Research Article

Determining the Impact of the AM-Mycorrhizosphere on "Dwarf" Sunflower Zn Uptake and Soil-Zn Bioavailability

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An *in vivo* compartmental pot greenhouse experiment involving "dwarf" sunflower and an arbuscular mycorrhizal (AM) fungus was designed to assess the contribution of non-AM roots (rhizosphere), AM roots and extraradical hyphae (mycorrhizosphere), or strictly extraradical hyphae (hyphosphere) on plant growth, plant metal uptake, and soil parameters using the micronutrient zinc (Zn) as a typical metal contaminant. We observed that, at high soil-Zn concentrations, the mycorrhizosphere treatments had lower Zn concentrations (especially in shoots and flowers) and a lower incidence of leaf chlorosis than the rhizosphere treatments. These phytoprotective effects are believed to be related to AM-induced biosorption processes that reduce soil metal bioavailability to delay the onset of plant metal toxicity. We also observed that the presence of extraradical hyphae causes a slight alkalinisation of the proximal soil environment whereas roots tended to acidify it, this having significant consequences toward metal bioavailability. Altogether, the AM symbiosis is considered to be a key component of ecosystem function involved in buffering plant growth conditions due to the processes of metal biosorption and hyphal alkalinisation which could contribute in enhancing the soil's resiliency.

1. Introduction

The arbuscular mycorrhizal (AM) symbiosis—a mutually beneficial association between the roots of most herbaceous plants and Glomeromycotan fungi [1]—is primarily recognized for increasing the mineral status of plants via the mycorrhizosphere (i.e., combined surface area of AM roots and extraradical hyphae). It has also been suggested that the mycorrhizosphere plays a key role in the regulation of soil metal bioavailability through biosorption processes, then contributing to the alleviation of plant metal toxicity and nutrient imbalances [2–6]. Recently, we proposed a conceptual model derived from meta-analytical findings depicting the impact of the AM symbiosis on plant metal uptake in relation to soil conditions ranging from low (trace) to high (toxic) metal exposure levels [7–9], a model we further investigated using an *in vitro* carrot root-organ

culture system [10]. From these studies, we demonstrated that the AM fungi play a dual role in metal acquisition: first by increasing nutrient uptake at low metal exposure levels, and then lessening it at high levels through metal biosorption. Notably, in our *in vitro* study [10], we suggested that the "enhanced uptake" and "metal biosorption" processes occur independently in shaping plant metal uptake and, also, likely hold an important role in enhancing soil resiliency despite such metal toxicity conditions. To expand on these notions, we present here an *in vivo* compartmental pot growth system designed to assess the contribution of non-AM roots (rhizosphere), AM roots and extraradical hyphae (mycorrhizosphere), or strictly extraradical hyphae (hyphosphere) on plant growth and Zn uptake, soil Zn bioavailability, and soil-pH. The compartmental pot system was selected to isolate each of these "sphere environments" and subsequently determine their impact on various plant

TABLE 1: Pre-experimental soil characteristics.

Parameter	Unit	Value (SE)	
P*	ppm	20	(0.71)
K[†]	ppm	90.2	(0.86)
Mg[‡]	ppm	214	(5.42)
Organic Matter	%	3.8	(0.25)
Na[†]	ppm	58.3	(7.87)
Ca[†]	ppm	1646	(250)
CEC K	meq/100 g[‡]	0.2	(0)
CEC Mg	meq/100 g	1.8	(0.05)
CEC Ca	meq/100 g	8.2	(1.26)
CEC Na	meq/100 g	0.3	(0.03)
CEC Total	meq/100 g	10.6	(1.36)
Base Saturation K	%	2.3	(0.21)
Base Saturation Mg	%	17.6	(1.50)
Base Saturation Ca	%	78.1	(1.67)
Base Saturation Na	%	2	(0.50)

*$NaHCO_3$ Extractable;
[†]NH_4 Acetate Extractable;
[‡]milli-equivalent per 100 g

physiological and edaphic factors in relation to increasing soil metal exposure levels. In this regard, the "dwarf" sunflower cultivar *Helianthus annuus* L. var. Pacino and the AM fungus *Glomus intraradices* Schenck & Smith (isolate DAOM-181602) were selected as suitable experimental organisms for the study and the essential micronutrient Zn as a typical metal contaminant which is known to reach toxic levels in the environment due to extensive fertilizer application, metal galvanization, and rubber vulcanization [11–13].

2. Materials and Methods

2.1. Experimental Design. Dwarf sunflower plants were grown from seeds (McKenzie Seeds, Brandon, MB, Canada) for 10 weeks in two-compartment pot systems containing a previously autoclaved, low mineral soil mixture (sand:potting soil, 1 : 1 v/v) which was then inoculated (or not) with fungal propagules of *G. intraradices* (Myke Pro Endo, Premier Tech, Rivière-du-Loup, QC, Canada). The fungal inoculum was integrated as a 3 cm thick substrate layer containing 15 propagules g^{-1} dry substrate (200 g inoculum substrate dose having 2750–3000 total propagules *per* pot) in the AM treatments (mycorrhizosphere and hyphosphere plants), whereas the non-AM treatments (rhizosphere plants) received an equivalent volume of the same substrate without any propagules. Each experimental pot system (Figure 1(a)) was composed of a central compartment (7 L capacity) containing soil in which seeds were sown and a surrounding peripheral compartment (7 L capacity) containing the pre-sterilized soil treated with one of the four soil-Zn concentrations (0, 50, 200, 400 mg Zn kg^{-1} dry soil), as determined from a previous study [14]. The two compartments were separated by either a 50 μm or a 2000 μm pore-size nylon filter bag

