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Contribution of AM symbiosis to *in vitro* root metal uptake: From trace to toxic metal conditions

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Abstract: This *in vitro* study investigated the role of arbuscular mycorrhizal (AM) symbiosis in root metal acquisition and stress tolerance from two experiments using a carrot root-organ culture system and involving the essential micronutrient zinc as a typical metal contaminant. We demonstrated that the AM symbiosis plays a dual role in root metal acquisition by increasing nutrient uptake via mycorrhizal 'enhanced uptake' at low (trace) metal concentrations in the growth medium, but then lessening the uptake through 'metal-binding' processes at high (toxic) concentrations. Furthermore, we also observed the relative contribution of hyphal uptake and translocation to roots which led us to suggest that the enhanced uptake and metal-binding processes likely occur simultaneously and/or independently. Ultimately symptoms of metal toxicity toward both the roots and AM fungi at the highest Zn exposure concentrations was observed. From this finding, a critical toxicity burden likely exists arising from conditions ranging from trace to toxic metal extremes.

Keywords: *Daucus carota* L., *Glomus intraradices*, metal bioavailability, critical toxicity burden, stress avoidance

38 **Introduction**

39 The arbuscular mycorrhizal (AM) symbiosis, an association between the roots of most
40 herbaceous plants and the Glomeromycota fungi (Schüßler et al. 2001), is a key component of
41 ecosystem functioning involving the exchange of soil resources for plant carbohydrates.
42 Whereas this association is most widely recognized for improving root acquisition of nutrients
43 and water by means of the combined absorptive surface area (*e.g.* roots and fungal hyphae) of the
44 mycorrhizosphere (Lambers et al. 2007; Subramanian and Charest 2009), recent studies also
45 indicate an involvement in alleviating metal toxicity and related growth challenges such as
46 nutrient imbalances and changes in soil-pH (Audet and Charest 2006, 2007a; Leyval et al. 1997).
47 In this latter role, the mycorrhizosphere is believed to regulate growth conditions through metal-
48 binding processes including the exudation of ligands (Joner et al. 2000; Jeffries et al. 2003).
49 From previous meta-analyses (Audet and Charest 2007b, 2008), a hypothetical model (Fig.1)
50 was developed to depict the impact of AM symbiosis on plant metal acquisition suggesting that
51 AM fungi:

52 (a) Increase metal uptake via the extensive mycorrhizospheric absorptive network at
53 low (trace) metal exposure concentrations; and
54 (b) Reduce metal bioavailability through metal-binding at high (toxic)
55 metal concentrations, thereby decreasing plant uptake and enhancing
56 tolerance through stress-avoidance.

57 Using the essential micronutrient zinc (Zn) as a typical metal contaminant, we tested this model
58 using an *in vitro* carrot (*Daucus carota* L.) root-organ system in dual-culture with an AM fungus
59 (*Glomus intraradices* Schenck & Smith). With this experimental design, our goal was to
60 determine the patterns of metal uptake by AM and non-AM root-organs, discern the relative
61 contribution of hyphal uptake and translocation to host roots, and determine any subsequent

62 effects of metal uptake on root mass, AM colonization, and fungal spore development. This
63 culture system was selected for studying mycorrhizal interactions as it enables the direct
64 investigation of the nutrient uptake scenarios described above as well as determining the
65 relative contribution of hyphal metal uptake and translocation to roots (Fortin et al. 2002, 2005;
66 Declerck et al. 2003; Rufyikiri et al. 2003, 2004;).

67 **Materials and methods**

68 In our two experiments, Ri T-DNA transformed carrot roots were inoculated, or not, with the
69 AM fungus *G. intraradices* (DAOM 181602) and grown in a low mineral medium in one- or
70 two-compartment Petri plates (100 mm x 15 mm) according to St-Arnaud et al. (1995, 1996).
71 The medium contained sucrose as the source of carbon for roots and was amended with one of
72 four Zn concentrations (0, 0.1, 0.5, and 1.0 mM Zn) ranging from trace to toxic conditions.
73 Here, the 0 Zn control treatment contained a baseline concentration of 9 µM Zn.

74 **Experimental design**

75 In the first experiment designed to determine the overall patterns of AM and non-AM root-organ
76 metal uptake (Fig.2a and 2b), one-compartment plates were filled with 25 mL of the medium
77 solution (pH 5.5) containing one of the four Zn concentrations and solidified with 0.4 % (m/v)
78 Gelrite® gellan gum (Sigma-Aldrich™, Canada). Each plate consisted of two 3 cm roots
79 aseptically excised from a culture collection, transferred onto the medium, and inoculated (AM)
80 or not (non-AM control) with ~50 fungal propagules of *G. intraradices* consisting of spores and
81 hyphae which were verified by microscopic examination. The plates were then sealed, inverted,
82 and incubated (124 L Incubator, Conviron®, Canada) in darkness at 25 °C for eight weeks
83 allowing for the growth and colonization of roots, proliferation of fungal hyphae, and production
84 of spores. To ensure adequate biomass for further analyses, we pooled five plates per replicate

