTIES

'Strength of binding to hard ligands' is important

Any QICAR-based approach needs to consider what physicochemical property best explains the effect of cations in relation to the physiological characteristic of interest. We have focused on the 'strength of binding to hard ligands', just one of numerous properties that could be considered. Wolterbeek and Verburg [11], for example, used electrochemical potential, ionization potential, electronegativity, covalent index, hydrated radius, and the log of the first hydrolysis constant (amongst others). We have chosen to focus on 'strength of binding to hard ligands'

because (i) this property provides a superior relationship with other physicochemical properties (including strength of binding to soft ligands) [12, 13], (ii) it is related to other physicochemical properties which also give good relationships (see below), and (iii) the strength with which metal cations bind to hard ligands provides a coherent mechanistic understanding of various, otherwise apparently disparate, observations.

Following on the work of Kinraide and Yermiyahu [12], Kinraide [14] proposed a normalized hard ligand scale (HLScale) from the strength of cation binding to 13 hard ligands, including oxalate, citrate, hydroxide, carbonate, and others. In this process, the binding strengths between metal cations and ligands were normalized so that for the HLScale a value of 0.0 represents the mean binding strength among the 64 cations considered whilst values of -1.0 and 1.0 represent values one standard deviation below or above the mean, respectively. Thus the HLScale values of –1.88 for Cs^{+}, –0.09 for Cu^{2+}, and 1.99 for Zr^{4+} indicate very weak binding of Cs^{+}, an approximately average binding of Cu^{2+}, and very strong binding of Zr^{4+} to hard ligands.

Although we have used primarily ‘strength of binding to hard ligands’ in our analyses, the possibility cannot be excluded that the observations described hereafter could also be explained using other physicochemical properties. In certain regards, the exact physicochemical property to which the effects of metal cations are related is not of the highest importance. For example, ‘binding strength to hard ligands’ (HLScale) is closely correlated with both ‘log of the first hydrolysis constant’ ($R^2 = 0.950$, excluding H^{+}) and ‘hydrated radius’ ($R^2 = 0.765$) when calculated using the physicochemical properties of the 50 cations listed by Kinraide and Yermiyahu [12]. It is not unexpected that the log of its first hydrolysis constant is closely

123 correlated to the strength with which a cation binds to hard ligands. However, some
124 physicochemical properties are not closely related, and differentiation among the effects of the
125 various physicochemical properties is indeed important. For example, a linear regression between
126 strength of binding to hard ligands and strength of binding to soft ligands yields an R^2 value of
127 only 0.203. Therefore, an understanding of the nature of these observed correlations may
128 elucidate the effects that metal cations may have in plants.
129
130 *Ligands in plant tissues*
131 In reference to ligands, the terms 'hard' and 'soft' were originally defined by Pearson
132 [15]. Soft ligands have high polarizability, low electronegativity, large radii, empty orbitals of
133 low energy, and are easily oxidisable, whilst hard ligands have the opposite properties [16].
134 Therefore, in biological systems, hard ligands often include oxygen or nitrogen (such as $R\text{-}COO^-$,
135 $R\text{-}PO_4^-$, $R\text{-}NH_2$, or $R_2\text{-}NH$) whilst soft ligands often include sulfur (such as $R\text{-}S^-$ or $R_2\text{-}S$).
136
137 There are numerous ligands in plant root tissues to which metal cations could potentially
138 bind [17-19]. For example, compounds with $R\text{-}COO^-$ functional groups (hard ligands) are
139 important in the cell wall, specifically the galacturonic acid residues of the pectic matrix which
140 provides the majority of the cell wall's cation binding capacity [20]. $R\text{-}COO^-$ functional groups
141 occur in the vacuole, which contains organic ligands such as citrate or malate, in plant hormones
142 (such as in auxin [IAA^-]), in proteins (including most enzymes), and in transport proteins. The R-
143 PO_4^- functional groups (hard ligands) are important at plasma membrane (PM) surfaces
144 (phosphatidic acid), for transport across membranes (ATPases), in phytic acid, and as $R\text{-}OPO_2O\text{-}$
145 R in DNA and RNA. The $R\text{-}NH_2$ functional groups (hard ligands) are important in proteins. In

contrast to the hard ligands, the N-sites on the nucleobases A, C, G, T, or U of DNA and RNA are intermediate ligands to which metal cations could bind. Similarly, $R\text{-}S^-$ functional groups (soft ligands) are important in the vacuole (cysteine-containing compounds, such as metallothionein or glutathione).

RELATIONSHIPS BETWEEN CATION PHYSICOCHEMICAL PROPERTIES AND THEIR TOXIC EFFECTS IN PLANTS

1. Rhizotoxicity is related to the strength of binding to hard ligands

Most published studies have examined the toxicities of only a few metals at most, but a limited number of investigations have compared the toxicities of a large number of cations to plant roots. Interpretation of these multi-element studies, however, is often difficult due to deficiencies in experimental technique. As a specific instance of this general problem, Wheeler et al. [21] examined the rhizotoxicities of eight metals (Al, Cu, Fe, Ga, La, Mn, Sc, and Zn) in wheat (*Triticum aestivum* L.). The concentrations of several metals far exceeded their solubility, with measured concentrations of some metals at the end of the experiment < 50 % of the nominal values. Thus, valid comparisons among metal effects are not possible since growth was related to nominal rather than measured concentrations. Similarly, Wong and Bradshaw [22] compared the rhizotoxicity of Al, Cd, Cu, Fe(II), Hg, Mn, Ni, Pb, and Zn in ryegrass (*Lolium perenne* L.), with solutions adjusted to pH 7.0. At this pH, hydrolysis and precipitation would ensure that concentrations of many metals in solution would be substantially lower than the nominal values, again invalidating comparisons among metal toxicities.

After discarding studies we considered flawed, there remained only a limited number of studies with data that warrant consideration. Kinraide and Yermiyahu [12] examined the relationship between the physicochemical properties of 19 metal cations and their rhizotoxicity in wheat. A reanalysis of their data using the HLScale (as defined by Kinraide [14]) shows that cations become increasingly toxic as the strength of binding to hard ligands increases (Fig. 1A). Kinraide and Yermiyahu [12] suggested that the binding strength of cations sets a "lower limit" for toxicity. These authors proposed that a metal cation will be at least as toxic as the strength with which it binds to hard ligands – this being a 'common' mechanism whereby metal cations exert toxic effects proportional to their binding strength (i.e. a non-specific mechanism). This is in contrast to 'specific' toxicity, where the mechanism of toxicity would vary and there would not be a single factor that would determine the toxic effects of any given metal cation. Kinraide and Yermiyahu [12] found that additional mechanisms may sometimes increase the toxicity above this 'lower limit', with three metal cations, Tl^{+}, Ag^{+}, and Cu^{2+}, substantially more toxic than predicted based upon their strength of binding to hard ligands (Fig. 1A). Presumably this 'additional' toxicity results from other mechanisms, such as strong binding to soft ligands or interference with the metabolism of essential ions.