(Industrial Filter Manufacturing Ltd., Penetanguishene, ON, Canada) which permitted either the proliferation of non-AM roots (Figure 1(b)—rhizosphere—2000 μm), both AM roots and hyphae (Figure 1(c)—mycorrhizosphere—2000 μm), or strictly extraradical hyphae (Figure 1(d)—hyphosphere—50 μm) into the peripheral compartment. In this regard, the 50 μm filter was deemed large enough to allow fungal hyphae to proliferate into the peripheral compartment, but small enough to restrict roots, whereas the 2000 μm filter was permeable to both. The soil-Zn treatments added to the peripheral compartment were achieved by weighing appropriate volumes of the pre-autoclaved soil, adding Zn from a $ZnSO_4 \cdot 7H2O$ stock solution, homogenizing the mixture with an industrial mixer, and air-drying the soil. This soil pretreatment method was used to reduce the likelihood of metal diffusion from the peripheral compartment into the central compartment, compared to other point-source experimental amendment strategies [15]. The factorial design of the study (1 plant *sp.* $\times 3$ sphere treatments (non-AM rhizosphere, AM mycorrhizosphere, or AM hyphosphere) $\times 4$ soil-Zn concentrations (0, 50, 200, or 400 mg kg^{-1} DS) $\times 4$ reps) provided a total of 48 plants, 1 plant *per* pot. Four replicates of unseeded pots were also prepared for each of the soil-Zn treatments to compare the pre- and postexperimental soil-Zn bioavailability ($NaNO_3$ extractable) as well as the pH of the bare soil. The greenhouse conditions were maintained at 25°C/23°C (day/night) with a 16 hour photoperiod under halogen lighting, an average daytime light intensity of 364.2 μmol m^{-2} s^{-1}, and a 65% relative humidity using an Argus greenhouse control system (Argus Control Systems Ltd., White Rock, BC, Canada). Over the course of the experimental period, all plants as well as the unseeded pots were watered daily avoiding any leaching and fertilized biweekly (100 ml *per* week) from weeks 4 to 10 using a low zinc Long-Ashton nutrient solution, pH 4.4 [16]: 2.0 mM K_2SO_4, 4.0 mM $CaCl_2$ anhydride, 1.5 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM $NaH_2PO_4 \cdot H_2O$, 5.0 μM NH_4NO_3, 0.01 mM $MnSO_4 \cdot 4H_2O$, 1.0 μM $CuSO_4 \cdot 5H_2O$, 1.0 μM $ZnSO_4 \cdot 7H_2O$, 0.05 mM H_3BO_3, NaCl 0.09 mM, 0.5 μM $Na_2MoO_4 \cdot 2H_2O$, and 0.1 mM EDTA-Fe.

2.2. Plant Physiological and Chemical Analyses. After the 10-week growth period, plants were harvested, the roots clean-rinsed with tap water, and the length of shoots and number of healthy versus chlorotic (e.g., spotted) leaves recorded. Plants were then partitioned as flowers, shoots (e.g., leaves and stems), and roots, oven-dried at 70°C for 72 h, and weighed separately. To determine plant-Zn concentrations, 100 mg of dried flower, shoot, or root samples were ground and placed in acid-washed Teflon bombs (Nalgene, Rochester, NY, USA). Each sample was dissolved in 2 mL of 16 M HNO_3, heated for 10 h in an 80°C water-bath, and diluted with 20 mL pure grade H_2O (EMD Chemical Inc., Darmstadt, Germany). Five samples of apple leaves (Standard Reference Material #1515) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and five blanks consisting solely of HNO_3 and H_2O were also prepared to ensure the quality and accuracy of the metal analyses.

TABLE 2: Plant growth parameters.

Soil-Zn (mg kg^{-1} DS)	Sphere treatments	Dry mass (g)				Leaves		Shoot height (cm)
		Flowers	Shoots	Roots-CC†	Roots-PC‡	Total no.	% Chlorotic	
0	Hyphosphere	0.86 (0.09)[b]	1.57 (0.43)	0.77 (0.03)[b]	0 (0.0)[b]	14.5 (1.19)	0.76 (0.51)[c]	16.3 (1.84)[b]
	Mycorrhizosphere	1.65 (0.04)[a]	1.98 (0.51)	1.05 (0.21)[a]	0.19 (0.07)[a]	16.25 (0.25)	1.52 (0.78)[c]	18.3 (2.24)[ab]
	Rhizosphere	1.76 (0.19)[a]	2.01 (0.33)	0.98 (0.12)[a]	0.20 (0.04)[a]	18.5 (2.21)	0.00 (0.00)[d]	23.5 (2.9)[a]
50	Hyphosphere	0.82 (0.14)[b]	1.68 (0.11)	0.89 (0.06)[ab]	0 (0.0)[b]	14.3 (0.85)	3.13 (2.00)[b]	17.9 (1.07)[b]
	Mycorrhizosphere	1.58 (0.12)[a]	2.15 (0.32)	1.00 (0.07)[a]	0.19 (0.03)[a]	17.0 (1.14)	4.75 (1.88)[b]	21.89 (1.88)[ab]
	Rhizosphere	1.76 (0.20)[a]	2.24 (0.34)	0.96 (0.04)[a]	0.24 (0.06)[a]	17.5 (2.30)	8.13 (4.18)[b]	23.75 (2.26)[a]
200	Hyphosphere	1.01 (0.12)[b]	1.79 (0.28)	0.68 (0.09)[b]	0 (0.0)[b]	13.8 (0.62)	3.13 (2.00)[b]	16.13 (1.98)[b]
	Mycorrhizosphere	1.35 (0.33)[a]	2.23 (0.17)	0.94 (0.17)[a]	0.18 (0.02)[a]	16.5 (0.64)	27.1 (6.60)[a]	19.1 (2.56)[ab]
	Rhizosphere	1.50 (0.17)[a]	2.08 (0.09)	0.93 (0.04)[a]	0.18 (0.04)[a]	17.0 (2.12)	40.1 (11.6)[a]	22.0 (2.38)[a]
400	Hyphosphere	0.74 (0.08)[b]	1.45 (0.16)	0.61 (0.11)[b]	0 (0.0)[b]	13.5 (1.83)	7.60 (4.63)[b]	15.5 (1.04)[b]
	Mycorrhizosphere	1.51 (0.29)[a]	2.06 (0.15)	0.72 (0.06)[b]	0.24 (0.05)[a]	13.8 (1.03)	36.0 (10.7)[a]	17.8 (1.53)[b]
	Rhizosphere	1.41 (0.31)[a]	1.76 (0.26)	0.68 (0.10)[b]	0.19 (0.02)[a]	14.5 (0.96)	45.6 (10.9)[a]	21.75 (2.67)[a]
F-values and levels of significance								
	Soil-Zn (Zn)	0.63^{ns}	1.31^{ns}	3.63*	0.26^{ns}	2.05^{ns}	78.5***	1.41^{ns}
	Sphere treatments (S)	16.4***	0.14^{ns}	3.33*	37.6***	1.42^{ns}	63.2***	3.5*
	ZnxS	0.73^{ns}	1.27^{ns}	0.85^{ns}	0.95^{ns}	0.70^{ns}	18.3***	0.54^{ns}
	Block	1.28^{ns}	2.04^{ns}	0.85^{ns}	0.11^{ns}	0.58^{ns}	0.35^{ns}	1.41^{ns}

