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**Getting to the Roots of Plant Metal Stress Tolerance
Examining the Role of the AM Symbiosis in Plant Metal Uptake and Soil Metal Bioavailability**

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Getting to the roots of plant metal stress tolerance

**Examining the role of the AM symbiosis in
plant metal uptake and
soil metal bioavailability**

Patrick J. Audet

**A thesis submitted to
The Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy**

**Ottawa-Carleton Institute of Biology
University of Ottawa**

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“I’m a bit run down, but I’m okay”

– Ron Sexsmith, Hard Bargain

Abstract

This doctoral thesis investigated the impact of the arbuscular mycorrhizal (AM) symbiosis, an ubiquitous and beneficial association between plant roots and soil fungi, toward plant growth, stress tolerance, and metal uptake in relation to extrinsic metal conditions ranging from low (*e.g.* trace) to high (*e.g.* toxic) exposure levels. The investigative strategy is divided into two main parts: (1) statistical meta-analysis and (2) experimental analysis. In the first part, an extensive literature review in the field of metal phytoremediation was conducted in order to construct a meta-dataset consisting of various plant physiological and soil ecological parameters which were ultimately extracted from nearly 30 published works. Meta-analytical statistical tools were then used to examine general trends and perspectives in metal phytoextraction and metal stress tolerance (Chapter 2), to establish an inherent role for the AM symbiosis therein (Chapter 3), and to discuss the potential for plant investment in symbiotic associations as an extrinsic stress tolerance strategy in complement to the plant's intrinsic stress resistance mechanisms (Chapter 4). From these findings, a series of conceptual models were proposed depicting the plant growth and metal uptake in relation to increasing metal exposure levels by integrating the primary AM-induced mechanisms of 'enhanced uptake' and 'metal biosorption'. In the second part, *in vitro* root-organ (Chapter 5) and greenhouse culture systems (Chapters 6 and 7) were designed using the micronutrient zinc (Zn) as a typical metal contaminant to test various parameters of AM-plant growth and metal uptake, for which the proposed conceptual models were used as a framework for developing new

hypotheses regarding plant-soil interactions. The methods and analytical techniques included Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for the determination of soil- and plant-Zn concentrations, differential root staining and microscopic analysis for the assessment of AM-root colonization, and standard physiological metrics for the determination of plant health status. In brief, I showed that the AM symbiosis plays a dynamic role in plant development and stress tolerance first by enhancing the uptake of limiting metal nutrients, and then by reducing the uptake of potentially toxic metal contaminants due to metal biosorption under metal toxicity conditions. Accordingly, I also suggested that such mechanisms contribute in buffering the proximal growth environment, and thereby increasing soil's resiliency. Altogether, I consider the revised models depicting the impact of the AM symbiosis on plant and soil systems to be a relevant tool in environmental remediation practices.

Résumé

Cette thèse de doctorat visait à étudier l'impact de la symbiose mycorhizienne arbusculaire (MA), association mutualiste entre des champignons et les racines des plantes, sur la croissance des plantes, leur tolérance au stress et leur accumulation de métaux lorsque soumises à de basses (traces) jusqu' à de hautes (en excès) concentrations. Notre approche a été conçue en deux parties : (1) des méta-analyses statistiques et (2) des analyses expérimentales. Dans la première partie, nous avons réalisé une revue de la littérature dans le domaine de la phytoremédiation des métaux afin de développer une banque de données comportant plusieurs paramètres sur la physiologie des plantes et l'écologie des sols à partir d'une trentaine d'études scientifiques. À l'aide de méthodes méta-statistiques, nous avons identifié diverses tendances et perspectives par rapport à la phytoextraction des métaux et à la tolérance des plantes au stress (Chapitre 2), établi et intégré des rôles potentiels de la symbiose MA dans cette perspective (Chapitre 3), et discuté de la probabilité de l'investissement des plantes dans la symbiose mycorhizienne en temps que stratégie extrinsèque pouvant ainsi compléter les mécanismes de résistance intrinsèque des plantes (Chapitre 4). À partir de ces résultats, nous avons proposé des modèles conceptuels afin de décrire les effets de la symbiose MA sur la croissance des plantes et l'accumulation des métaux ; des modèles axés principalement sur des processus mycorhiziens de «l'absorption améliorée» et de «la biosorption des métaux». Dans la deuxième partie, des systèmes de culture in vitro des champignons MA sur racines excisées (Chapitre 5) ainsi que des systèmes de culture en serre (Chapitres 6 et 7) ont

ete conçus en utilisant le zinc (Zn) comme contaminant métallique typique afin d'étudier divers aspects de l'impact des champignons MA sur la croissance des plantes et leur accumulation de métaux. Plus précisément, les modèles conceptuels proposés dans notre première partie ont été utilisés pour énoncer de nouvelles hypothèses quant aux interactions entre les plantes et le milieu édaphique. À cet égard, nos méthodes et techniques ont inclus l'analyse 'ICP-OES' pour la détermination des teneurs en Zn dans les substrats et les tissus végétaux, la coloration des racines suivie par leur observation au microscope pour la détermination des taux de colonisation, et l'analyse de paramètres physiologiques pour l'évaluation de l'état de santé des plantes. En bref, nous avons démontré que la symbiose MA joue un rôle dynamique dans le développement et la tolérance des plantes grâce au mécanisme «d'absorption améliorée» qui contribue à supplémer l'absorption de métaux essentiels en conditions limitantes, mais qui réduit cette absorption en conditions toxiques en raison de «la biosorption des métaux». Nous suggérons donc que de tels processus contribuent à équilibrer les milieux de croissance, améliorant ainsi leur résilience. En conclusion, nous considérons que nos modèles conceptuels qui décrivent l'impact de la symbiose MA sur la croissance des plantes et le fonctionnement des milieux pourraient s'avérer fort utiles dans l'aménagement d'écosystèmes contaminés