In another study, Kopittke et al. [13] examined the elongation of cowpea (*Vigna unguiculata* (L.) Walp.) roots, and related the toxic effects of 26 metal cations to a range of their physicochemical properties (including Pauling electronegativity, standard electrode potential, covalent index, and binding to hard ligands). It was found that rhizotoxicity increased as the strength of binding to hard ligands increased, i.e., the concentration required to cause a 50% reduction in root elongation rate (EC50) decreased (Table 1, Fig. 1B). This further suggests that

191 the binding of metal cations to hard ligands is an important non-specific mechanism of toxicity.
192 Again, however, three notable exceptions to this rule were observed – Tl^+, Ag^+, and Cs^+ appeared
193 to be substantially more toxic than expected based upon the strength of their binding to hard
194 ligands (*c.f.* Tl^+, Ag^+, and Cu^{2+} as found in wheat roots by Kinraide and Yermiyahu [12]) (Fig. 1
195 and Table 1).
196
197 We reanalysed the data reported in a meta-analysis by Kopittke et al. [23] who examined
198 the toxicity of eight metal cations using data from 119 studies conducted in solution culture.
199 Using a Weibull-type equation, a highly significant relationship ($R^2 = 0.703$) was found between
200 the median concentrations reported to be toxic and the strength with which the metal cations bind
201 to hard ligands (Fig. 1C). Toxicity was also plotted against Pauling electronegativity ($R^2 =$
202 0.663), log of the first hydrolysis constant ($R^2 = 0.612$), the standard electrode potential ($R^2 =$
203 0.603), strength of binding to soft ligands ($R^2 = 0.438$), hydrated radius ($R^2 = 0.422$), and
204 ionization potential ($R^2 = 0.059$) (data not presented). Given the close inter-correlations between
205 some of these variables, it is not surprising that several of the physicochemical properties had
206 similar R^2 values when examining these eight metal cations. For example, the strength of binding
207 to hard ligands is closely related to the log of the first hydrolysis constant ($R^2 = 0.966$). (Note that
208 the metal cations previously found to be 'exceptions', Ag^+, Cs^+, Cu^{2+}, and Tl^+, were not included
209 in the study of Kopittke et al. [23]). These data, obtained across a wide range of species and
210 across a large range of experimental conditions, indicate that the toxicities of these eight metal
211 cations are related to their physicochemical properties.
212

213 Statistical associations provide some insight, but this question remains: What is the
214 underlying mechanism by which metal cations cause this non-specific reduction in root growth?
215 Various hypotheses are worthy of investigation. For instance, the strength of binding to hard
216 ligands may influence the speciation (and hence toxicity) of metal cations through binding to
217 DNA, lipids, etc. Alternatively, the strength of binding to hard ligands may influence the
218 formation of reactive oxygen species.
219
220 *2. Metal distribution and speciation are related to the strength of binding to hard ligands*
221 Given that at least some mechanisms of toxicity appear to be non-specific (Table 1), it
222 might be assumed that the level of toxicity is perhaps a function of the degree with which any
223 given metal cation interacts with ligands at the 'site' of toxicity. Therefore, we investigated the
224 distribution of metals within root tissues to determine if there is a pattern relating toxicity to
225 interactions with particular sites. Indeed, cowpea data show (Fig. 2) that Cu (HLScale -0.09) is
226 located almost entirely in the rhizodermis and outer cortex while Zn (HLScale -0.41) moves
227 farther into the root cylinder. Lombi et al. [24] found that Zn and Ni (HLScale -0.41) behave
228 similarly in moving into the root cylinder.
229
230 To examine this further, the distribution of five metals (Cu, Hg, Mn, Ni, and Zn), with
231 HLScale values ranging from 0.86 for Hg to -0.41 for Zn (Table 1), were investigated in fresh
232 hydrated roots of cowpea using in situ synchrotron-based μ-XRF [25]. The results of that study
233 showed rhizodermal concentrations of these cations to be positively correlated with the HLScale
234 (Fig. 3). The concentrations of Hg (250 μg g^{-1}) and Cu (160 μg g^{-1}) in the rhizodermis were ca. 2
235 to 4 times higher than those of Mn, Ni, or Zn (63 to 88 μg g^{-1}) even though bulk solution

236 concentrations were ca. 4 to 150 times lower [i.e. Cu (1.5 µM), Hg (1.0 µM), Mn (150 µM), Ni
237 (5.0 µM), Zn (40.0 µM)] (Fig. 3). Thus, the data suggest that the strong binding of Hg and Cu
238 resulted in the accumulation of these metals in the rhizodermis. Furthermore, and in accordance
239 with expectations, Wang et al. [25] reported that the concentration of Hg and Cu in the
240 rhizodermis (i.e. 250 and 160 µg g^{-1}) was 6.8 and 3.6 times higher than in the cortex (i.e. 36 and
241 44 µg g^{-1}), respectively. In contrast, concentrations of Mn, Ni, and Zn in the rhizodermis tended
242 to be lower than those in the inner tissues.
243
244 Many studies that examined the radial distribution of metals have only examined a single
245 metal. This limits direct comparison among cations under constant experimental conditions.
246 However, Marienfeld and Stelzer [26] studied roots of oat (*Avena sativa* L.) and found that even
247 after exposure to Al (HLScale of 0.67) for 10 d, concentrations of Al were substantially higher in
248 the rhizodermis (ca. 10 mM) than in the cortex (ca. 4 mM), endodermis (ca. 2 mM), and stele (ca.
249 1 mM) (see also Lazof et al. [27]). Similarly, examining the radial distribution of Al in roots of
250 *Zea mays* L. and *Vicia faba* L., Marienfeld et al. [28] found that concentrations were ca. 4- to 30-
251 fold higher in the rhizodermis and outer cortex than in the inner cortex after exposure to 50 µM
252 Al for 3 h, the magnitude of the difference being greater for the dicot than the monocot. It was
253 concluded that "the inhibition of the elongation of inner cortex cells must be mediated indirectly
254 by Al injury to the outer cortical cells". However, Kinraide et al. [29] used haematoxylin staining
255 in wheat roots and found that whilst Al accumulated predominately in the rhizodermis of cv.
256 Atlas (Al-tolerant), it accumulated predominately in the cortex of cv. Scout (Al-sensitive).
257 Clearly, some differences exist among genotypes, and the difficulty of obtaining accurate

quantitative data regarding the spatial distribution of Al in roots has hindered progress in this regard.