Means ($n = 4$) and SE (inside parentheses) are shown.
Shared letters within each column designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests.
†Central Compartment; ‡Peripheral Compartment.
"ns" nonsignificant; *$P < .05$; **$P < .01$; ***$P < .001$.

All plant samples were analyzed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES—730-ES, Varian Inc., Palo Alto, CA, USA). The instrumental limit of detection calculated from the procedural blanks and reference material was 0.0036 μg g^{-1}.

2.3. Soil Chemical Analyses. The pre-experimental (e.g., prior to Zn treatment) soil characteristics (Table 1) were determined from five 10 g soil samples dissolved in NaHCO$_3$ or NH$_4$ acetate solutions and analyzed using Atomic Absorption Spectrometry (Accutest Laboratories, Ottawa, ON, Canada). Further to these analyses, soil samples for all treatments were also collected after the experimental period from both the central and peripheral compartments at harvest, and the unseeded pots sampled prior to and after the experimental growth period. The soil-pH of these samples was analyzed directly from 5 g of soil suspended in pure grade H$_2$O with a soil:solution ratio of 1 : 2.5 (m/v) [17]. To determine soil-Zn concentrations, 5 g soil from each treatment were suspended in 0.1 M NaNO$_3$ with a soil:solution ratio of 1 : 2.5 (m/v), filtered across an 11 μm grade #1 cellulose filter paper (Whatman Inc., Piscataway, NJ, USA), and analyzed with ICP OES [18]. Five samples of Buffalo River sediment (Standard Reference Material #8704) from NIST along with five blanks consisting solely of 0.1 M NaNO$_3$ were also analyzed to ensure the quality and accuracy of the metal analysis. As with the plant tissue analysis,

TABLE 3: AM root colonization.

Soil-Zn (mg kg^{-1} DS)	Sphere treatments	Fungal structures					
		Hyphae		Vesicles		Arbuscules	
		% Frequency	% Root length	% Frequency	% Root Length	% Frequency	% Root length
0	Hyphosphere	28.5 (1.7)	50.5 (10.9)[a]	21.0 (3.1)	36.5 (8.8)	21.5 (2.9)	26.0 (1.8)[a]
	Mycorrhizosphere	29.0 (6.2)	57.5 (4.8)[a]	25.5 (7.3)	29.0 (3.3)	17.5 (3.2)	30.0 (2.0)[a]
50	Hyphosphere	34.0 (8.6)	56.3 (2.8)[a]	22.0 (2.6)	27.5 (6.3)	27.5 (3.3)	32.5 (2.5)[a]
	Mycorrhizosphere	26.5 (5.6)	30.0 (4.1)[b]	23.5 (3.0)	30.0 (4.1)	13.8 (4.4)	25.0 (2.9)[a]
200	Hyphosphere	22.0 (5.2)	29.5 (10.0)[ab]	19.5 (5.6)	29.5 (5.0)	20.5 (5.1)	28.5 (1.5)[a]
	Mycorrhizosphere	32.5 (3.8)	21.5 (14.5)[ab]	9.5 (3.4)	21.5 (6.5)	18.5 (3.0)	16.3 (2.4)[b]
400	Hyphosphere	30.5 (7.7)	35.0 (6.5)[ab]	22.5 (4.6)	35.0 (6.5)	19.5 (3.9)	25.0 (6.5)[ab]
	Mycorrhizosphere	33.5 (5.2)	30.5 (10.5)[ab]	17.5 (4.9)	20.5 (3.9)	24.5 (5.7)	31.3 (3.1)[a]
	F-values and levels of significance						
	Soil-Zn (Zn)	0.94[ns]	0.28[ns]	2.13[ns]	0.64[ns]	0.17[ns]	1.55[ns]
	Sphere Treatments (S)	0.04[ns]	0.55[ns]	0.67[ns]	3.31[ns]	1.62[ns]	0.98[ns]
	ZnxS	0.27[ns]	3.16*	1.41[ns]	0.86[ns]	1.78[ns]	3.47*
	Block	0.17[ns]	1.63	3.99*	2.44[ns]	0.83[ns]	0.13[ns]

Means ($n = 4$) and SE (inside parentheses) are shown.
Shared letters within each column designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests.
"ns" nonsignificant; $*P < .05$.

TABLE 4: Regression models for soil-Zn as a function of soil-Zn treatment.