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Section I

Introduction

Chapter 1

General objectives and research strategy

1.1 Metals in the environment

As is the case for all living organisms, plants require mineral nutrients for growth and development. Depending on the plants' relative growth requirements, mineral nutrients are referred to as either macro- or micronutrients (Marschner 1995). Alternatively, macro- and micronutrients can also be classified according to their physicochemical properties to define them respectively as either non-metals (nitrogen, sulfur, phosphorus, boron, chlorine) which have a negative valence, or metals (potassium, calcium, magnesium, iron, manganese, zinc, copper, molybdenum, nickel) which have a positive valence. While some non-metals are often required in high quantities as structural components of proteins, carbohydrates and lipids, metals are required in much lower (even trace) quantities due to their primary role as enzyme activation cofactors and prosthetic constituents (Foy et al. 1978; Larcher 2003). Consequently, metals are considered to be no-less critically involved in plant growth and development than non-metals. Soil metal bioavailability (*i.e.* referring to the physicochemical status of metals in which they are readily taken up and internalized by living organisms) is determined by many factors including the initial bulk soil structure (*i.e.* soil that is not penetrated by roots), its cation exchange capacity (*i.e.* the capacity for ion exchange between the soil and soil-solution; used as a measure of nutrient retention), pH (*i.e.* relative measure of metal solubility), mineral content, and composition of soil microorganisms (Dean 2009; McBride 1994). As

such, the soil metal bioavailability can span a wide exposure range (from trace to toxic levels) depending on various environmental and anthropogenic factors. In particular, metal deficiency conditions can arise as a result of plant nutrient depletion associated with intensive agricultural practices, leaching due to flooding and erosion, and (or) reciprocal antagonisms caused by soil metal imbalances (Altieri 1999, Baligar et al 2001, Vitousek 1982). By contrast, metal toxicity conditions can arise as a result of extensive agricultural biosolid application (*e.g.* application of treated sewage sludge), metal galvanization, rubber vulcanization, and other industrial processes leading to an influx of metal rich waste effluents into the environment (Bradl 2005, Prasad 2001).

As illustrated by Marschner (1995), plants respond to metals in the environment in a ubiquitous manner showing a symmetrical growth pattern across the entire metal exposure range (Fig 1.1). When metal bioavailability and total metal uptake (*i.e.* referring to metal content in plant tissues) are low, visible symptoms of plant nutrient deficiency typically occur which result in critical developmental challenges (Table 1.1). Similarly, when metal bioavailability and total metal uptake are high, non-specific symptoms of toxicity (*e.g.* tissue chlorosis and necrosis) can arise and incur severe growth challenges. Between these critical deficiency and toxicity ranges, the metal bioavailability and metal uptake are considered 'non-limiting' resulting in optimal plant growth (90-95% of maximal relative growth). This view of plant development is consistent with Kirk's (2002) depiction of metal uptake in relation to soil metal exposure ranging from deficiency to toxicity conditions (Fig 1.2). Here, plant metal uptake increases linearly in relation to increasing soil metal

bioavailability until reaching a plateau when the roots attain their maximum relative uptake capacity. Ultimately, the uptake and overall plant growth both decline due to the physiological burden associated with high soil metal exposure, for instance cellular oxidative stress, fluctuations in soil-pH, and soil nutrient imbalances (Christie et al 2004, Schützendübel and Polle 2002). Despite these considerable challenges, plants have developed key physiological adaptations such as phytochelation and metallothionein metabolisms enabling them to effectively mobilize, take up, and sequester metals (Cobbett 2001, Cobbett and Goldsbrough 2002). From an environmental perspective, these characteristics can contribute to the mitigation of industrial and agricultural pollution referring to phytoremediation (gr. plant-remedy). Consisting in the combined processes of phyto-extraction, -stabilization, -degradation, and (or) volatilization (Fig. 1.3), phytoremediation has gained acceptance as a cost-effective and non-invasive technology for reducing the persistent toxic effects of organic and inorganic pollutants (Chaney et al 1997, Meagher 2000, Pilon-Smits 2005, Salt et al 1995, 1998). In this PhD thesis, we used meta-analysis as a quantitative literature review method to evaluate trends in metal phytoremediation (particularly phytoextraction) among a wide array of essential and non-essential metals (Section II). Using the essential micronutrient Zn as a model of metal contaminants (Barceloux 2001, Chaney 1999), we then conducted targeted experimental studies to further assess plant adaptive strategies regarding metal uptake and stress tolerance under *in vitro* root-organ culture and whole-plant greenhouse conditions (Section III). As emphasized throughout this body of work, we believe that soil microorganisms play an integral role in plant metal stress tolerance and also contribute in enhancing soil

resiliency when faced with soil metal exposure levels ranging from trace to toxicity conditions.

Table 1.1 Symptoms of deficiency and toxicity in herbs and broadleaved woody plants, From Foy et al. 1978, Larcher (2003), and Marschner (1995)

Mineral nutrient	<i>Symptoms of deficiency</i>
N	Stunting (dwarfism), scleromorphism, decrease in shoot/root ratio, premature yellowing of old leaves
P	Disturbance of reproductive processes (delayed flowering), spindly appearances, dark green or bronze-violet discoloration of leaves and stalks
S	Similar to N deficiency, intercostal chlorosis of young leaves
K	Disturbed water relations (tip drying), curling of edges of older leaves
Ca	Disturbance in growth by division (small cells), tip drying, leaf deformation, impaired root growth
Mg	Stunted growth, intercostal chlorosis of older leaves
Fe	Straw yellow intercostal chlorosis, in extreme cases young leaves turn white (veins green), apical bud formation suppressed
Mn	Inhibition of growth, chlorosis and necroses on young leaves
Zn	Stunted growth, white-green discoloration of older leaves, disturbances in fructification
Cu	Tip drying, leaves curl, spotty chlorosis of young leaves
B	Impaired growth (meristem necroses), diminished root broanching, phloem necroses, disturbances in fructification
Mineral nutrient	<i>Symptoms of toxicity</i>
Mn	Spotty chlorosis of older leaves
B	Tip necrosis and marginal scorching
Non-specific	Stunting, decrease in shoot/root ratio, impaired root growth, general necrosis and chlorosis

Figure 1.1 Conceptual model of relative plant growth as a function of extrinsic metal exposure ranging from trace to toxicity levels (From Marschner 1995).