85 and included seven replicates per treatment (1 root-organ x 2 $AM^{+/-}$ x 4 $Zn^{[0, 0.1, 0.5, 1.0]}$) for a total
86 of 56 pooled replicates (280 total plates).
87 In the second experiment (Fig.2c and 2d), two-compartment plates (100 mm x 15 mm)
88 divided by a median partition were filled with 20 mL of the medium containing 0 mM Zn in the
89 root-hyphal compartment (RHC) and 20 mL of the medium containing one of the four Zn
90 concentrations in the hyphal compartment (HC). As described above, roots were transferred into
91 the RHC, inoculated with fungal propagules, and incubated for eight weeks; non-AM roots
92 serving as a control group were prepared in the same manner. In order to promote the crossing
93 and proliferation of extraradical hyphae from the RHC to the HC, only the RHC medium
94 contained sucrose whereas the HC did not and the HC medium was generated on a 45°
95 downward slope (Fortin et al. 2002; Cranenbrouck et al. 2005). Over the experimental period,
96 only the extraradical hyphae were permitted to cross the dividing wall to proliferate throughout
97 the HC. Accordingly, all roots were monitored regularly and trimmed as required to prevent
98 them from crossing the partition. We pooled five plates per replicate and included seven
99 replicates per treatment (1 root-organ x 1 AM x 4 $Zn^{[0, 0.1, 0.5, 1.0]}$ + 1 non-AM $Zn^{[0]}$) for a total of
100 35 pooled replicates (175 total plates).
101 **Biomass and metal uptake**
102 After an eight-week growth period, pooled replicates (n=4) of AM and non-AM roots from
103 randomly selected treatment blocks were chosen to measure the dry mass and metal
104 concentrations for both the one- and two-compartment experiments. The roots were carefully
105 removed from the growth medium, rinsed with dH_2O, frozen with liquid nitrogen, and
106 lyophylized at -60 °C in a partial vacuum for 72 h (Unitrap 10-100 Virtis Inc., Gardiner, USA).
107 Freeze-dried roots were then weighed, ground, and each sample (~20-120 mg) was transferred
108 into acid-washed Teflon® bombs (Nalgene® Oak Ridge, USA). Samples were dissolved in 2 mL

109 of 16 M HNO_3, heated for 10 h in a 80 °C water bath, and diluted with 20 mL pure grade H_2O
110 (EMD, Germany). In the same manner, ten samples of Apple Leaves (Standard Reference
111 Material #1515) from the National Institute of Standards and Technology (NIST, USA) and five
112 blanks consisting solely of HNO_3 and H_2O were also prepared to ensure the quality and accuracy
113 of the metal analysis. All sample solutions were analyzed via Inductively Coupled Plasma
114 Optical Emission Spectrometry (730-ES, Varian Inc., Canada) to determine the Zn
115 concentrations. We obtained 97.6 ± 1.2 % mean Zn recovery from the standards. In addition,
116 the instrumental limit of detection calculated from the standard deviation of repeated
117 measurements was 0.0033 $\mu g\ g^{-1}$, the HNO_3 dissolution method limit of detection calculated
118 from procedural blanks was 0.0256 $\mu g\ g^{-1}$, and the instrumental limit of quantification within the
119 dynamic range of the calibration curve was 0.0111 $\mu g\ g^{-1}$.

120 Further to assessing mean root dry mass, we used liberal (Wilkins 1978) and conservative
121 (Wilson 1988) tolerance indices as indicators of relative growth. The Wilkins (1) and Wilson (2)
122 tolerance indexes, calculated in relation to control treatments within each treatment block (n=4),
123 are defined as:

124
$$\frac{DryMass_{\exp}}{DryMass_{control}} \quad (1)$$
125

126
$$\frac{DryMass_{\exp} + DryMass_{\exp}}{DryMass_{control} + DryMass_{\exp}} \quad (2)$$
127

128 The index values approaching 0 indicate decreasing Zn tolerance (or increasing toxicity) in
129 relation to the control treatments.
130
131
132

133 **Root colonization and spore production**

134 Roots from the remaining pooled replicates (n=3) were carefully removed, rinsed, and stained
135 with aniline blue 0.02% dye solution (6.78 mM aniline blue; 500 ml glycerol; 450 ml dH_2O; 50
136 ml 1 % HCl) according to Dalpé (1993). Fifty ~1-2 cm root segments per replicate were
137 mounted on slides and examined at 100X and 400X magnification using a compound microscope
138 (CX41, Olympus Inc., Canada). Mycorrhizal colonization was estimated according to
139 Giovannetti and Mosse (1980) and Dalpé (1993) by determining the % frequency of
140 colonization, % length of root colonization, and relative density (# mm^{-1} root length) of fungal
141 structures (e.g. vesicles). The equations for the % frequency (3), % length of root colonization
142 (4), and relative density (5) are defined as:

143 $$\frac{\#Segment_{AM}}{\#Segment_{total}} x100\% \quad (3)$$

144

145 $$\frac{RootLength_{AM}}{RootLength_{total}} x100\% \quad (4)$$

146

147 $$\frac{\#AMstructures}{RootLength_{AM}} \quad (5)$$

148

149 Once the roots used in Zn and AM colonization analyses were harvested, the growth
150 medium from plates was collectively pooled (5 per replicate, n=7) and analyzed for the
151 determination of fungal spore production by cutting the medium to fragment the hyphal network
152 and dissolving it in 10 mM sodium citrate buffer, pH 6.0 (Doner and Bécard 1991). The
153 dissolved medium solutions were then vacuum-filtered across a 20 µm nylon filter apparatus
154 (Millipore™, USA) to isolate the spores which were later re-suspended in 50 mL dH_2O. Ten 1
155 mL sub-samples of the re-suspended spores were taken from each replicate and examined under
156 the compound microscope using a Sedgewick-Rafter counting cell (Wilco®, USA) to extrapolate

157 the total spore production. For the two-compartment experiment, the growth medium from each
158 compartment was analyzed separately to specifically compare the spore production between the
159 RHC and HC.

160 **Statistical analyses**

161 One- and two-way analyses of variance (ANOVA) with Bonferonni and Scheffé studentized
162 range tests were performed for mean comparison analyses (Zar 1999). Kolmogorov-Schmirnoff
163 and Levene's tests were used, respectively, to verify the normality of distribution and the
164 homogeneity of residual variance. The data were Log-transformed as required to meet the
165 assumptions of parametric analyses. All of the Fisher statistics (F), degrees of freedom (df), and
166 p-value estimates were calculated using S-Plus 8.0 statistical software (Insightful Corp. 2007).