In contrast to the general findings with Al, other studies have found that the decrease in concentration from rhizodermis to inner tissues for metal cations that bind only weakly to hard ligands is not as pronounced or is not present at all. For example, Claus et al. [30] found that the apoplastic concentration of Zn (HLScale -0.41) in the inner root tissues of *Arabidopsis thaliana* was similar to that in the rhizodermis. Under some scenarios, apoplastic Zn was slightly higher in the rhizodermis but in others slightly lower; symplastic Zn was higher in the inner tissues than in the rhizodermis. Similarly, Terzano et al. [31] found that the highest concentration of Zn in soil-grown roots of *Eruca vesicaria* L. was at the endodermis and inner cortex. Seregin et al. [32] found that the concentration of Ni (HLScale of -0.41) was qualitatively higher in the inner cortex than in the rhizodermis and outer cortex. An exception seems to be the concentration of Cd (HLScale of -0.48), which is generally highest in the rhizodermis and decreases gradually through the cortex [33, 34]. However, the magnitude of this accumulation in the rhizodermis is less than that reported for metals such as Al.

Overall, therefore, published information indicates that the extent to which metal cations move into the root cylinder is influenced by the strength with which they bind to hard ligands. Specifically, metal cations that bind strongly to hard ligands accumulate predominately in the rhizodermis and outer cortex, whilst those that bind weakly to hard ligands move farther into the root cylinder.

Besides differences in radial movement, differences exist also among metal cations in their subcellular distribution: those that bind strongly to hard ligands bind predominantly to sites within the cell wall. Taylor et al. [35] used a highly sensitive ^{26}Al technique to study Al taken up by single cells of *Chara corallina*. Exposure to 50 µM Al resulted in < 0.5 % of the total Al in the protoplasm. Similarly, Rangel et al. [36] reported that ca. 80 % of the Al in roots of *Phaseolus vulgaris* exposed to 20 µM Al for 4 to 24 h was bound to the cell wall. Wang and Greger [37] reported that 80% of the Hg (HLScale 0.86) was associated with the root cell walls of *Salix* spp., and Nishizono et al. [38] reported that 70% to 90% of the Cu (HLScale -0.09) in roots of *Athyrium yokoscense* was located in the cell wall. It is noteworthy that the majority of the Cu was bound to polygalacturonic acid within the rhizodermis and outer cortex of hydrated cowpea roots after 24 h exposure [39].

Movement into the protoplasm appears to be more important for metal cations that bind less strongly to hard ligands. The dominant forms of Zn (HLScale -0.41) within roots of *Eruca vesicaria* L. were Zn phytate and Zn citrate (i.e. presumably within the protoplasm) [31], whilst ca. 60-85% of the Zn was present as Zn phytate in roots of cowpea [39]. Furthermore, ≥ 80 % of the Mn (HLScale of -0.62) was associated with citrate in roots of cowpea [40] and Cd (HLScale of -0.48) was found to be bound entirely to thiol groups in roots of maize (*Zea mays* L.) [41].

In summary, therefore, the strength of binding to hard ligands influences both the cellular and subcellular location of metals as well as the dominant chemical species within the root. Furthermore, the inter- and intra-cellular distribution of ligands with the highest affinity for certain metal cations determines their distribution within tissues and cells. Of course, the

304 dominant ion-ligand species of a metal is not necessarily the metal species that exerts the greatest
305 physiological effect within the root – caution is needed in this regard.
306
307 *3. Development of toxicity symptoms is related to the strength of binding to hard ligands*
308 If the strength with which a metal cation binds to hard ligands influences its (i) toxicity,
309 (ii) radial movement through the root cylinder, and (iii) speciation, then it might be assumed that
310 binding strength would also be associated with common toxicity symptoms. Indeed, toxicity
311 symptoms in roots (caused by excess levels of cations) may provide insights into the mechanisms
312 by which metal cations exert their toxic effects.
313
314 Metal cations have been reported to have a range of visible toxic effects on plant roots
315 and be involved in the kinetics of symptom development. In this study, we are particularly
316 interested in similarities in symptoms and those that form after only short periods of exposure. In
317 a relatively early study, Clarkson (1965) examined the effects on cell division and root elongation
318 of Al, Ga, In, and La in roots of onion (*Allium cepa* L.) and stated that that the "results were
319 similar in every respect". We initially noted that Al and La cause ruptures in the rhizodermis and
320 outer cortex in roots of mungbean. We noted also that Cu causes ruptures in roots of Rhodes
321 grass (*Chloris gayana* Kunth.), cowpea, Sabi grass (*Urochloa mosambicensis* Hack.), and
322 camphor (*Cinnamomum camphora* (L.) J. Presl.). As summarised by Kopittke et al. [9] and
323 Osawa et al. [42], these ruptures (sometimes referred to as "cracks") are common in Al-exposed
324 roots of pea (*Pisum sativum* L.), maize, camphor, soybean (*Glycine max* L.), cowpea, *Lotus*
325 *corniculatus*, and ahipa (*Pachyrhizus ahipa* (Wedd.) Parodi.).
326

Using cowpea as a model species, we previously investigated these ruptures in detail and found that 10 metal cations (Ag^{+}, Al^{3+}, Cu^{2+}, La^{3+}, Ga^{3+}, Gd^{3+}, Hg^{2+}, In^{3+}, Ru^{3+}, and Sc^{3+}) cause the formation of markedly similar ruptures, whilst 16 metal cations (Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cs^{+}, H^{+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, Pb^{2+}, Sr^{2+}, Tl^{+}, and Zn^{2+}) did not [9, 43, 44] (Fig. 4). Rupturing appears to be related to the strength of binding to hard ligands (Table 1, Fig. 1B, and Fig. 4), except in the case of Ag^{+} and H^{+} (as will be discussed later). In some instances, the ruptures were sufficiently severe that almost 50 % of the root diameter had torn apart – this surely causing an almost complete loss of root function (Fig. 4).

The ruptures often form within 2 h of exposure [43]; typically they form initially 2-5 mm from the apex (i.e. within the elongation zone of the root) (Fig. 4, also see Kopittke et al. [9]); and they are apparently caused by the tearing and separation of the outer cellular layers whilst the inner tissues continue to elongate [9, 42]. As concluded by Marienfeld et al. [28], we are unable to suggest any alternative process that would produce the formation of these ruptures. So why is the elongation of the outer cells inhibited whilst the inner cells continue to elongate? Cell elongation results from the controlled relaxation of the cell wall with internal turgor providing the driving force for expansion [45]. Cell wall acidification is necessary for cells to expand in response to the action of auxin [46], an effect confined, initially at least, to cells in the elongation zone [47, 48]. It is in this zone that Al has the opposite effect, decreasing root growth [48]. We conclude that the strong binding of metal cations to the rhizodermis inhibits the controlled loosening of the walls of these cells as required for root growth.