Figure 1.1

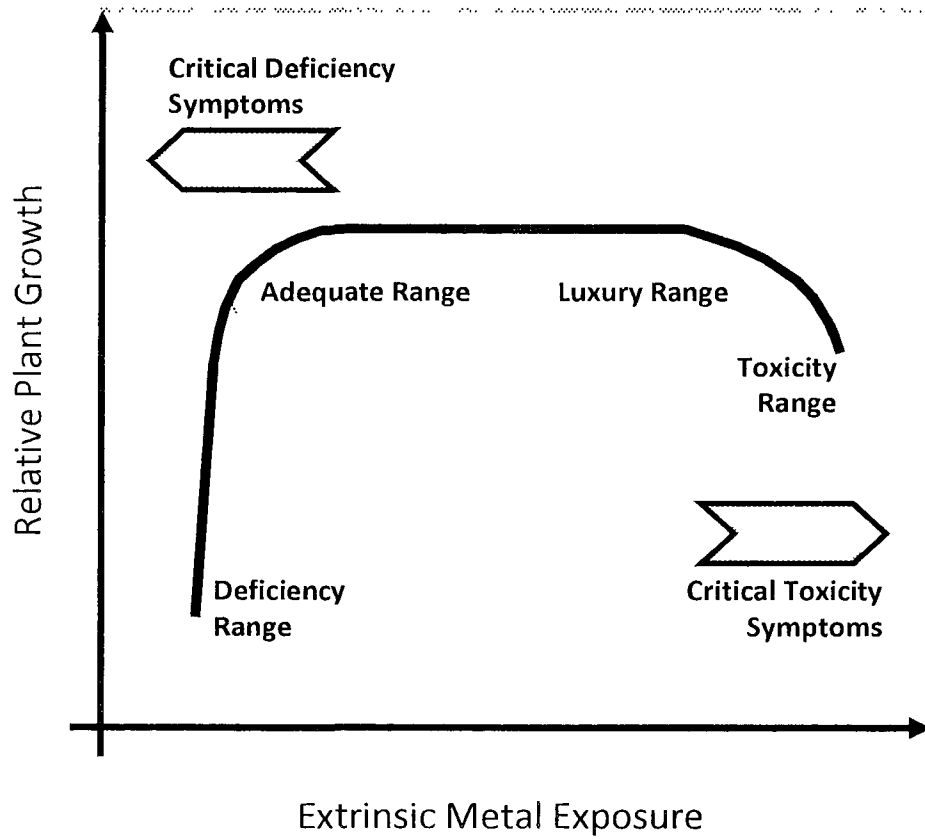


Figure 1.2 Conceptual model of plant metal uptake as a function of extrinsic metal exposure ranging from trace to toxicity levels (From Kirk 2002).

Figure 1.2

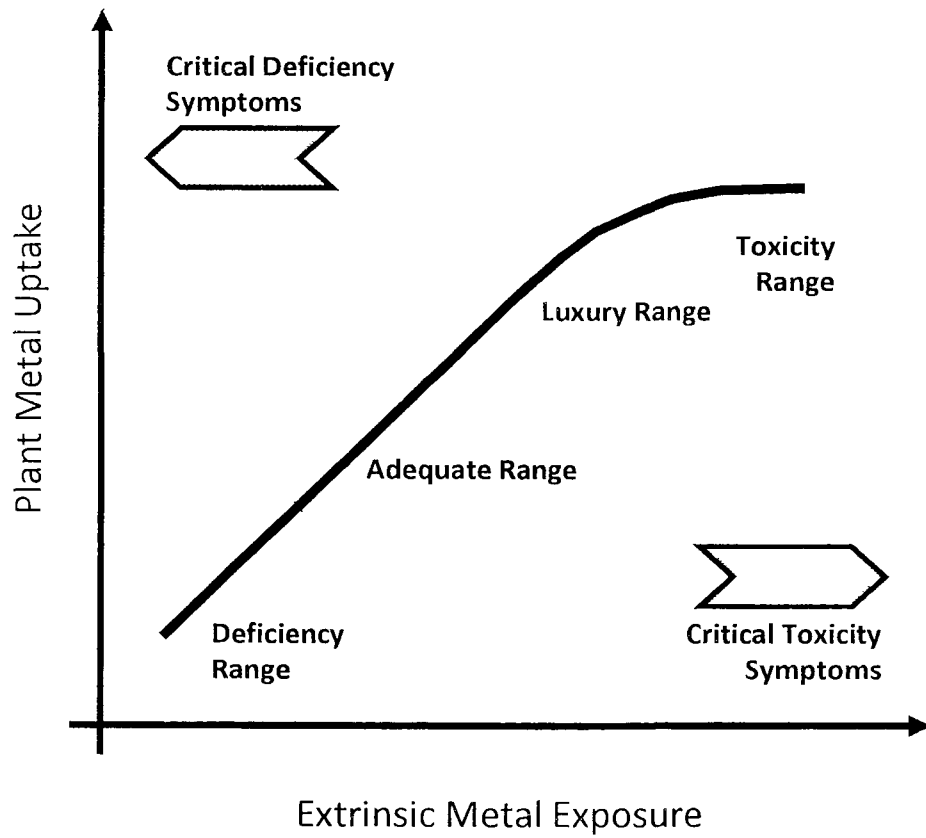
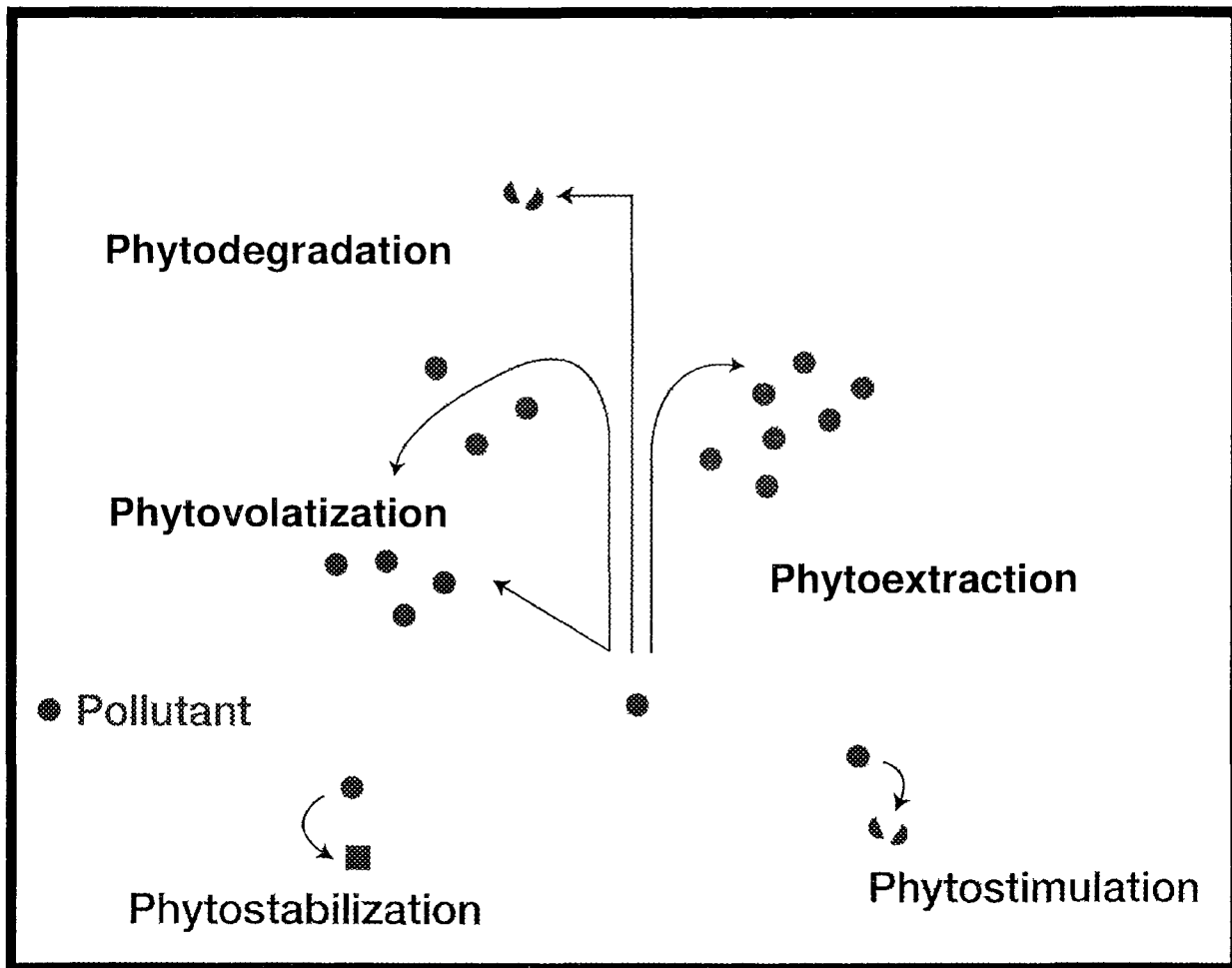