167 **Results**

168 In the one-compartment experiment, we observed a change in AM root Zn uptake compared to
169 non-AM roots as Zn concentrations in the medium increased (Fig.3a and 3b). Overall, Zn uptake
170 was nearly two-fold greater among AM (31.7 $\mu g\ g^{-1}$ DM) than non-AM roots (17.9) at the low
171 rates of exposure, but then two-fold lower at the high rates (890 to 1919 in AM versus 1508 to
172 4160 in non-AM roots). These results were also found for the total Zn content of roots, albeit
173 showing a high degree of statistical variance. In general, root growth was deleteriously affected
174 by metal uptake whereby dry mass declined by six-fold (Fig.4) and noticeable stress (*e.g.* root
175 browning) gradually appeared as Zn concentrations in the medium increased. Despite these
176 detrimental effects, AM root dry mass at low Zn treatments (0 and 0.1mM) tended to be 10 to 20
177 % greater than non-AM roots as corroborated by both indices of growth tolerance (Table 1),
178 although these trends were not statistically significant. As for AM root colonization (Table 2),
179 the % frequency and % length of root colonization also decreased, most markedly between the
180 0.1 to 0.5 mM Zn treatments. The visual examination of AM roots indicated that the hyphal

181 network was prolific among all the Zn treatments (data not shown), although the extraction of
182 spores from the growth medium revealed a five- and then fifty-fold decrease in spore production
183 as Zn levels increased (Table 2).

184 In the two-compartment experiment, the root Zn concentration was measured to
185 determine the contribution of hyphal metal uptake and translocation from the HC to the host
186 roots (Fig.5a and 5b). Visual examination revealed that AM roots were well colonized and that
187 the extraradical hyphae had crossed the median partition to proliferate throughout the HC (data
188 not shown). Here, hyphal uptake and translocation resulted in over two-fold higher Zn
189 concentration and content among AM than non-AM roots at the 0 mM Zn control treatment (69.5
190 $\mu g\ g^{-1}$ DM & 6.5 μg in AM versus 23.8 & 2.1 in non-AM roots), followed by a linear increase in
191 AM root Zn uptake with increasing Zn concentration in the HC medium (up to 455.0 $\mu g\ g^{-1}$ DM
192 and 53.6 μg). Despite this increase, the root dry mass did not differ significantly between any of
193 the AM or non-AM treatments which ranged between 91.0 and 101.2 mg. Likewise, the overall
194 AM root colonization did not show any remarkable trend among the treatments. Moreover,
195 approximately six-fold greater number of spores were measured in the RHC than the HC (Fig.6).
196 In this case, the number of spores in the RHC did not differ between the Zn treatments, whereas
197 a marked decline (from 1854 to 148) was found in the HC as Zn concentrations in this
198 compartment increased.

199 **Discussion**

200 Consistent with our hypothetical model, we report on the impact of AM fungi (AMF) on root
201 metal acquisition characterized by the dual processes of enhanced uptake and metal-binding. In
202 our one-compartment experiment, these processes contributed to a two-fold greater metal uptake
203 in AM than non-AM roots at low Zn concentrations in the medium followed by a two-fold lower
204 uptake at high concentrations. At low metal exposure, the extensive extraradical hyphae
205 enhanced metal uptake by taking up and translocating metal ions to roots in exchange for
206 photosynthates (Koide 1990; Smith and Smith 1990). Correspondingly, molecular analyses of
207 *Medicago truncatula* and *Glomus intraradices* have shown that the regulation of specific ion
208 transporters (*e.g.* ZIP protein family) accounts for the active uptake of Zn and other
209 microelements (Burleigh et al. 2003; Gonzalez-Guerrero et al. 2005). Plants benefit from this
210 enhanced uptake mechanism by supplementing their elemental requirements and then improving
211 their health status; in our study, an effect which may account for the 10 to 20% greater biomass
212 among AM than non-AM roots at the low metal treatments. By contrast, the impact of AM
213 symbiosis shifts at high treatments due to the binding properties of fungal tissues (Gonzalez –
214 Guerrero et al. 2008) and their exudation of ligands, particularly owing to the immobilization of
215 excess metal ions to hydroxide, oxyhydroxide, and sulfhydryl constituents (Gadd 1993; Galli et
216 al. 1994; Gonzalez-Chavez et al. 2002). In this case, metals may be sequestred by the mycelium
217 through binding to the hyphal walls and then diffusing into hyphal cells, or precipitated as metal-
218 ligand complexes in the proximal growth environment (Joner et al. 2000; Jeffries et al. 2003).
219 Consequently, this fungal metal-binding process is believed to decrease plant metal uptake by
220 reducing the bioavailability of potentially toxic metals and then delaying the onset of metal
221 phytotoxicity (Leyval et al. 1997; Audet and Charest 2006, 2007b). In addition to these
222 properties, mycorrhizospheric proliferation is also recognized for defining edaphic conditions by