Parameter	Compartment	Analysis	Treatment	$f(x)$	F	r^2	df	P
Soil-Zn	Peripheral	Pre-Experimental	Unseeded	$1.015x - 1.45$	5060	0.99	1,14	$<10^{-3}$
		Post-Experimental	Unseeded	$-(4.7^e - 4)x^2 + 0.89x - 1.46$	2573	0.99	1,14	$<10^{-3}$
			Hyphosphere	$-(4.7^e - 4)x^2 + 0.86x - 3.76$	618.8	0.98	2,13	$<10^{-3}$
			Mycorrhizosphere	$-(7.5^e - 4)x^2 + 0.97x - 8.48$	475.9	0.98	2,13	$<10^{-3}$
			Rhizosphere	$-0.001x^2 + 0.91x - 5.20$	249.7	0.97	2,13	$<10^{-3}$
	Central	Pre-Experimental	Unseeded	$(1.0^e - 7)x + 0.06$	0.10	0.01	1,14	.75
		Post-Experimental	Unseeded	$(1.0^e - 7)x + 0.04$	1.29	0.08	1,14	.27
			Hyphosphere	$(1.0^e - 7)x + 0.03$	1.46	0.09	1,14	.25
			Mycorrhizosphere	$(1.0^e - 7)x + 0.03$	1.52	0.10	1,14	.24
			Rhizosphere	$(1.0^e - 7)x + 0.07$	0.01	0.01	1,14	.96

Polynomial equations [$f(x)$], Fisher values [F], coefficients of determination [r^2], degrees of freedom [df], and estimates of P-value are shown.

the instrumental limit of detection calculated from the procedural blanks and reference material was 0.0036 μg g^{-1}.

2.4. Root Colonization Assay. At harvest, 2 g of fresh root samples from each replicate were carefully excised from the root-zone surrounding the tap-root and stained with an aniline blue 0.02% dye solution (6.78 mM aniline blue—500 ml glycerol—450 ml H$_2$Od —50 ml 1% HCl) [19]. Fifty ~1-2 cm long root segments *per* replicate were mounted on slides and examined at 100x and 400x magnification using a compound microscope (CX41, Olympus Inc., Markham, ON, Canada). Samples of non-AM roots were also observed to ensure their non-mycorrhizal status. Mycorrhizal colonization was estimated by determining the % frequency of fungal structures and % length of root colonization as evidenced by the presence of hyphae, vesicles and arbuscules

TABLE 5: Regression models for soil-pH as a function of soil-Zn treatment.

Parameter	Compartment	Analysis	Treatment	$f(x)$	F	r^2	df	P
Soil-pH	Peripheral	Pre-Experimental	Unseeded	$-0.002x + 6.31$	65.5	0.82	1,14	$<10^{-3}$
		Post-Experimental	Unseeded	$(3.8^e - 6)x^2 - (2.8^e - 6)x + 5.78$	205.1	0.97	2,13	$<10^{-3}$
			Hyphosphere	$(3.7^e - 6)x^2 - (2.6^e - 6)x + 5.71$	205.1	0.95	2,13	$<10^{-3}$
			Mycorrhizosphere	$(2.8^e - 6)x^2 - 0.0021x + 5.63$	76.5	0.92	2,13	$<10^{-3}$
			Rhizosphere	$(1.9^e - 6)x^2 - (1.6^e - 6)x + 5.49$	87.9	0.93	2,13	$<10^{-3}$
	Central	Pre-Experimental	Unseeded	$(1.0^e - 4)x + 6.34$	1.72	0.11	1,14	.21
		Post-Experimental	Unseeded	$(1.0^e - 4)x + 5.78$	1.65	0.10	1,14	.25
			Hyphosphere	$(3.7^e - 5)x + 5.59$	0.30	0.02	1,14	.59
			Mycorrhizosphere	$(3.2^e - 5)x + 5.59$	0.24	0.02	1,14	.64
			Rhizosphere	$(2.8^e - 5)x + 5.57$	0.15	0.01	1,14	.71

Polynomial equations [$f(x)$], Fisher values [F], coefficients of determination [r^2], degrees of freedom [df], and estimates of P-value are shown.
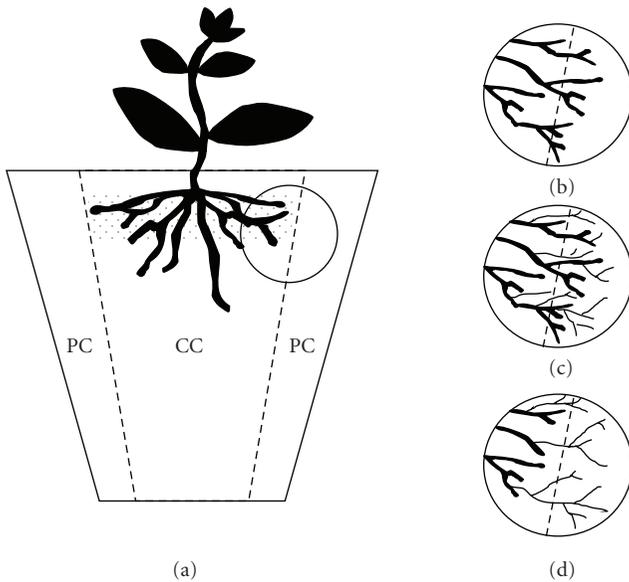

FIGURE 1: Schematization of the compartmental pot system (a) showing the proliferation of hyphae across the dividing filter. The inoculum (or control) substrate layer is shown. Seeds were sown in the central compartment (CC) while the soil-Zn treatments were incorporated in the surrounding peripheral compartment (PC) representing either the rhizosphere (b), mycorrhizosphere (c), or hyphosphere environments (d).