Figure 1.3 Possible fate of environmental pollutants during phytoremediation: the pollutant (represented by red circles) can be stabilized or degraded in the rhizosphere, sequestered or degraded inside the plant tissue, or volatilized (From Pilon-Smits 2005).

Figure 1.3



1.2 AM symbiosis

Aptly referred to as “fungus-roots” (Frank 1885), the mycorrhizae are mostly non-pathogenic soil fungi living intimately with terrestrial plant roots (Kirk et al. 2001). Together, the roots and fungi form a symbiotic mutualism characterized by the formation of the mycorrhizosphere (*i.e.* combined roots and extraradical hyphae – Fig. 1.4) which enables the increased access to soil resources (e.g. mineral nutrients and water) in exchange for plant carbohydrates (Allen 1991; Harley and Smith 1983). The mycorrhizae are ubiquitous organisms having adapted to and successfully colonized nearly all known terrestrial ecosystems (Peterson et al. 2004), and are classified into three primary assemblages (Fig. 1.5) depending on their respective morphology, life history strategy, and specific plant host: the ectomycorrhizae (associated with Pinaceae, Fagaceae, Betulaceae, and Salicaceae species), the endomycorrhizae (associated with the majority of angiosperms and some gymnosperms), and the ectendomycorrhizae (associated with Orchidaceae and Ericaceae species). Falling within the class of the endomycorrhizae, the present work focuses on the arbuscular mycorrhizal (AM) fungi and their symbiosis with herbaceous plants. Having originated over 450 million years ago, the AM fungi comprise species of the Glomeromycotan phylum which are believed to form associations with up to 90% of all herbaceous plants (Remy et al. 1994; Schüßler et al. 2001). In this regard, the AM fungi are well studied in the field of plant physiology and mycology, and widely recognized for benefitting their hosts when subjected to various environmental stress factors (Table 1.2). As typified by a unique morphology consisting of intra- and extraradical hyphae, arbuscules and vesicles (Fig. 1.6), the AM fungi are peculiar compared to other

mycorrhizal phyla in that they penetrate the cortical cells of vascular plant roots in order to develop an intercellular exchange network. They are considered 'true' mutualists due to their 'host obligate' status which requires that they maintain an active symbiosis in order to complete their life cycle (Johnson et al. 1997; Jones and Smith 2004).

Over the past decades, numerous advances have been made demonstrating the beneficial role of AM fungi in plant physiology and soil ecology (Allen 1991; Harley and Smith 1993), particularly relating to the dual mycorrhizospheric processes of 'enhanced uptake' and 'metal biosorption'. Here, 'enhanced uptake' refers to the role of extraradical hyphae in actively increasing the root absorptive capacity by forming the mycorrhizosphere which actively increases resource acquisition compared to the rhizosphere alone (Fig. 1.7). This mechanism is most widely recognized for preferentially enhancing plant nutrient use efficiency to circumvent the challenges of soil metal deficiency (Jeffries et al. 2003; Koide 2006; Tillman et al. 2002). By contrast, 'metal biosorption' refers to the regulation of soil metal bioavailability in the mycorrhizosphere due to its enhanced metal-binding capabilities (Fig. 1.8) (Leyval et al. 1997). In this case, extraradical hyphae have been shown to bind metal ions directly to their negatively charged surface constituents (*e.g.* carboxyls, hydroxides, oxy-hydroxides, sulfhydryls) and exude organic chelators which can precipitate metal-ligands in the soil solution (Apak 2002; Gadd 1993; Gonzalez-Chavez et al. 2002). Together, these processes contribute in reducing soil metal bioavailability to decrease plant metal uptake and potentially delay the onset of metal phytotoxicity. As such, the AM fungi contribute to metal phytoremediation by protecting plants from metal

toxicity and buffering the proximal soil environment by enhancing the process of metal phytostabilization

Further to the objectives described in Chapter 1 1, the meta-analytical and experimental components in this thesis were used to investigate the impact of the AM symbiosis in plant metal uptake, stress tolerance and soil resiliency particularly relating to the processes of 'enhanced uptake' and 'metal biosorption' In this regard, the 'enhanced uptake' hypothesis predicts that AM plants have greater metal uptake than non-AM plants, potentially leading to increased metal toxicity when metal exposure is high On the other hand, the 'metal biosorption' hypothesis predicts that AM plants have lower metal uptake than non-AM plants, then delaying metal toxicity under these conditions By specifically testing these hypotheses, I aim to outline the dynamic role of AM fungi in ecosystem function by highlighting its impact on agricultural and phytoremediation management practices in relation to metal exposure conditions ranging from trace to toxic levels