223 enhancing soil-moisture retention (Augé et al. 2001; Piotrowski et al. 2004), stabilizing the soil-
224 structure matrix (Bearden 2001; Miller and Jastrow 1990), and enriching soil-microbial diversity
225 (Filion et al. 1999; St-Arnaud and Elsen 2005). Taken as a whole, these characteristics
226 contribute in supplementing plant nutrient uptake, but also lessening the uptake of toxic metals in
227 complement to other intrinsic stress resistance mechanisms such as metallotheinin and
228 phytochelatin metabolism (Cobbett 2000; Cobbett and Goldsbrough 2002; Maier et al. 2003).
229 Whereas the one-compartment culture system has enabled us to identify a general pattern
230 of AM root metal uptake characterized by increased uptake at low treatment levels and decreased
231 uptake at high levels compared to non-AM roots, the two-compartment system has allowed us to
232 discern the contribution of mycorrhizal enhanced uptake and translocation to host roots. In this
233 case, AM root colonization resulted in an over two-fold greater Zn uptake compared to non-AM
234 roots followed by a linear increase in AM root metal uptake with increasing metal concentration
235 in the hyphal compartment. Upon isolating this effect, it may be implied that enhanced uptake
236 actively contributed in increasing metal uptake despite the contributions of metal-binding
237 processes which likely reduced the metal bioavailability in the hyphal compartment. Thus, it is
238 probable that both of these fungal processes should occur simultaneously and/or independently.
239 This scenario is supported conceptually from the perspective that each phenomenon influences
240 different aspects of root metal uptake: the first acting on the uptake capability of host roots, and
241 the second on the bioavailability of metals in the mycorrhizosphere. The resulting pattern of AM
242 root metal uptake may, therefore, change compared to non-AM roots depending on the
243 prominence of these mycorrhizal-induced physico-chemical adaptations which then shape
244 subsequent growth conditions (Hinsinger 2001a,b ;Lombi et al. 2001).
245 Beside the impact of AM symbiosis on root metal acquisition, we recognize the
246 challenges associated with conditions ranging from trace to toxic metal extremes which often

247 result in a compromise between plant and fungal growth versus stress-tolerance (Audet and
248 Charest 2007a, 2007b, 2008). As such, we measured an approximate five-fold decrease in root
249 dry mass and observed noticeable root browning symptomatic of metal phytotoxicity at the
250 highest extrinsic Zn concentrations. Unlike our predictions, the growth status of AM roots did
251 not significantly benefit from fungal metal-binding which was previously assumed to reduce
252 metal toxicity through stress avoidance. This finding points out the probable existence of a
253 critical phytotoxicity burden arising either directly due to cellular oxidative stress caused by
254 metal uptake (Baccouch et al. 1998; Schützendübel and Polle 2002; Cho and Seo 2005) or
255 indirectly due to elemental nutrient imbalances arising in the rhizosphere (McBride 1994;
256 Kabata-Pendias 2007). Alternatively, the decrease in root dry mass could also be inherent to the
257 culture system itself since there is no possibility of translocating metals to shoots, thereby
258 potentially alleviating stress in roots. Nevertheless, decreases in AM root colonization
259 frequency, length of root colonized, and fungal structure density were ultimately observed at the
260 highest extrinsic metal concentrations in addition to a dramatic fifty-fold decrease in spore
261 production. Here, we suspect that the AMF are themselves burdened by high metal exposure as
262 expressed by a severe depression in their proliferation (Pawlowska and Charvat 2004;
263 Janoušková and Vosatká 2005). Despite these challenges, survey studies of metal-contaminated
264 field sites attest to the remarkable resilience of plants and AMF when subjected to such
265 environmentally stressful conditions, especially nutrient imbalances and fluctuations in pH (Del
266 Var et al. 1999; Pawlowska et al. 2000).

267 **Conclusion**

268 The carrot root-organ culture system has enabled us to demonstrate the contribution of AM
269 symbiosis to root metal acquisition as characterized by the enhanced uptake and metal-binding
270 phenomena. Furthermore, by discerning the relative contribution of hyphal uptake and
271 translocation to roots, we revised our model to suggest that these fungal processes likely occur
272 independently. Thus, it is our view that the model would be a relevant tool in remediation
273 management practices, meanwhile outlining the potential growth challenges stemming from
274 from trace to toxic metal extremes.

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464 **7. Figure Captions**

465

466 Figure 1. Conceptual model illustrating the impact of AM symbiosis on plant metal
467 acquisition and relative plant growth. Here, AM root colonization increases plant
468 metal uptake via 'enhanced uptake', but later decrease it via 'metal-binding' to
469 improve the plant's growth status. Reproduced from Audet and Charest (2007b)
470 courtesy of Environmental Pollution, Elsevier.
471
472 Figure 2. Top-view of schematized non-AM (a) and AM colonized (b) Ri T-DNA carrot
473 root-organs in one-compartment plates with the latter showing extraradical
474 hyphae and fungal spores. Top- (c) and side-views (c) of AM roots in two-
475 compartment plates showing roots and hyphae in the root-hyphal compartment
476 (RHC) and hyphae and spores only in the hyphal compartment (HC).
477
478 Figure 3. Zn concentration (a) and content (b) in non-AM (empty bars) and AM roots (solid
479 bars) from one-compartment plates. Pooled means (n=4) and standard errors are
480 shown. Shared letters designate treatments that are not significantly different
481 according to Bonferonni and Scheffé mean comparison ($p<0.05$).
482
483 Figure 4. Dry mass of non-AM (empty bars) and AM roots (solid bars) from one-
484 compartment plates. Pooled means (n=4) and standard errors are shown. Shared
485 letters designate treatments that are not significantly different according to
486 Bonferonni and Scheffé mean comparison ($p<0.05$).
487
488 Figure 5. Zn concentration (a) and content (b) in non-AM and AM roots translocated from
489 two compartment plates. Pooled means (n=4) and standard errors are shown.
490 Shared letters designate treatments that are not significantly different according to
491 Bonferonni and Scheffé mean comparison ($p<0.05$).
492
493 Figure 6. Number of spores extracted from the root-hyphal (RHC - solid bars) and hyphal
494 compartments (HC - empty bars) from two-compartment plates. Pooled means
495 (n=7) and standard errors are shown. Shared letters designate treatments that are
496 not significantly different according to Bonferonni and Scheffé mean comparison
497 ($p<0.05$).