4. The generation of reactive oxygen species is related to the strength of binding to hard ligands and the standard electrode potential

It has been suggested that reactive oxygen species (ROS) may be involved in various stresses, including those caused by excess metals. Interestingly, Kinraide and Yermiyahu [12], who reanalysed the data of Kawano et al. [49] with tobacco (*Nicotiana tabacum* L.) suspension-culture cells, found that the cation-induced generation of the superoxide anion ($O_2^{\bullet-}$) was highly correlated to the strength of binding to hard ligands. Similarly, Kinraide et al. [50] found that pro-oxidant activity is closely related to the standard electrode potential (E^o) of 25 cations. Interestingly, however, all metals, whether biologically redox-active (such as Mn, Fe, Co, Ni, or Cu) or non-active (such as Na or Ca), induced oxidative stress that was closely correlated with E^o – apparently redox-active metals did not cause oxidative stress by generating ROSs through Fenton-type reactions (i.e. redox cycling). Therefore, the data of Kinraide et al. [50] suggest that metal cations induce oxidative stress through a separate underlying mechanism, and that this mechanism is itself related to E^o or the strength of binding to hard ligands [12, 50]. It is also important to note the metal cation concentrations required to induce measureable increases in pro-oxidant activity are often substantially higher than those required to induce toxic effects as explained by Kinraide et al. [50] and references therein. It would therefore seem possible that oxidative stress may result after non-oxidative intoxication by other mechanisms as suggested by Yamamoto et al. [8] with respect to Al.

A HYPOTHESIS

We propose a hypothesis for further investigation based upon the preceding discussion related to the various observations outlined above. However, we are also aware that there are some observations that do not fit with this hypothesis, as detailed in the next section, so further work is clearly required. It is proposed that the binding of metal cations to hard ligands is an important, non-specific mechanism that dictates, at least to some extent, the effects that metal cations have on plant roots. We suggest that the effects of metal cations on plant roots occur along a continuum (Fig. 1) with the magnitude of their effects dictated by the strength with which they bind to hard ligands. This hypothesis is illustrated below with separate explanations of (i) a metal cation that binds strongly to hard ligands and (ii) a metal cation that binds only weakly to hard ligands. However, different mechanisms would be required to explain why some metal cations (Tl^{+}, Ag^{+}, Cs^{+}, and Cu^{2+}) are sometimes more toxic than expected by the strength of their binding to hard ligands.

Firstly, consider metal cations which bind strongly to hard ligands. (1) These metal cations accumulate predominately in the cell wall by binding to hard ligands, especially the carboxyl groups of polygalacturonic acid in the pectic matrix of primary cell walls [39] which accounts for ca. 70-90 % of the charge in root cell walls [51]. (2) Because of this strong binding to the cell walls, these metal cations accumulate primarily in the rhizodermis and outer cortex of the root apex and elongation zone (Fig. 3). (3) This strong binding of metal cations to the cell wall exerts a toxic effect on root growth at relatively low levels (Fig. 1). (4) The toxic effect of these metal cations is caused directly by an inhibition of the controlled relaxation of the cell wall as required for cell elongation [45]. (5) Finally, this inhibition of cell wall relaxation (primarily in the rhizodermis of the elongation zone and in the outer cortex where the binding of the metals is

the highest) causes, and is evidenced by, the rupturing and tearing of these cells due to the continued elongation of the inner cells in a range of plant species (Fig. 4).

In contrast, metal cations that bind only weakly to hard ligands (1) bind only weakly to the cell wall carboxyl groups, (2) inhibit cell wall relaxation only at high concentration (typically $\gg$ 100 μM), (3) do not cause ruptures to form (due to their weaker binding), and (4) move into the root cylinder. Nevertheless, metal cations whether binding strongly or weakly to hard ligands fall, on a continuum related to the HLScale (Fig. 1).

In emphasising the potential importance of 'strength of binding to hard ligands', it must be noted that a similar proposal was made by Kinraide and Yermiyahu [12] when studying the rhizotoxicity of 19 metal cations in wheat (see earlier discussion). These observations (and the resultant hypotheses) are interesting because they suggest that many metal cations are toxic, not due to interference with transport channels, but due to binding to lower-affinity functional groups. In this regard, it would be interesting to determine whether this relationship between HLScale and toxicity exists in other organisms also. Notably, Stockdale et al. [52] and Iwasaki et al. [53] proposed that the accumulation and toxicity of metals in aquatic macro-invertebrates is related to their binding to non-specific ligand sites, and that these ligand sites could largely be represented as oxygen-containing ligands (i.e. hard ligands).

What is the mechanism by which the strong binding of cations reduces root elongation and causes rupturing, and why is the cell wall important?

We suggest that the importance of binding to the pectic matrix in the cell wall (i.e. as the 'hard ligand' to which cation binding causes toxicity) is because (i) the effects of cation-binding on the cell wall have been shown *in vivo*, in isolated cell walls, and in pectate gels, and (ii) the high pectin concentration in primary cell walls close to the apex accounts for most of the root's cation binding capacity [20]. For example, the binding of Al^{3+}, Cu^{2+}, or La^{3+} to pectin gels decreases their hydration and increases their strength relative to Ca-saturated gels [54-56]. Similarly, metal cations that bind strongly to hard ligands inhibit cell-wall autolysis (i.e. modification of the cell wall as required for elongation) in isolated cell wall material and decrease the enzymatic degradability of pectate gels [57].

The exact mechanism by which the strong binding of metal cations to hard ligands inhibits cell wall loosening and causes toxicity is not known precisely and requires further investigation, but there are several possibilities. Decreased cell wall loosening and the formation of the ruptures may occur directly through increased crosslinking of the pectic matrix. It is difficult, however, to envisage how a relatively weak gel could overcome the high turgor within cells [58]. Alternatively, changes to pectic gel structure, especially hydration, may decrease enzyme mobility and access to substrates [56]. Several possibilities exist in this regard, including (1) a restriction in the movement of expansin, thereby limiting controlled relaxation of the cell wall, (2) a decrease in auxin transport along with decreased cell wall acidification, and (3) decreased enzymatic attack on the pectin backbone [57]. Expansins allow controlled slippage of the load-bearing cellulose and xyloglucan fibres, recent evidence [59] showing that pectin is bound to xyloglucan thereby masking its binding with the LM15 molecular probe. The basipetal transport of auxin through the rhizodermal and outer cortical cells plays an important role in cell

elongation [19, 60]. Interestingly, several studies have recently indicated that auxin may be involved in the expression of Al toxicity [61-63]. Other mechanistic pathways, both direct and indirect, are possible also.

It is possible that the 'hard ligand' to which cation-binding causes toxicity is not actually the polygalacturonic acid in the cell wall as we have hypothesised. For example, perhaps the strong binding of cations to the $R\text{-}PO_4^-$ functional groups (hard ligands) of the PM causes toxicity. Indeed, Ishikawa et al. [6] suggested that an alteration of PM permeability is an important effect of Al toxicity. Similarly, the plant contains a large number of other hard ligands, and it is possible that the binding of metal cations to these other ligands may induce toxic effects. Regardless, any hypotheses regarding the mechanisms by which cation-binding induces toxic effects should be able to explain the observations outlined earlier.

It should also be noted that our suggestion that trace metals exert their toxic effects in cell walls is not new, particularly for Al, with substantial information available in this regard [7, 64].