[19, 20]. The equations for the % frequency (1) and % length of root colonization (2) are defined as

$$\frac{\text{No. Segments}_{AM}}{\text{No. Segments}_{Total}} \times 100\%, \qquad (1)$$

$$\frac{\text{Root Length}_{AM}}{\text{Root Length}_{Total}} \times 100\%, \qquad (2)$$

Together, these respective indicators provide insight into the relative distribution and intensity of the AM root colonization [21].

2.5. Statistical Analyses. One- and two-way Analyses of Variance (ANOVA) with Bonferonni and Scheffé studentized range tests were performed for mean comparisons of plant metal uptake, plant growth, and root colonization data whereas univariate regression models were used to calculate the slopes of soil-pH and soil-Zn data [22]. Analyses of Co-Variance (ANCOVA) were performed on the soil-pH and soil-Zn data to compare the slopes and intercepts of the regression equations between the treatments. The Kolmogorov-Schmirnoff and Levene's tests were used to verify the normality of distribution and the homogeneity of residual variance. The data were Log-transformed as required to meet the assumptions of each parametric analysis. All of the Fisher statistics (F), coefficients of determination (r^2), degrees of freedom (df), and P-value estimates were calculated using S-Plus 8.0 statistical software (Insightful Corp., Seattle, WA, USA).

3. Results

Significant differences were observed in flower, shoot, and root Zn concentrations between the hyphosphere, mycorrhizosphere, and rhizosphere treatments with increasing soil-Zn addition (Figures 2(a), 2(b), and 2(c)). Although the plant Zn concentrations did not vary among the treatments at the low soil-Zn levels, the rhizosphere treatments had 20% to 40% higher Zn concentrations in their tissues than the mycorrhizosphere treatments, and 55% to 75% higher concentrations than the hyphosphere treatments at both the 200 and 400 soil-Zn concentrations. As for plant growth (Table 2), the rhizosphere treatments generally had greater flower, shoot, and root dry masses and longer shoots than mycorrhizosphere and especially hyphosphere treatments; however, these plant growth parameters did not show any

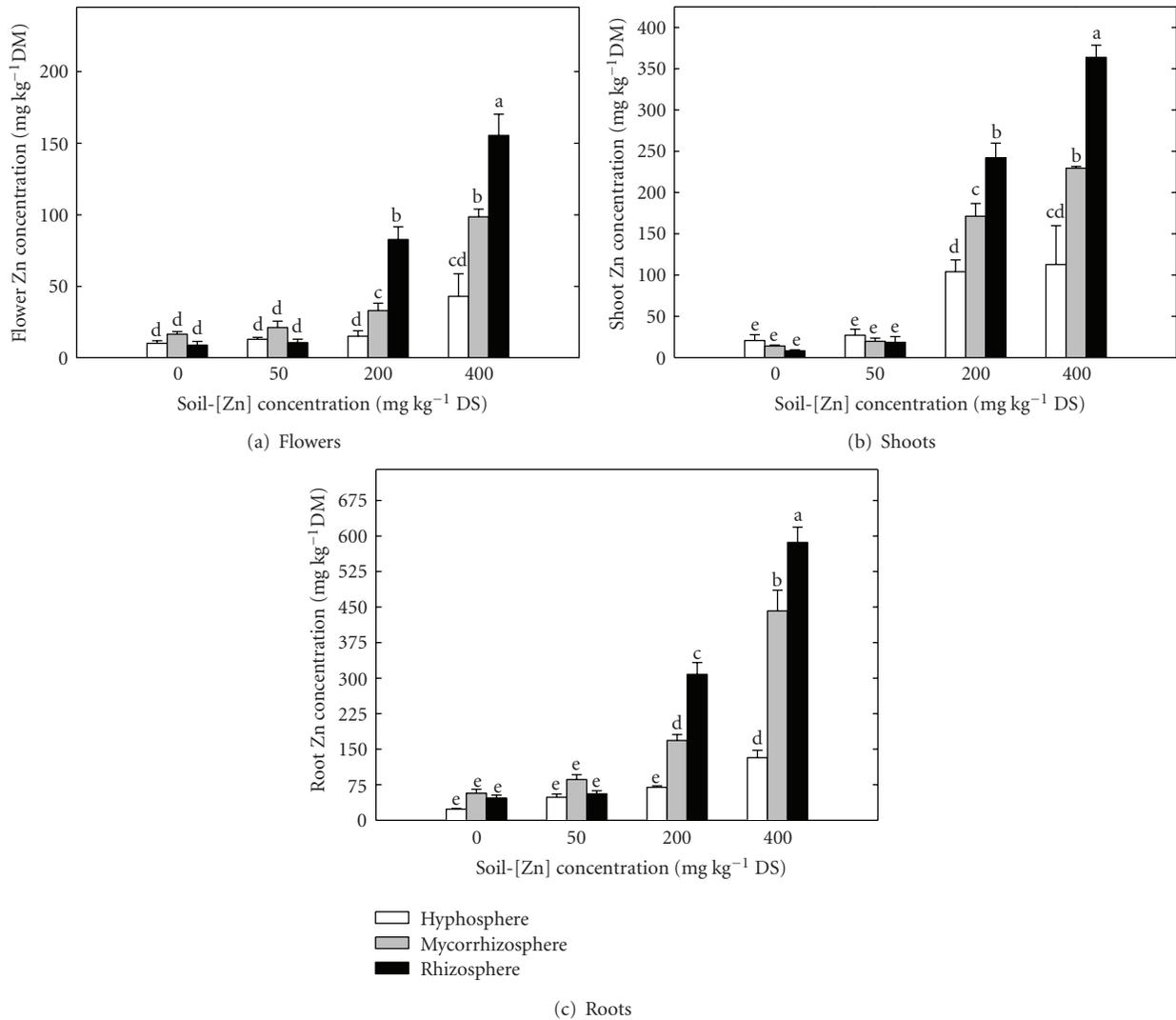(a) Flowers

(b) Shoots

(c) Roots

FIGURE 2: Flower (a), shoot (b), and root Zn concentrations (c). Means ($n = 4$) and standard errors for the rhizosphere (black), mycorrhizosphere (grey), and hyphosphere (white) treatments are shown. Shared letters designate treatments that are not significantly different according to ANOVA coupled with Bonferonni and Scheffé mean comparison tests ($P < .05$).