Figure 1

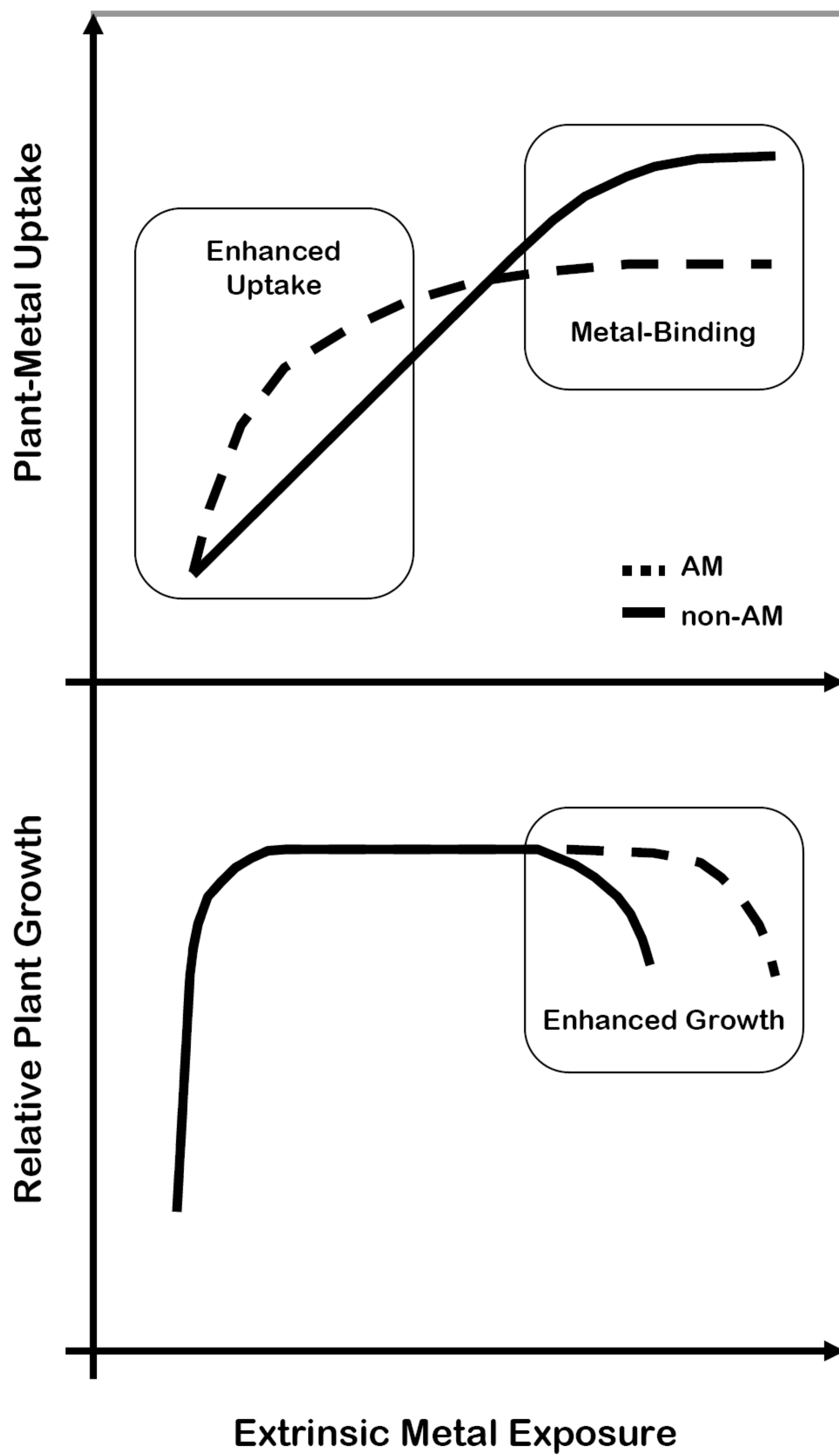

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Figure 2

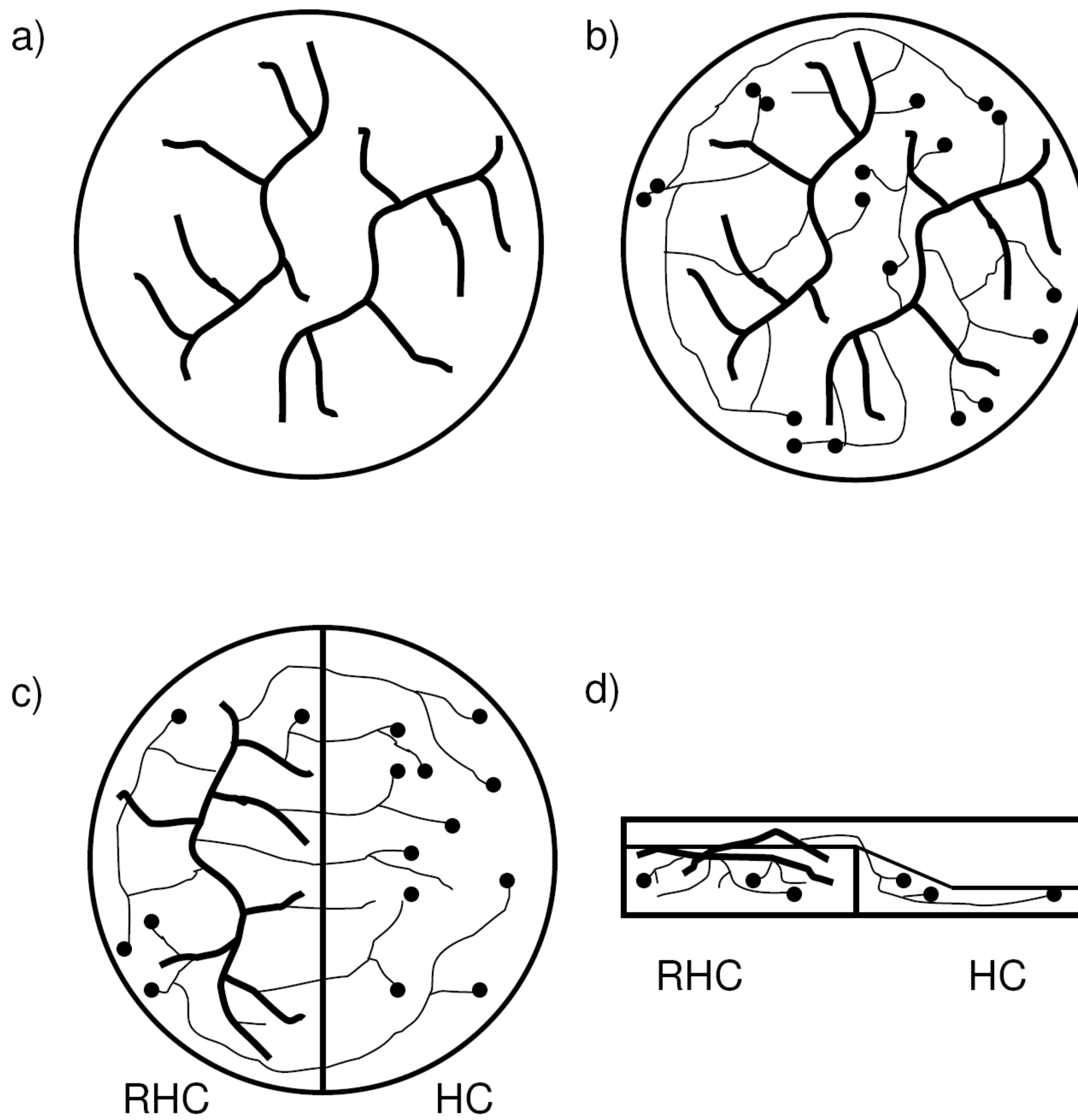

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Figure 3

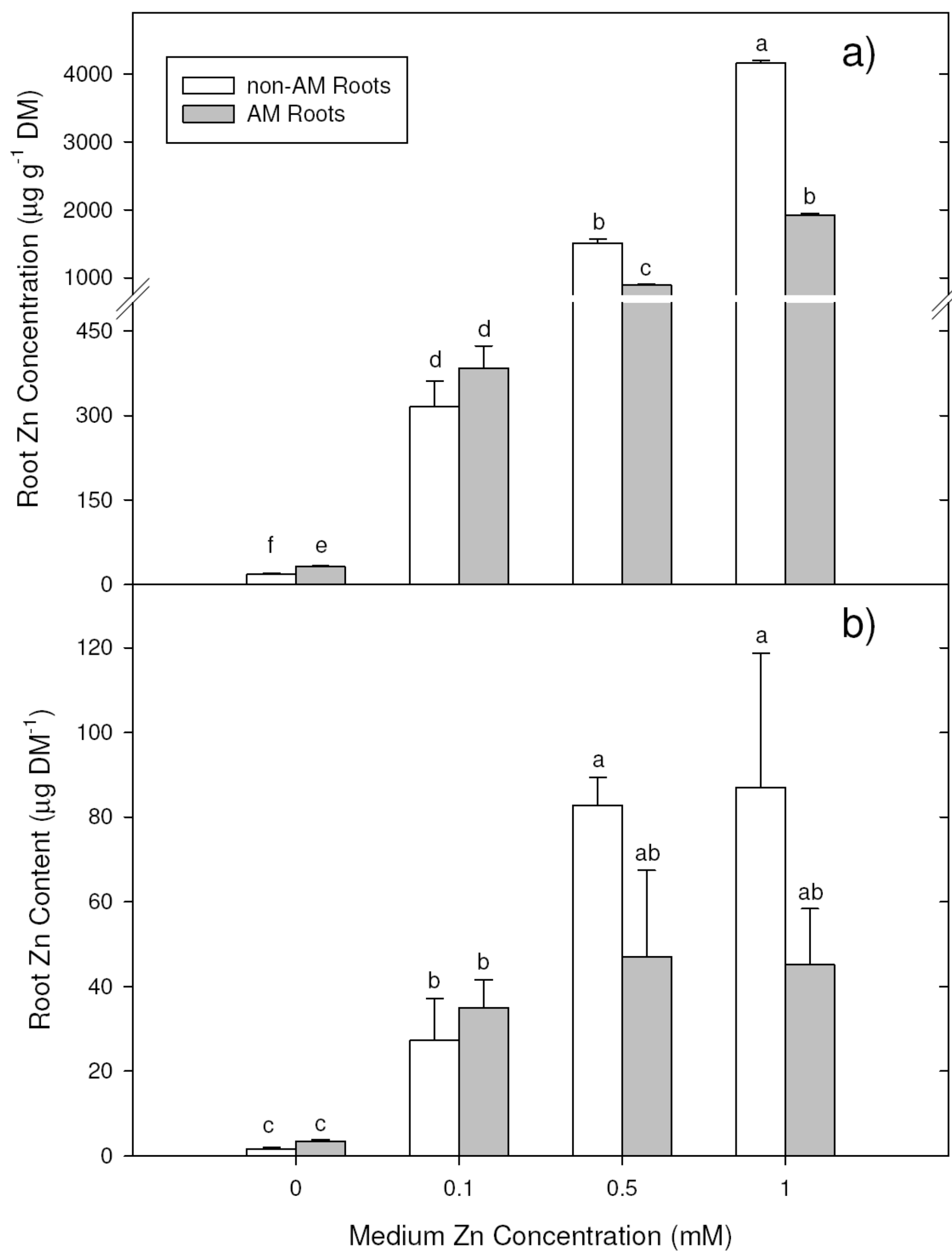

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Figure 4

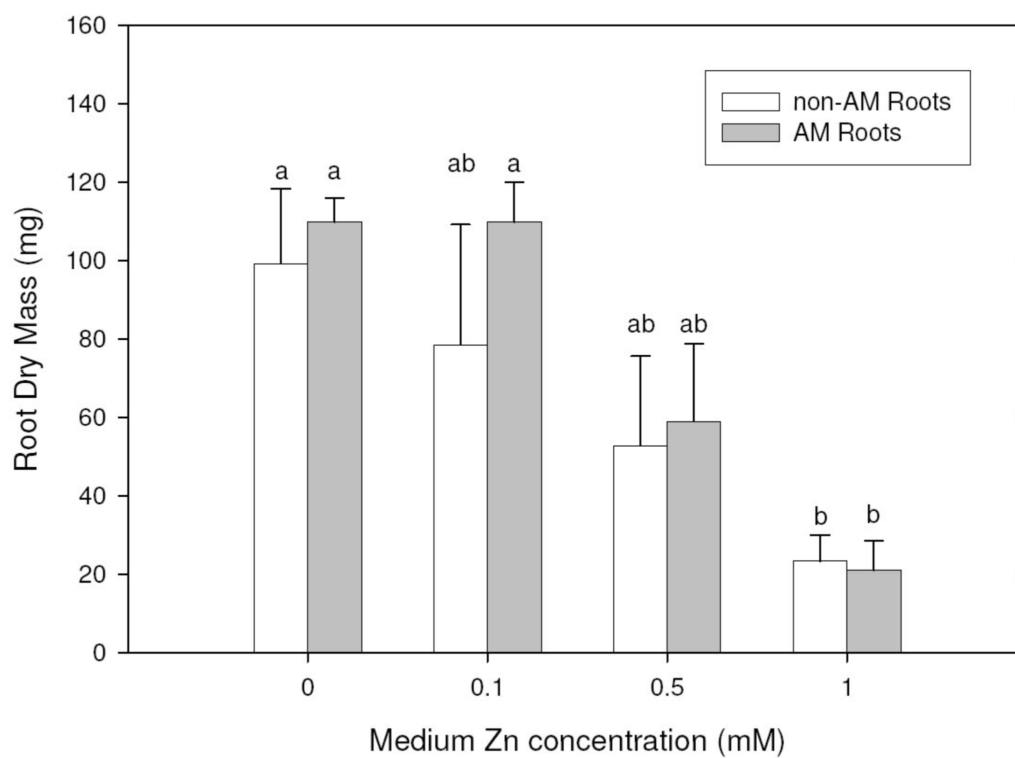

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Figure 5

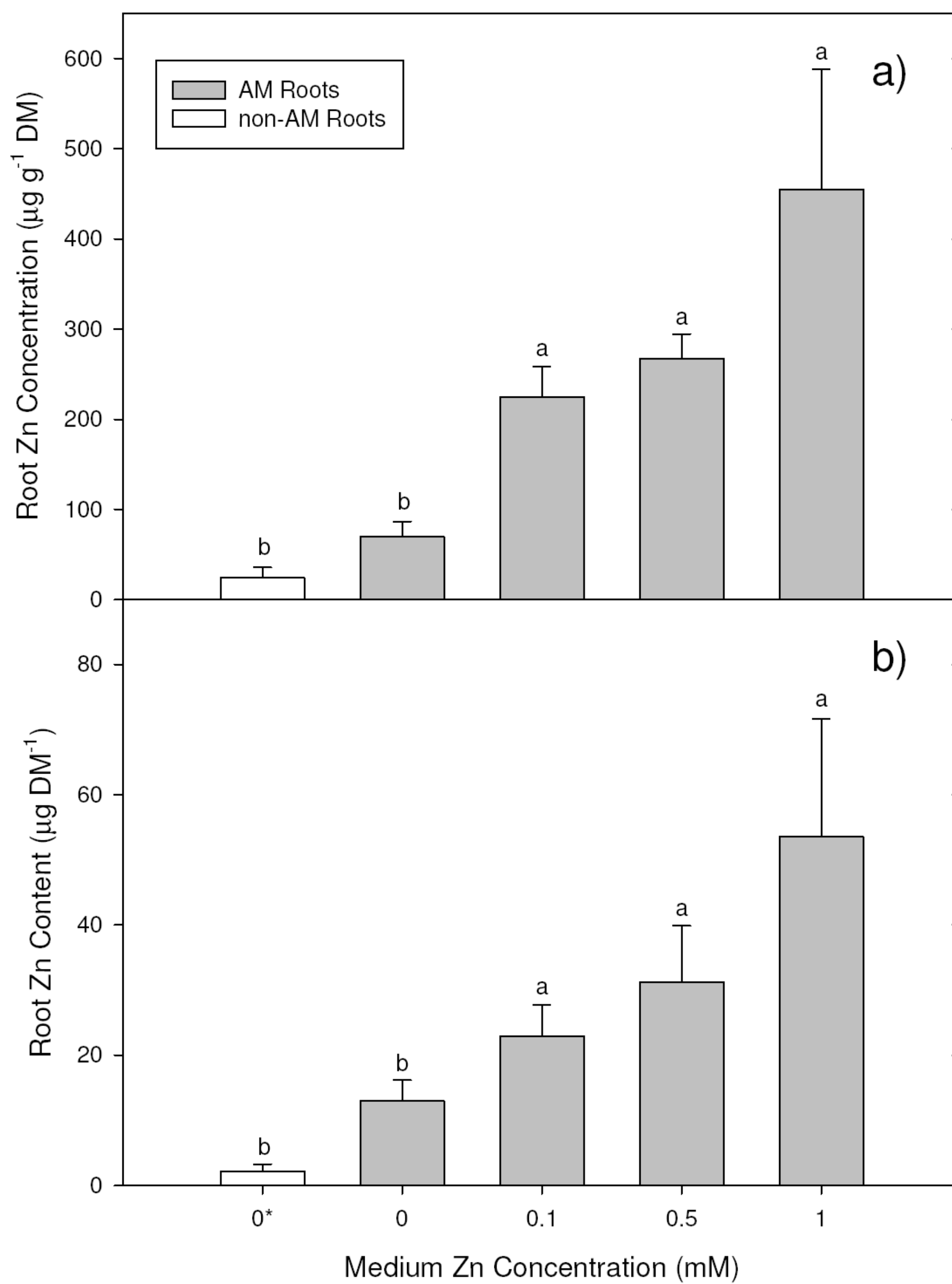

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Figure 6

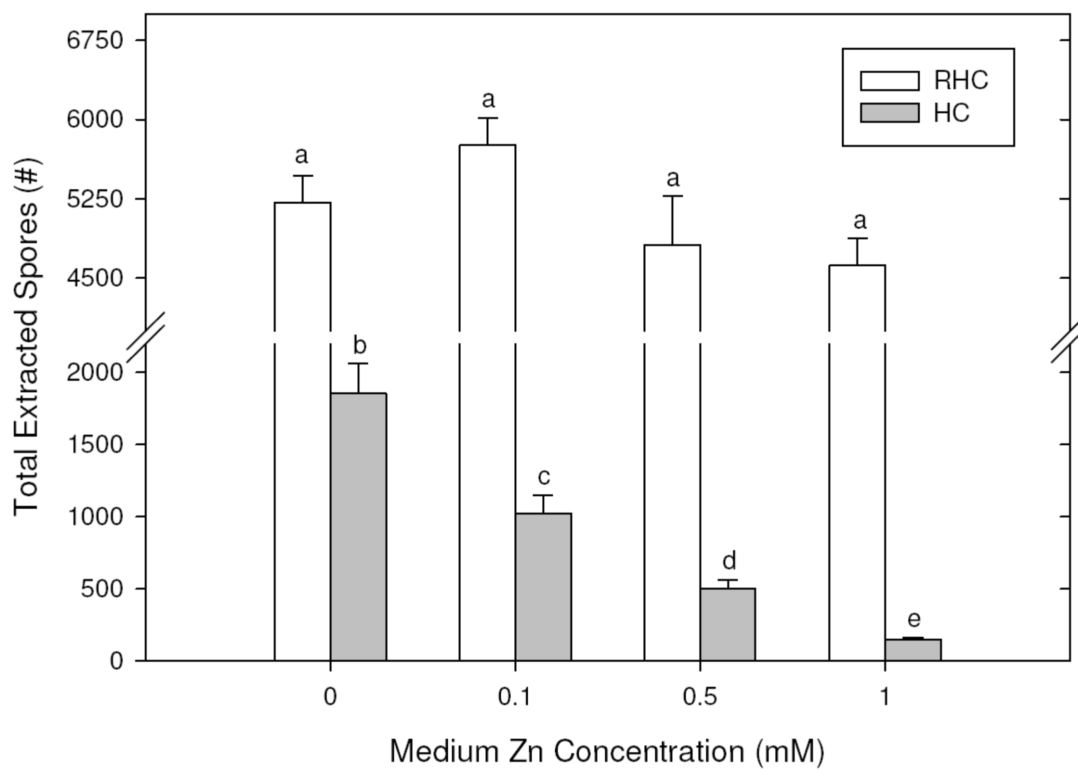