EXCEPTIONS AND SPECIAL CASES

Many observations provide evidence regarding a common behaviour of metal cations in plant roots that is based on the general relationship between the HLScale and rhizotoxicity. We are also aware that there are a number of exceptions that do not conform to this hypothesis. Further studies are needed, therefore, to understand the reasons for the anomalies. Whilst these exceptions do not preclude the validity of the hypothesis, it is essential that we consider them.

461 *Exception 1: Some cations have an unexpectedly high rhizotoxicity*

462 The minimum toxicity of metal cations appears to be related to the strength with which
463 they bind to hard ligands as first suggested by Kinraide and Yermiyahu [12]. Some cations such
464 as Cs^+, Tl^+, Ag^+, and Cu^{2+} sometimes appear to be toxic by additional mechanisms which
465 increase their toxicity substantially (Table 1 and Fig. 1). Several possible mechanisms may
466 explain why these metal cations are toxic at a concentration lower than expected based upon their
467 HLScale. For example, Ag^+ binds strongly to soft ligands; indeed, few cations bind more strongly
468 to soft ligands than does Ag^+ [14]. So it is possible that Ag is highly toxic because it binds to a
469 soft ligand and thereby interferes with a metabolic process. Similarly, Cs^+ interferes with K^+
470 metabolism by binding to K-binding sites on essential proteins (interestingly, small additions of
471 K^+ alleviate greatly the toxicity of Cs^+) [65]. Thus, for metal cations such as Ag^+ Cs^+, Tl^+, Cu^{2+},
472 toxicity is not necessarily always dictated by the strength with which they bind to hard ligands, as
473 is the case for most metal cations (Table 1 and Fig. 1), but can be dictated by other effects.
474
475 *Exception 2: Silver unexpectedly causes roots of cowpea to rupture*

476 Interestingly, Ag^+ is an exception in another (although presumably related) manner: it
477 causes rupturing of cowpea roots even though it binds only weakly to hard ligands [44] but
478 strongly to soft ligands. Many cations of environmental interest that bind weakly to hard ligands
479 also bind weakly to soft ligands [14]. Blamey et al. [44] suggested that it is possible that Ag^+
480 binds strongly to soft ligands within the cell wall (including sulfhydryl groups, olefins, or
481 aromatic groups [66]) or with the (hard) carboxyl groups of IAA. Both possibilities would
482 interfere with cell wall loosening, thereby causing ruptures. In bacteria, Ag toxicity results in a
483 detachment of the plasma membrane from the cell wall and a decrease in protein synthesis [67].

Indeed, Smith et al. [68] suggested that Ag^{+} inhibits ripening in tomato (*Solanum lycopersicum*) by inhibiting biosynthesis of polygalacturonase which is required for pectin breakdown and physical access of cell wall loosening enzymes to the cell wall.

Exception 3: Differences among plants in other physiological responses

Wheeler et al. [21] found that an Al-tolerant line of wheat was no more tolerant to toxicities of Cu, Sc, La, Ga, Zn, Fe, or Mn than was an Al-sensitive line, suggesting that at least some of the toxic effects of Al differ from the effects of other metals. Interestingly, however, these authors also stated that it is possible that "some of the toxic effects … may be similar to the toxic effects of Al". In a similar manner, although excess Al is known to elicit the efflux of organic ligands (such as malate), other metal cations that bind strongly to hard ligands do not have the same effect [69]. It is also noted that there are substantial differences in recovery when roots are transferred to toxicant-free solutions after being exposed to comparable doses measured in terms of their reduction of root elongation [70].

Interestingly, Sivaguru and Horst [71] and Kollmeier et al. [63] reported that Al applied to the distal transition zone inhibited growth of maize roots but that Al applied to the elongation zone had "no effect on root elongation". This observation is not in accordance with the proposed hypothesis where Al applied to the elongation zone would inhibit the loosening and elongation of these cells. Interestingly, Ryan et al. [72] reported that application of Al to the tip of maize roots inhibited growth more than did the application of Al to the elongation zone, although application to the elongation zone did reduce growth somewhat and caused "visual damage to the epidermal and cortical tissues". Clearly, further research is required in this regard. Finally, it has been

507 reported in wheat that the addition of Al causes swelling of epidermal cells [73]. According to
508 our hypothesis and observations, we do not understand why these cells swell.
509
510 *Exception 4: Are H^+ and Ca^{2+} special cases?*
511 It is noteworthy that H^+ has a high HLScale value (0.19) but does not cause roots of
512 cowpea to rupture (Table 1 and Fig. 1B). A possible reason for this anomaly is that H^+ differs
513 from all the metal cations that bind strongly to hard ligands because it is monovalent rather than
514 divalent or trivalent (Table 1). Indeed, H^+ is unique amongst monovalent cations because its
515 HLScale value is much greater than those of other monovalent cations (Table 1). This may result
516 primarily from its small ionic radius (0.0012 nm) compared to other monovalent cations with
517 0.167 nm for Cs^+, 0.102 nm for Na^+, and 0.138 nm for K^+ [12]. Furthermore, acidification of the
518 cell wall is necessary for cell-elongation [58] through the action of auxin [19]; expansins have an
519 acid pH optimum also [45]. Indeed, acid solutions rapidly increase the rate of root elongation [47,
520 48].
521
522 We suggest that Ca is a special case also because a continuous supply of Ca is required
523 for roots to grow [74], being essential for cell wall and plasma membrane integrity. Calcium is
524 required at low concentration in the absence of other cations but at high concentration in their
525 presence, since ion competition can cause a net decrease in Ca binding. Importantly, Ca^{2+} is the
526 major cross-linking cation of the pectic component of the cell wall [20], and the binding of Ca^{2+}
527 to the cell wall plays an important role in regulating cell elongation [75]. Cell wall tension is
528 regulated by Ca^{2+} and H^+, the former by limiting and the latter by promoting cell wall relaxation.
529

It is likely that the physicochemical properties of Ca^{2+} and H^+ in their roles in the pectic matrix may explain their effects on cell expansion and root elongation. Pectin, with a pKa of ca. 3.4, is soluble in water but both Ca^{2+} and an increase in pH to > ca. 5 are required for gel formation [76]. As a monovalent cation with HLScale value = 0.19, H^+ readily displaces Ca^{2+} (HLScale = -0.89) from the pectic gel. This H^+-displacement of Ca^{2+} was shown by Ryan et al. [77] with intact *Chara* cells and isolated cell walls as the bulk solution was decreased from pH 7.0 to 4.6 (an increase in H^+ activity from 0.1 to 40 μM). Interestingly, a similar magnitude of Ca^{2+} efflux occurred upon the addition of K^+ to the bathing solution, but at much higher concentration (0.2 to 10 mM K) as would be expected with the K HLScale value = -1.75. Finally, decreasing solution pH to < 3.5 results in flaccid roots as would be expected on displacement of sufficient Ca^{2+} to denature the pectic gel and destroy cell wall integrity.