particular trends in relation to soil-Zn amendment. Still, as a symptom of metal toxicity, rhizosphere treatments had increasingly higher percentages (up to 45.6%) of chlorotic leaves compared to mycorrhizosphere (up to 36.0%) and hyphosphere treatments (up to 7.6%). Moreover, roots collected in the peripheral compartment of rhizosphere and mycorrhizosphere treatments had similar dry masses; meanwhile, it was noted that no roots were found in this compartment among the hyphosphere treatments. All roots from the mycorrhizosphere and hyphosphere treatments were shown to be well colonized as evidenced by the presence of hyphae, vesicles, and arbuscules (Table 3). More specifically, a higher % frequency of hyphae than vesicles and arbuscules was observed among all the treatments, but a slight decline in % hyphal-root length colonized in relation with increasing soil-Zn levels. In addition, rhizosphere treatments were confirmed to be nonmycorrhizal.

The soil-Zn concentrations were measured in the peripheral (Figure 3(a)) and central compartments (Figure 3(b)) and regression models calculated for each treatment (Table 4). The pre-experimental soil-Zn concentration in the peripheral compartment indicated a linear increase in relation with increasing soil-Zn amendment reaching up to 404.1 mg kg^{-1} dry soil. The postexperimental soils indicated polynomial (e.g., hyperbolic) profiles in the descending order of unseeded bare-soil (272.5 mg kg^{-1} dry soil), hyphosphere (263.4), mycorrhizosphere (247.2), and rhizosphere treatments (199.2). By contrast, there were no significant trends (e.g., no slope) for soil-Zn in the central compartment with values ranging between 0.01 and 0.07 mg kg^{-1} dry soil. The soil-pH was also measured in the peripheral (Figure 4(a)) and central compartments (Figure 4(b)) and regression models calculated for each treatment (Table 5).
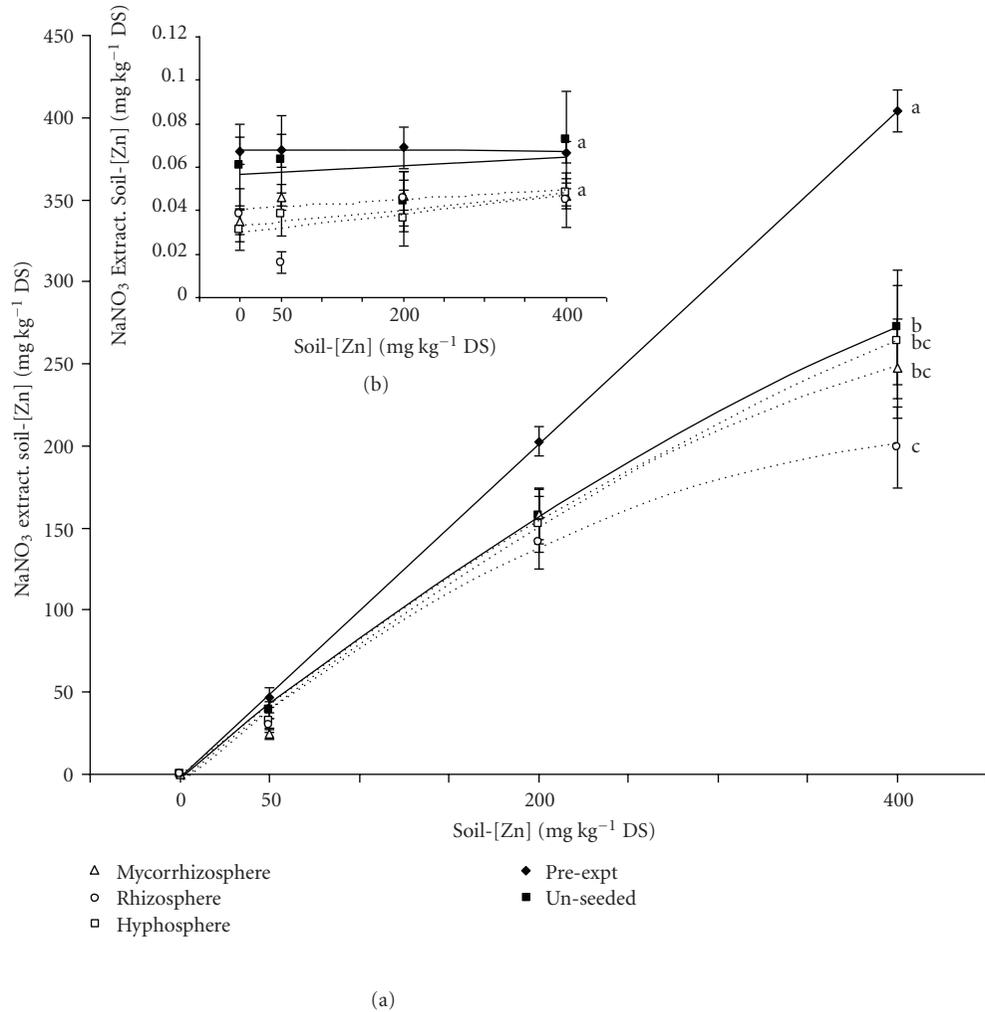
Figure 3: Soil-Zn concentrations measured in the peripheral (a) and central compartments (b). Means ($n = 4$) and standard errors for the pre-experimental unseeded (black diamond), post-experimental unseeded (black square), hyphosphere (white square), mycorrhizosphere (white triangle), and rhizosphere (white circle) treatments are shown. Shared letters designate regression equations having slopes that are not significantly different according to ANCOVA ($P < .05$).