Table 1.2 Summary of the impact of AM symbiosis on plant physiology and soil ecology

Mechanism	Target	Description	Reference
Enhanced Resource Acquisition Capability	Essential soil resources (Non-metals, metals, and water)	Preferential uptake of nitrogen (NO ₃ /NH ₄) and phosphorus (P _i)	Bolan (1991)*, Chapman et al (2006)*, Mosse (1973)*
		Mobilization and uptake of trace essential elements having low bioavailability (i.e.), particularly under nutrient deficiency conditions	Jeffries et al 2003*, Koide (1990)*, Marschner & Dell (1994)*
		Enhanced water use efficiency and drought-recovery	Auge et al (2001), Auge (2002)*, Subramanian and Charest 2009*
Soil Structure Stabilization	Metal Bioavailability	Metal-binding due to negatively charged surface constituents of extraradical hyphae (i.e. hydroxides, oxy-hydroxides, sulfhydryls), Reduction of plant metal uptake to delay phytotoxicity, particularly at high soil exposure levels (i.e. Zn, Pb, Cd, Ni)	Leyval et al (1997)*, Galli et al (1994)*, Gadd (1993)*
	Soil Retention Capacity	Enhanced soil aggregation properties, increased water and nutrient retention capacity, decrease of nutrient leaching	Auge et al (2001), Auge (2002)*, Bearden (2001, 2002), Miller & Jastrow (1990)
Mycorrhizal Exudation	Soil Microbia	Promotion of beneficial bacteria (i.e. nitrogen fixation), competitive exclusion of soil pathogens	Chapman et al (2006)*, Barea et al (1995)*, Johanson et al (2004)*
	Metal Bioavailability	Precipitation of metal-ligands, modulation of soil-pH	Leyval et al (1997)*, Galli et al (1994)*, Gadd (1993)*

*Denotes review publications

Figure 1.4 Schematization of the rhizosphere, mycorrhizosphere, and rhizosphere.

Figure 1.4

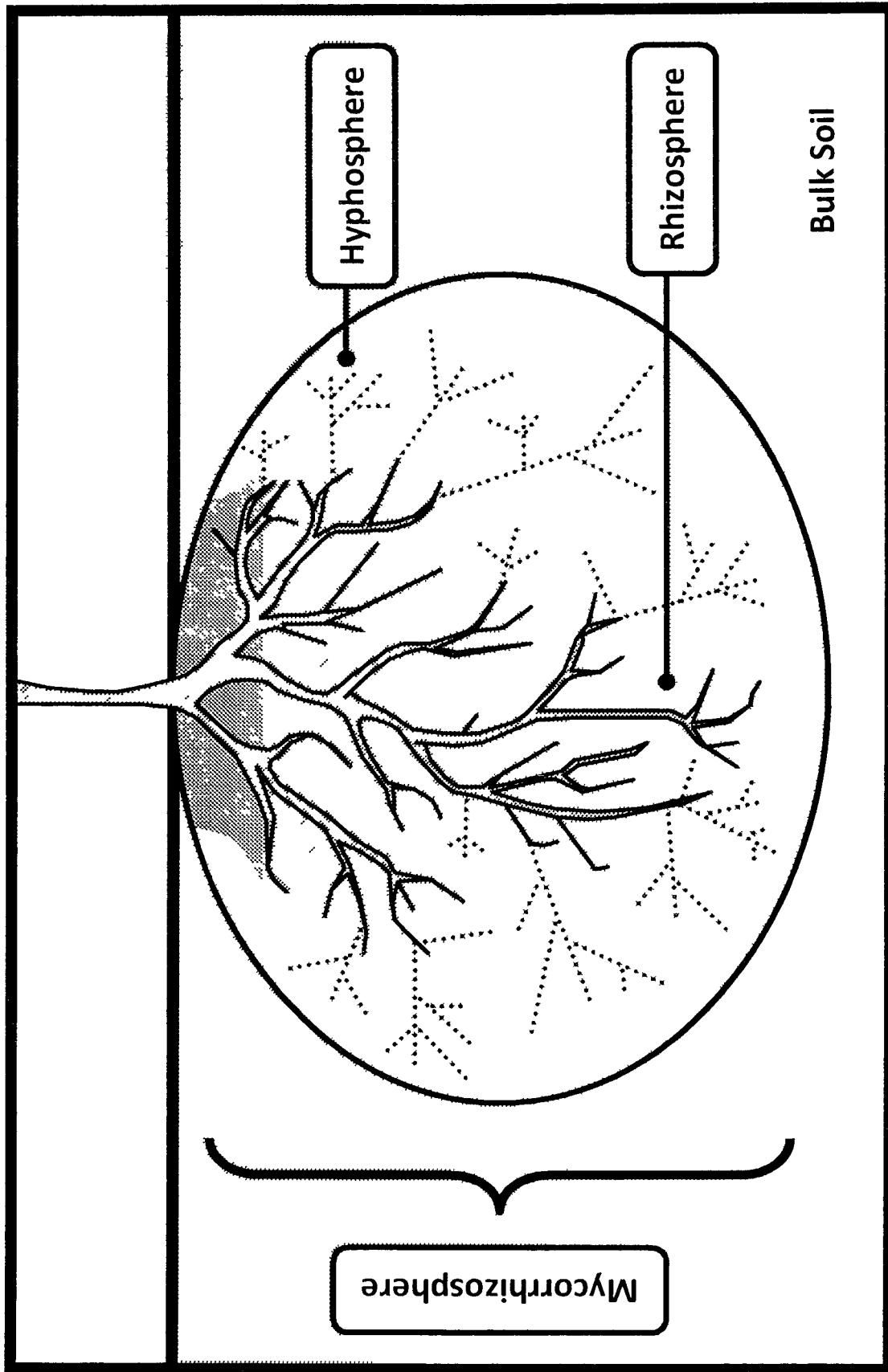


Figure 1.5 Root transect schematization showing ectomycorrhizae (a), endomycorrhizae (b), and ectendomycorrhizae (c). From Furlan (1995).