CONCLUSIONS

We have examined the physiological effects of metal cations and related them to their physicochemical properties, specifically their HLScale values [14]. The associated relationship with the HLScale included (i) the extent of cation binding to the cell wall, (ii) movement into the root cylinder, (iii) rhizotoxicity, and (iv) the development of toxicity symptoms. We have suggested a hypothesis that accounts for these observations; specifically, that the binding of metal cations to hard ligands is an important, non-specific mechanism that dictates, at least to some extent, their rhizotoxic effects. Metal cations that bind strongly to hard ligands (1) accumulate primarily in the rhizodermis and outer cortex of cells in the elongation zone, (2) bind predominately to the pectic matrix in the cell wall, (3) exert a toxic effect on root growth at low concentrations, and (4) cause toxicity directly by inhibiting the controlled relaxation of the cell

wall as required for cell elongation. In contrast, metal cations that bind only weakly to hard ligands (1) bind weakly to the cell wall, (2) inhibit relaxation of the cell wall only at high concentrations (typically $\gg$ 100 µM), (3) do not cause ruptures to form (due to their weaker binding), and (4) move into the root cylinder.

It would appear that the toxicity of most metal cations conforms to this general rule, but several exceptions or special cases have been identified. Firstly, some cations are unexpectedly toxic, perhaps through binding strongly to soft ligands. If these soft ligands were essential to metabolism, then toxicity would occur. Being a cation which binds weakly with hard ligands, Ag^{+} is an exception since it causes ruptures to rhizodermal and outer cells of the elongation zone. Secondly, cowpea roots are able to recover from the toxic effects of some metal cations which bind strongly to hard ligands (Al, Ga, and Ru) but not of others (Cu, Gd, In, La, and Sc), indicating some differences in their mechanism of toxicity [70]. Thirdly, organic acid secretion occurs upon exposure to Al^{3+}, but not upon exposure to other cations that bind strongly to hard ligands [69]. Finally, we regard H^{+} and Ca^{2+} to be special cases through their interplay in the integrity of the cell wall and plasma membrane.

Finally, we have focussed on the ‘strength of binding to hard ligands’, but do not necessarily exclude the possibility that another, perhaps related, ionic property is actually responsible for the commonalities observed. Similarly, we have suggested that polygalacturonic acid in the cell wall is the hard ligand for which this binding is important because of its location in primary cell walls. These observations have allowed us to suggest a mechanism to describe the observed physiological effects of metal cations at toxic levels. There are, however, numerous

other hard ligands in plant tissues to which binding could be important. Regardless, the evidence remains that many important effects of metal cations appear to be non-specific and related to their physicochemical properties.

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782

783

Table 1. The effects of 26 metal cations on plant root growth, sorted by the strength with which they bind to hard ligands (HLScale), where a value of 0 is the mean and +1 / -1 are one standard deviation above or below the mean. Note that as HLScale values decrease (strength of binding to hard ligands decreases), the following trends are generally evident: (i) toxicity decreases, and (ii) the likelihood that cowpea roots will rupture decreases. Four exceptions (Ag^+, Cs^+, H^+, and Tl^+) are shaded in grey and are discussed in the main text.

Cation	HLScale[1]	EC_{50} (µM)[2]	Ruptures[3]
Ru^{3+}	1.44	1.2	Yes
Sc^{3+}	0.88	1.8	Yes
Hg^{2+}	0.86	0.59	Yes
Ga^{3+}	0.81	0.92	Yes
In^{3+}	0.69	0.59	Yes
Al^{3+}	0.67	22	Yes
Gd^{3+}	0.28	1.7	Yes
H^+	0.19	49	No
La^{3+}	0.18	2.2	Yes
Cu^{2+}	-0.09	0.29	Yes
Pb^{2+}	-0.20	2.7	No
Ni^{2+}	-0.41	0.85	No
Zn^{2+}	-0.41	16	No
Cd^{2+}	-0.48	1.8	No
Co^{2+}	-0.48	2.4	No
Mn^{2+}	-0.62	720	No
Mg^{2+}	-0.88	14000	No
Ca^{2+}	-0.89	48000	No
Sr^{2+}	-1.01	1900	No
Ba^{2+}	-1.13	1700	No
Ag^+	-1.28	0.024	Yes
Tl^+	-1.50	0.007	No
Li^+	-1.57	6400	No
Na^+	-1.71	58000	No
K^+	-1.75	98000	No
Cs^+	-1.88	1.9	No

[1] HLScale: The strength with which cations bind to hard ligands, defined by Kinraide [14].
[2] EC_{50}: The concentration (µM) of cations in the bulk solution reducing root elongation by 50 %, taken from Kopittke et al. [13] for roots of cowpea.
[3] Ruptures: Indicates whether or not the cation at elevated concentration causes roots of cowpea to rupture (the separation and tearing of the rhizodermis and outer cortex), as reported by Blamey et al. [70], Kopittke et al. [9], and Kopittke et al. [43].

FIGURE LEGENDS

Fig. 1. (A) Concentrations of 19 metal cations at the outer surface of the plasma membrane that reduce elongation of wheat roots by 50 % ($EC_{50,PM}$) plotted against the strength with which they bind to hard ligands (HLScale, Kinraide [14]). Three metal cations (Tl^{+}, Ag^{+}, and Cu^{2+}) are excluded from the regression analysis – these cations are substantially more toxic than predicted from their strength of binding to hard ligands. Data are taken from Kinraide and Yermiyahu [12]. (B) Measured concentrations of 26 toxic cations in the bulk solution causing a 50 % reduction in the elongation of cowpea roots ($EC_{50,b}$). The dotted vertical line represents an arbitrary HLScale value of -0.15 which separates cations binding strongly to hard ligands causing ruptures and cations binding weakly not causing ruptures (Ag^{+} and H^{+} are the only exceptions). Three cations (Tl^{+}, Ag^{+}, and Cu^{2+}) are excluded from the regression. Error bars are shown although are often smaller than the symbols. Data from Kopittke et al. [13], Blamey et al. [44], and Kopittke et al. [43]. (C) Relationship between HLScale values and concentrations of eight cations in the bulk solution that have been found to induce toxic effects in solution culture. The points are the median toxic concentration with the 25th percentile shown using error bars (the 75th percentile is not shown to maintain clarity). Data are reanalysed from Kopittke et al. [23] who collected data from 119 individual studies published between 1975 and 2009 for a wide range of plant species.

Fig. 2. Distribution of Cu and Zn in roots of cowpea examined using synchrotron-based X-ray fluorescence microscopy (μ-XRF). Data were collected as described by Kopittke et al. [39]. Seedlings were grown for 24 h in solutions containing 1.5 μM Cu or 50 μM Zn. Brighter colours correspond to higher concentrations, but concentrations cannot be compared between the two images. The white bars are equal to 1 mm.