Unlike the soil-Zn concentrations, the pre-experimental soil-pH in the peripheral compartment indicated a linear decline from 6.30 to 5.53 with increasing soil-Zn levels. Meanwhile, the postexperimental soils also indicated significant decreases in pH, but profiles having polynomial trends in the descending order of unseeded bare-soil (from 5.80 to 5.28), hyphosphere (from 5.69 to 5.26), mycorrhizosphere (from 5.59 to 5.23), and rhizosphere treatments (from 5.52 to 5.17). Although there were no significant trends (e.g., no slope) for soil-pH in the central compartment, the range values were gradually lower in the order of pre-experimental unseeded bare-soil (6.32–6.42), postexperimental unseeded bare-soil (5.77–5.83), hyphosphere (5.57–5.62), mycorrhizosphere (5.59–5.61), and then rhizosphere treatments (5.56–5.60).

4. Discussion

Consistent with our conceptual model regarding the impact of AM symbiosis on plant growth and metal uptake [8, 10], the Zn concentrations of plants from the mycorrhizosphere treatments were generally lower than rhizosphere treatments at the highest soil-Zn levels which resulted in AM plants having a lower incidence of metal toxicity (e.g., leaf chlorosis). These findings are in line with other studies that detected lower Cd, Zn, and Pb uptake and an improved growth status in various AM (*Glomus sp.*) versus non-AM plants [23–26]. We attribute such enhanced metal-stress tolerance with AM-induced biosorption processes, including hyphal metal-binding and metal-ligand precipitation [27–29], that reduce metal bioavailability in the mycorrhizosphere to decrease plant metal uptake [3, 5, 6]. Correspondingly, further studies have shown that metals can be taken up and sequestered in fungal tissues instead of being transferred to roots [4, 30, 31], thereby reducing cellular oxidative stress
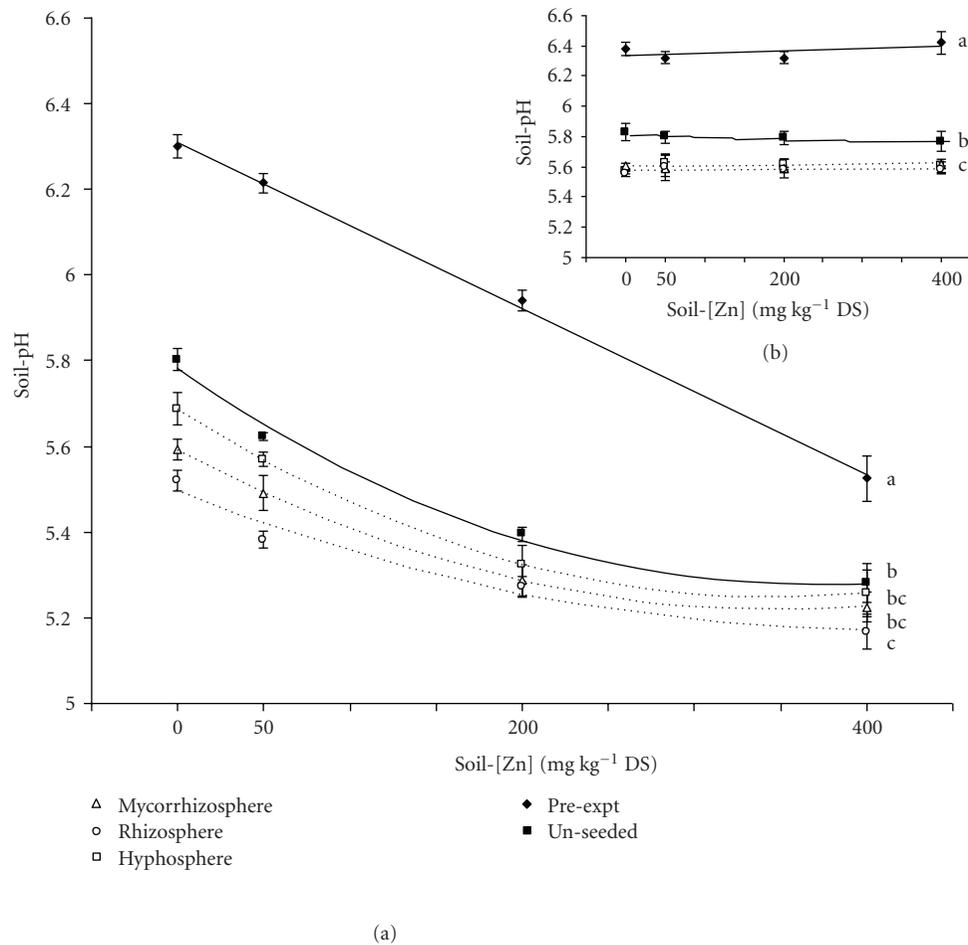
(b)

(a)

FIGURE 4: Soil-pH measured in the peripheral (a) and central compartments (b). Means ($n = 4$) and standard errors for the pre-experimental unseeded (black diamond), post-experimental unseeded (black square), hyphosphere (white square), mycorrhizosphere (white triangle), and rhizosphere (white circle) treatments are shown. Shared letters designate regression equations having slopes that are not significantly different according to ANCOVA ($P < .05$).

and delaying the onset of plant metal toxicity [32]. For these reasons, mycorrhizal biosorption and hyphal sequestration are considered to be important stress avoidance strategies that protect host plants in complement to their intrinsic detoxification mechanisms, such as metallotheinin and phytochelatin metabolisms [33]. In addition to comparing the metal uptake profiles of AM versus non-AM plants, our two-compartment pot system was specifically designed to determine the relative contributions of extraradical hyphae to host plant Zn uptake. Accordingly, in spite of having significantly lower Zn concentration levels than the rhizosphere and mycorrhizosphere treatments, plants of the hyphosphere treatments still had increasingly higher Zn concentrations even at the highest soil-Zn treatments. In agreement with our *in vitro* study [10], this finding indicates that hyphal metal uptake and its transfer to roots can contribute in increasing plant metal status despite the biosorption effect which typically reduces it, as in the case of other metals such as Ni, Cr, Cs, and Pb [34–37]. For this reason, we believe that the enhanced uptake and biosorption processes occur independently: the first increasing the absorptive capacity of

roots and the second regulating the bioavailability of metals in soils. Thus, it is their combined effects that shape the host plant's metal uptake profile.