Figure 1.5

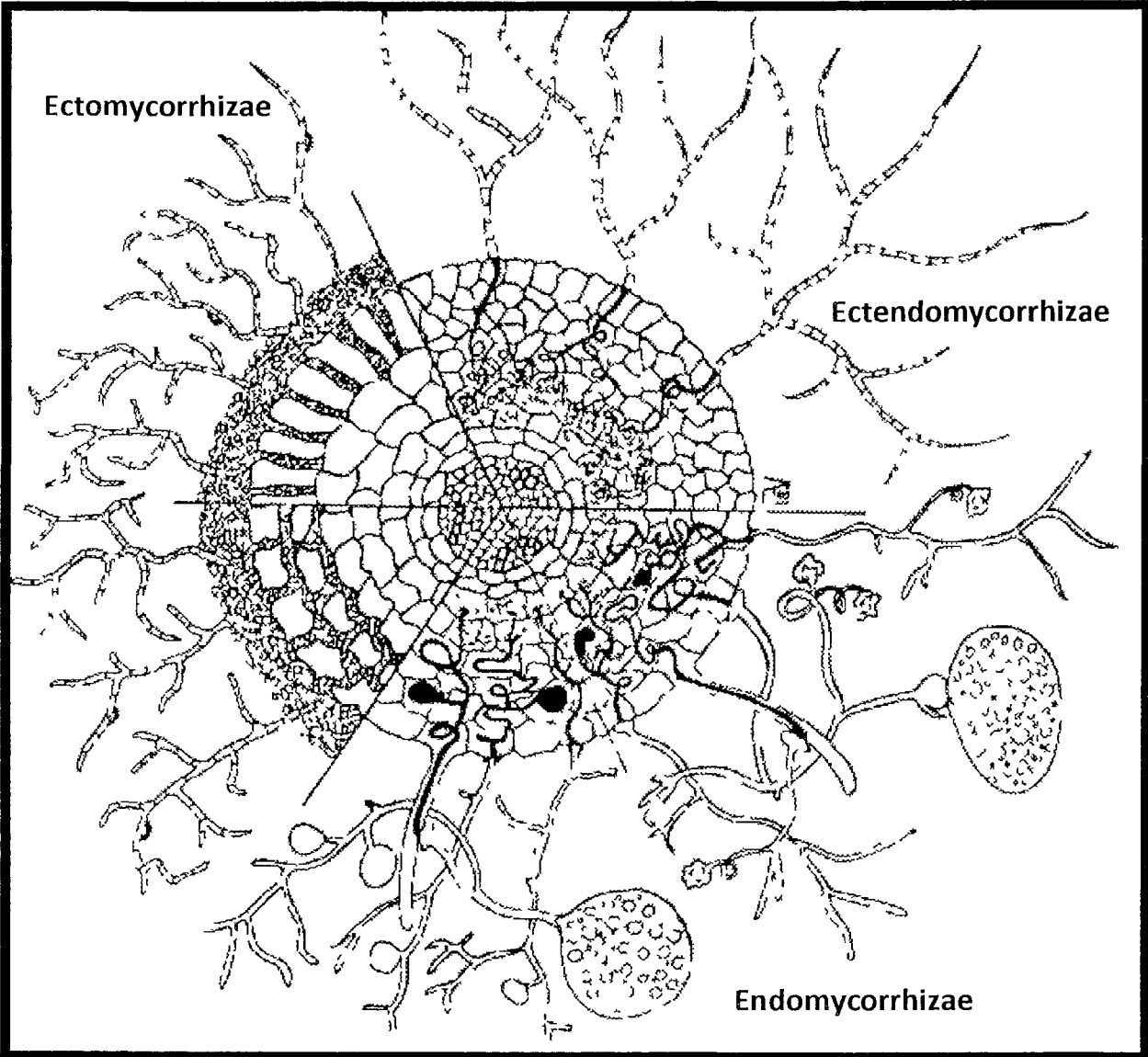


Figure 1.6 AM colonized Ri T-DNA carrot roots (HR) stained with Aniline Blue solution showing inter- (Ih) and extraradical hyphae (Eh), vesicles (Ve), arbuscules (Ar), spores (Sp), and spore clusters (Sc).