Fig. 3. The concentrations of five metals in the rhizodermis of cowpea using synchrotron-based X-ray fluorescence microscopy [25] plotted against the strength with which the metal cation binds to hard ligands (HLScale) [14]. Plants were grown in solutions containing toxic levels of cations (μM): 1.0 Hg, 1.5 Cu, 5.0 Ni,

40 Zn, or 150 Mn. Given that the concentrations in the bulk solutions varied from 1.0 to 150 µM, in (A) the concentrations of metals in the rhizodermis (µg g^{-1}) are divided by their concentrations in the bulk solution (µM).

Fig. 4. Scanning electron micrographs showing ruptures in roots of cowpea exposed to 40 µM Al, 2.4 µM Hg, 13 µM In, or 13 µM Ru for the times indicated. Data were collected as described by Kopittke et al. [9] and Kopittke et al. [43]. Note that for Ru (24 h), the root tip is almost entirely broken off – this was generally observed in severely ruptured roots which became very delicate and even the aeration bubbles rising through the nutrient solution had sufficient energy to break off the tip at the rupture.

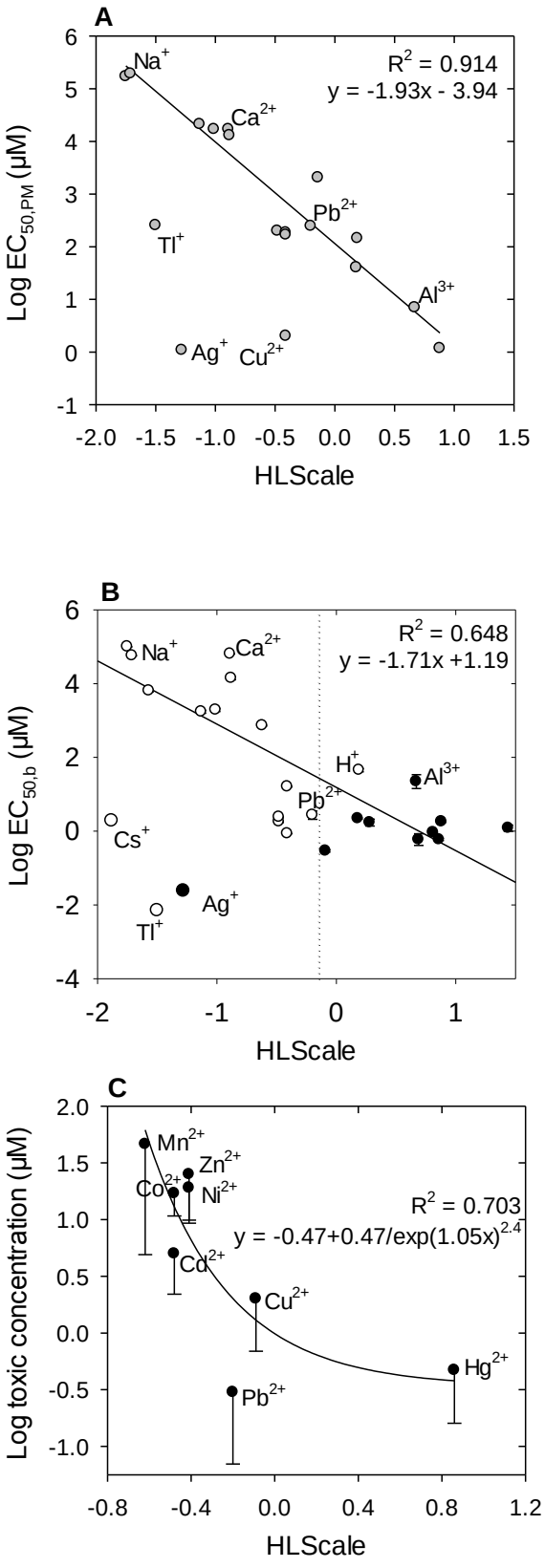
A
Log EC50,PM (μM)
HLScale
R2 = 0.914
y = -1.93x - 3.94
Na+
Ca2+
Pb2+
Tl+
Al3+
Ag+
Cu2+
B
Log EC50,b (μM)
HLScale
R2 = 0.648
y = -1.71x +1.19
Na+
Ca2+
H+
Al3+
Pb2+
Cs+
Ag+
Tl+
C
Log toxic concentration (μM)
HLScale
R2 = 0.703
y = -0.47+0.47/exp(1.05x)2.4
Mn2+
Zn2+
Co2+
Ni2+
Cd2+
Cu2+
Hg2+
Pb2+

Fig. 1.

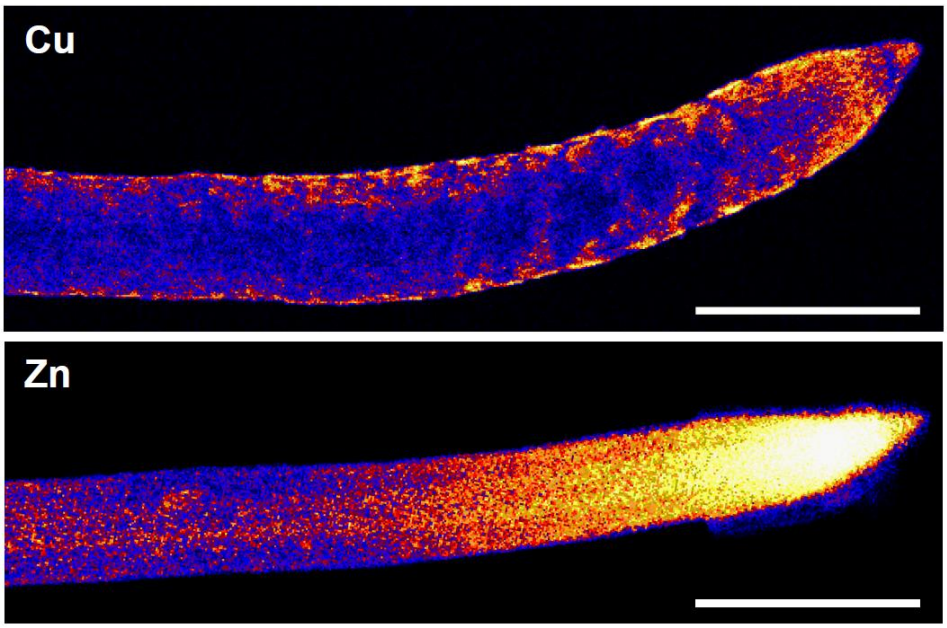

Fig. 2.