Contrary to our conceptual model, we did not detect any significant differences in plant Zn uptake nor any major symptoms of nutrient deficiency among plants of the 0-control and 50 soil-Zn levels. In this case, these soil-Zn treatments likely did not impose any perceptible nutrient deficiency stress or growth limitations which can otherwise lead plants to invest more in AM symbiosis to supplement their nutritional status [38, 39]. This can be attributed to the pre-experimental soil-Zn levels and fertilization regimes used in our study which likely provided sufficient Zn for an optimal plant growth among these treatments. As such, further experimental investigation may be needed to verify this facet of our proposed model which proposed that the AM symbiosis would enhance plant metal uptake to circumvent deficiency conditions. Still, we did observe the general trends of slightly lower dry masses, fewer leaves, and shorter shoots among the mycorrhizosphere and especially the hyphosphere treatments compared to the rhizosphere treatments, which

may have resulted from the metabolic cost of maintaining the symbiotic association through the transfer of plant carbohydrates to the AM fungus [40]. Corresponding with the considerable levels of AM root colonization reported here (e.g., as evidenced by the high frequency and distribution of fungal hyphae, arbuscules, and vesicles), the reduction in AM than non-AM plant biomass could be associated with the host plants' carbon allocation in developing the mycorrhizospheric infrastructure. Alternatively, this tendency could also be attributed, in part, to the design of our compartmental pot systems, especially among the hyphosphere treatments. In this case, the $50\,\mu m$ filter bags themselves may have limited the "rootable" volume of the hyphosphere plants to then reduce their overall growth [41]. Nevertheless, the similar levels of AM root colonization and abundance of all fungal structures among both the mycorrhizosphere and hyphosphere treatments would suggest that the symbiotic investment was similar between these treatments, and that the effect of our experimental design on plant growth and metal uptake was negligible.

As for the soil conditions, the addition of Zn in the form of $ZnSO_4$ caused a linear decrease of the pre-experimental soil-pH which was likely prompted by the proportional increase of SO_4^{2-} in the soil solution [24]. Furthermore, the daily watering and fertilization regimes also affected the soil conditions such that the soil-pH and soil-Zn levels all showed polynomial rather than linear profiles. Over time, the influx of SO_4^{2-} and H^+/H_3O^+ ions in the soil solution associated with the addition of Zn and water alters the soil's redox equilibrium and impacts the solubility of metal nutrients [42–45]: a process referred to as "metal ageing" [46]. Such inputs and metal ageing processes can detrimentally influence the soil's metal-binding capacity, resulting in an increased rate of metal leaching [45, 47, 48]. For these reasons, it is noteworthy that the presence of roots and (or) extraradical hyphae further affected soil conditions in a way that the soil-pH was increasingly more acidic and the soil-Zn bioavailability was gradually lower in the order of hyphosphere, mycorrhizosphere, and rhizosphere treatments. These tendencies are primarily attributable to the different rates of Zn uptake between the treatments and the subsequently different soil-Zn depletion zones. Notwithstanding, the exudation of organic chelators by roots and (or) extraradical hyphae may also have played a part in shaping the edaphic conditions, as in the case of phosphorus and nitrogen acquisition by mycorrhizae [49–53]. From these studies, it has been suggested that the extraradical hyphae can induce the moderate alkalinisation of their growth substrate in relation to P and N uptake whereas roots tend to acidify it. This could be relevant to our proposed conceptual model of AM-plant metal uptake since the process of hyphal alkalisation could favour metal biosorption to contribute in reducing metal bioavailability, unlike root acidification that may facilitate leaching and then increase metal solubility. Accordingly, we consider it intriguing for future experimental investigations to examine these mechanisms more closely (e.g., metal biosorption and hyphal alkalinisation) within the context of plant metal uptake in order to further elucidate the impact of the extraradical hyphae on soil metal bioavailability. Altogether, when taking into account the impact of the AM symbiosis in stabilizing the soil matrix and enhancing its water and nutrient retention capacity [54–56], the mycorrhizosphere should play a key role in enhancing the soil's resiliency in relation to metal stress conditions ranging from trace to toxicity exposure levels.

In conclusion and in line with our hypothetical model, we demonstrated that the AM symbiosis plays an important part in shaping host-plant Zn uptake, particularly by reducing plant tissue Zn concentrations at high exposure levels and thereby delaying the onset of Zn toxicity in shoots and flowers compared to non-AM plants. Accordingly, we also suggested that the mycorrhizosphere plays an equally important role in shaping the soil environment via biosorption processes and other AM-induced chemical changes which contribute in enhancing the soil's resiliency. As such, we consider the AM symbiosis to be an essential component of extrinsic stress tolerance and a key factor of ecosystem function. Meanwhile, we also consider our model depicting the impact of the AM symbiosis on plant and soil systems to be a relevant tool in environmental remediation practices by conceptually determining the fate of metal contaminants in relation with the activities of plants and soil microorganisms.

Abbreviations

AM: Arbuscular mycorrhiza
ICP OES: Inductively coupled plasma optical emission spectrometry
Zn: Zinc.

Acknowledgments

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