Figure 1.6

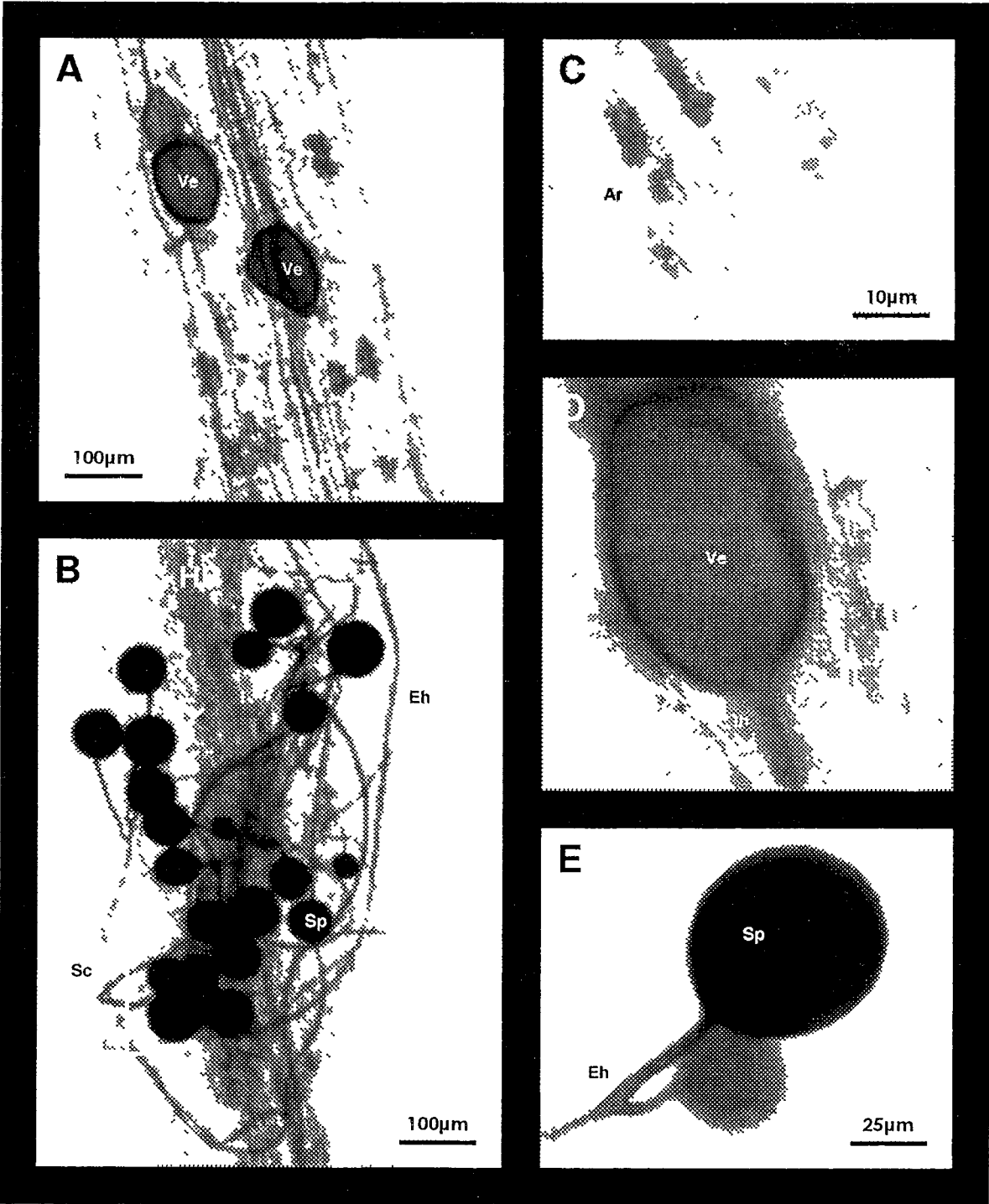


Figure 1.7 Schematization of the mycorrhizospheric process of 'enhanced uptake' (a) and its predicted effects toward soil metal bioavailability and plant uptake Capacity (b).

Figure 1.7

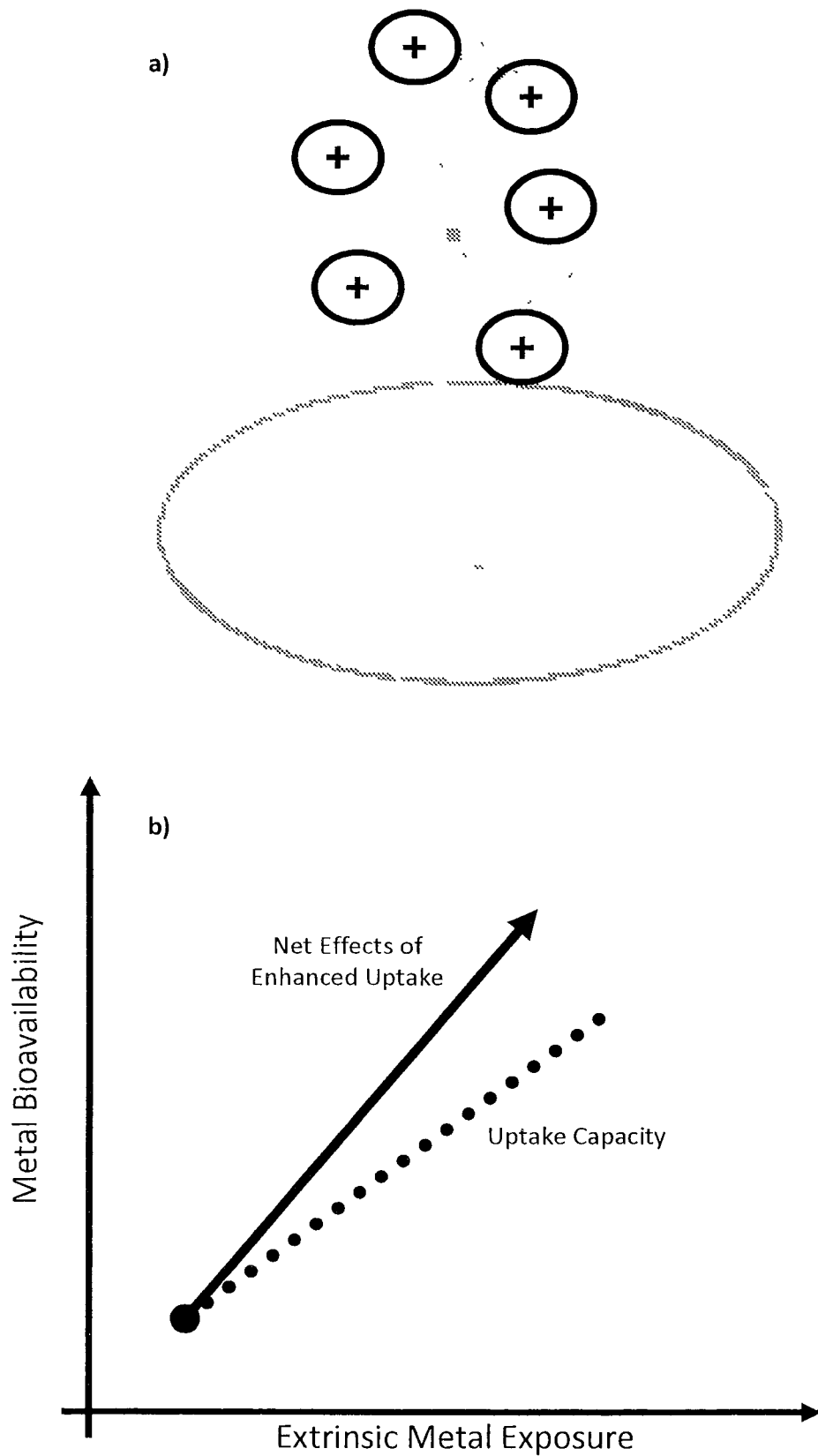
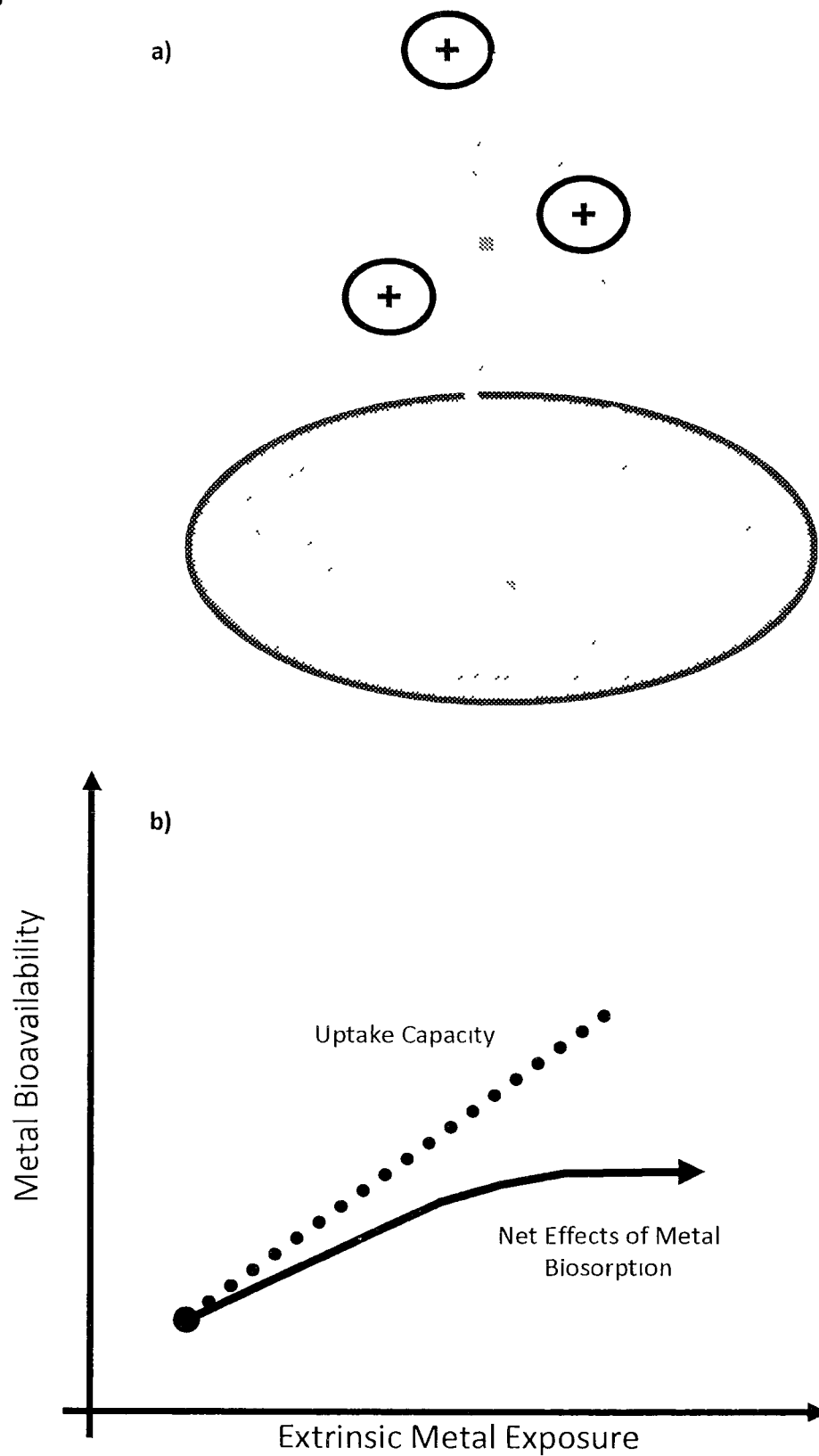