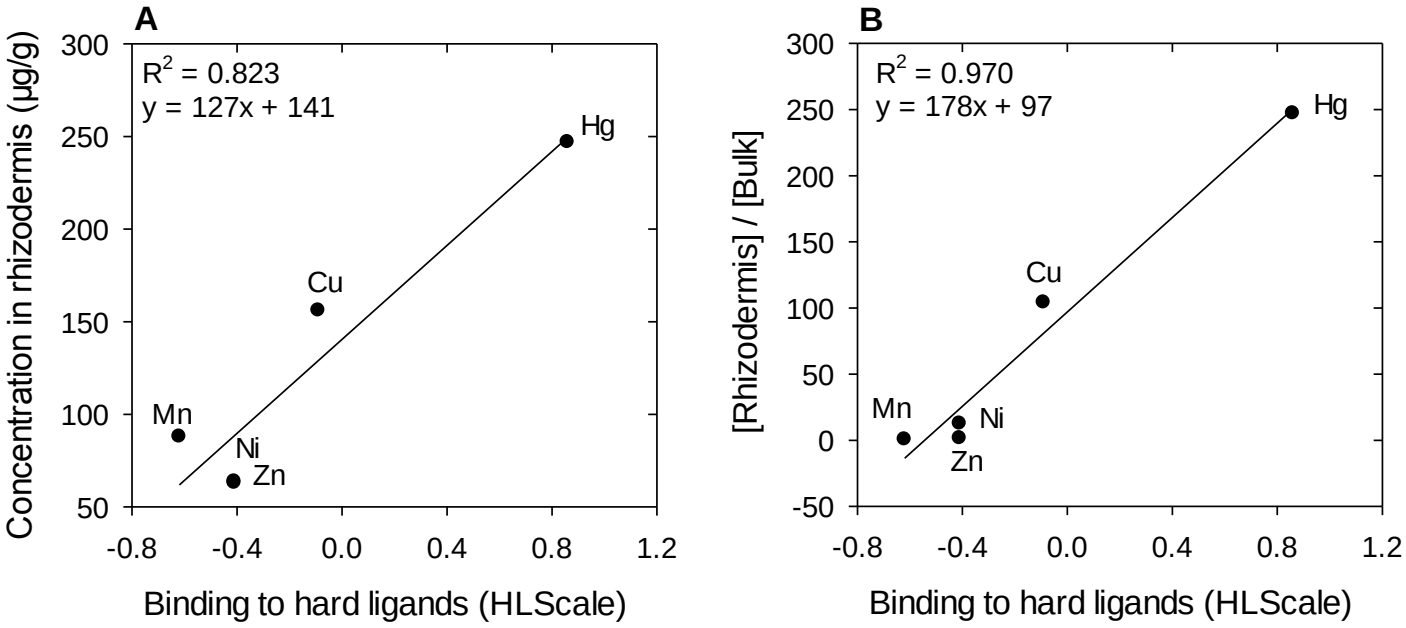
A
R^2 = 0.823
y = 127x + 141
Concentration in rhizodermis (µg/g)
Binding to hard ligands (HLScale)
Hg
Cu
Mn
Ni
Zn
B
R^2 = 0.970
y = 178x + 97
[Rhizodermis] / [Bulk]
Binding to hard ligands (HLScale)
Hg
Cu
Mn
Ni
Zn

Fig. 3.

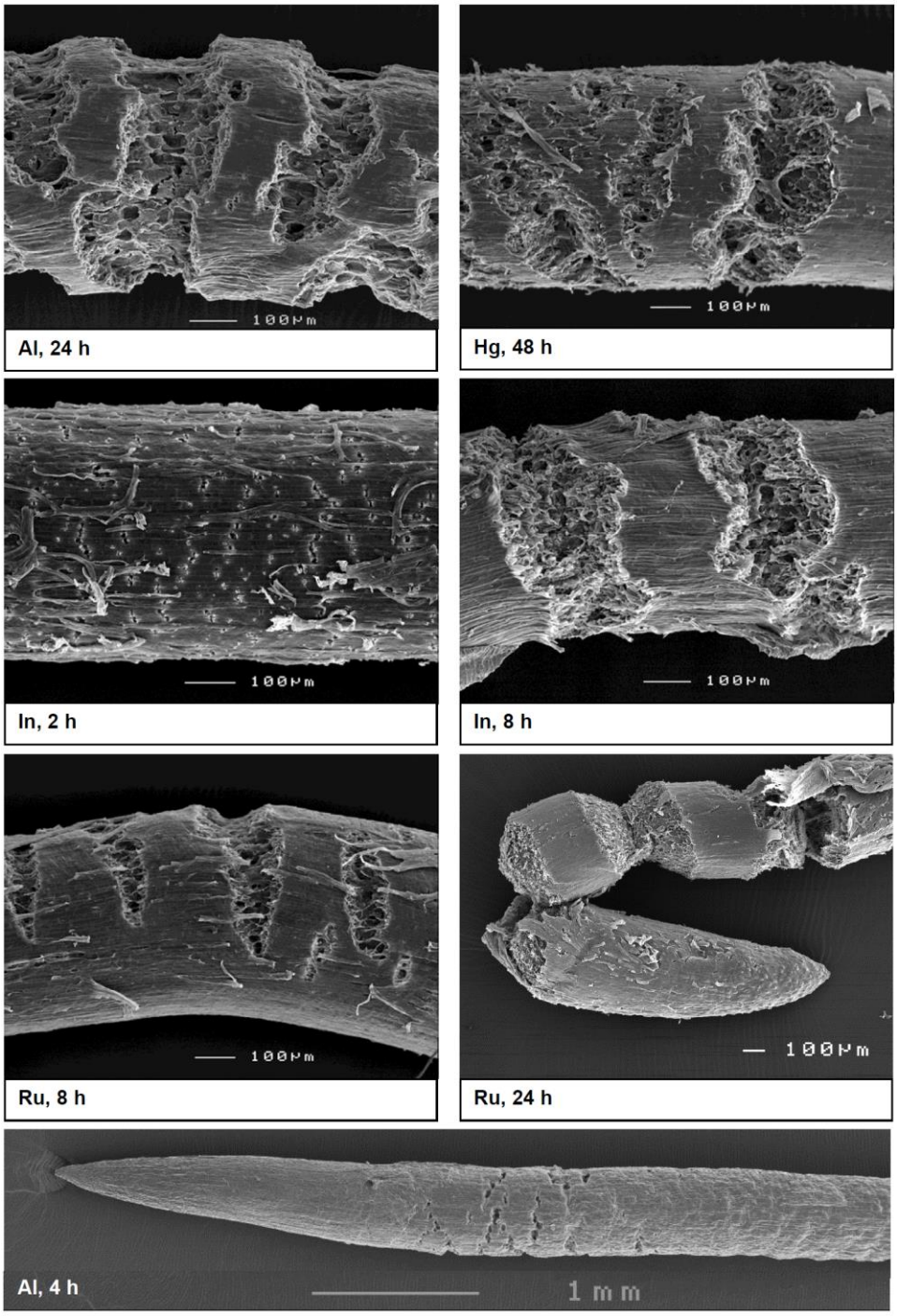
100µm
Al, 24 h
100µm
Hg, 48 h
100µm
In, 2 h
100µm
In, 8 h
100µm
Ru, 8 h
100µm
Ru, 24 h
Al, 4 h
1 mm

Fig. 4.