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Table 1. Indexes of root tolerance in one-compartment plates.

Zn concentration* (mmol·L^{-1})	Inoculation	Tolerance indexes: Wilkins	Tolerance indexes: Wilson
0.1	non-AM	0.79ab (0.31)	0.81b (0.20)
	AM	1.00a (0.06)	1.00a (0.03)
0.5	non-AM	0.53ab (0.23)	0.64b (0.18)
	AM	0.50ab (0.03)	0.66b (0.02)
1	non-AM	0.23b (0.07)	0.37c (0.08)
	AM	0.19b (0.07)	0.31c (0.10)
***F*-statistics**			
[Zn]		14.6***	17.4***
Inoculum		0.5 ns	5.6*
[Zn] × Inoculum		1.0 ns	4.9**

Note: Means (n = 3) and SE (inside parentheses) are shown. Shared letters in columns designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison; ns, not significant; ***, $p < 0.001$.

*The 0 mmol·L^{-1} concentration indexes do not appear, since they represent auto-correlation values of 1, owing to the nature of the equation.

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Table 2. AM root colonization and spore production in one-compartment plates.

	Root colonization*					
	Hyphae		Vesicles			Spore production†
Zn ($mmol \cdot L^{-1}$)	Frequency (%)	Root length (%)	Frequency (%)	Root length (%)	Relative density [No.·mm(-root length)$^{-1}$]	Total (No. per 5 plates)
0	91.5a (4.9)	55.4a (2.9)	34.9a (1.4)	22.9 (1.8)	13.5 (0.2)	18475a (2765)
0.1	91.5a (0.7)	58.4a (1.1)	32.6a (3.9)	22.8 (1.5)	15.2 (1.1)	3450b (1021)
0.5	77.2b (5.3)	52.7a (2.1)	17.1ab (11)	25.2 (7.8)	15.1 (1.2)	575c (175)
1.0	79.0b (4.9)	37.8b (4.2)	8.81b (1.9)	12.5 (6.3)	8.8 (4.5)	383c (122)
***F* statistics**	10.9*	8.3*	7.1*	1.2 ns	1.65 ns	38.2***

Note: Means (*, $n = 3$; †, $n = 7$) and SE (inside parentheses) are shown. Shared letters in columns designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison; ns, not significant; *, $p < 0.05$; ***, $p < 0.001$.

*Root length of colonized (AM) roots as a percentage of total root length.