Figure 1.8 Schematization of the mycorrhizospheric process of 'metal biosorption' (a) and its predicted effects toward soil metal bioavailability and plant uptake capacity (b).

Figure 1.8



1.3 Rationale

The rationale for this PhD thesis was that, although the AM fungi are recognized for beneficially impacting a number of individual plant physiological and soil ecological processes, there are no unified perspectives depicting these combined, multi-lateral effects across a broad-spectrum of environmental stress; for instance, from trace to toxic soil metal conditions. The case for AM-plant metal uptake is noteworthy since the AM fungi apparently hold two antithetical roles in plant metal uptake having different predicted outcomes depending on the soil metal conditions: (a) enhanced uptake which increases metal uptake and (b) metal biosorption which decreases it. For these reasons, the main questions that directed this research were:

- 1) *How do mycorrhizospheric processes benefit plant stress tolerance?*
- 2) *Under what environmental conditions do they occur?*
- 3) *What are the mechanisms that determine their function?*
- 4) *Do they occur simultaneously?*
- 5) *How do they impact the proximal soil environment?*

To address these questions and meet the general objectives stated above, my research strategy comprised two distinctive study components: statistical meta-analysis (Section II) and comparative experimental analysis (Section III). Meta-analysis was used to quantitatively review the current body of phytoremediation literature to assess general trends in plant metal uptake across a broad-spectrum of metal exposure (Chapter 2). Thereafter, the strategy was used to further assess the impact of AM symbiosis in plant metal uptake (Chapter 3), and to determine the role of resource allocation plasticity and metal partitioning in plant stress tolerance across

different plant guilds (Chapter 4) Overall, meta-analysis has served as a framework for testing hypotheses (e.g. 'enhanced uptake' and 'metal biosorption') and deriving integrative conceptual models of AM-plant interactions which are referenced throughout this thesis Besides meta-analysis, *in vitro* and *in vivo* experimental systems were selected to investigate the dynamics of AM symbiosis and plant stress tolerance within the context of the proposed conceptual models First, the *in vitro* carrot root organ culture system was used as a relevant experimental tool to directly test and ultimately revise our conceptual models of AM-plant interactions (Chapter 5) Then, two greenhouse culture systems were designed to determine the impact of AM fungi on whole plants and soil metal bioavailability, thereby expanding the scope of the proposed models to depict any broader role of the AM-mycorrhizosphere in ecosystem function (Chapters 6 and 7) Further to the core objectives of this thesis, I also present a supplementary study (Chapter 8) aimed at identifying potential experimental design biases in mycorrhizal pot-growth studies having important general implications to the field of comparative plant physiology In conclusion (Chapter 9), I provide a general discussion of our study stating the new contributions to the field of environmental plant physiology, and recommendations for future research

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Section II

Meta-Analysis

Introduction to meta-analysis

Most commonly used in epidemiological and clinical based research, meta-analysis is a second order (otherwise known as *Synthetic*) science referring to the process of combining and analyzing findings from multiple studies in the objective of describing general trends within a collective dataset (Glass et al 1981, Hedges & Olkin 1985, Lipsey & Wilson 2001) When sufficient studies are available, this quantitative literature review method can enhance the inferential power of the individual studies by enabling a post hoc statistical analysis which can identify broad-scale tendencies within a given research domain which may not be detected using other conventional literature review methods In this section (Chapters 2, 3, and 4), I have surveyed the current body of phytoremediation literature for the first time to quantify trends in plant growth, metal uptake, and the inherent role of the AM symbiosis therein Deriving from this meta analytical framework, I developed conceptual models depicting plant stress tolerance strategies when subjected to soil metal conditions ranging from trace to potentially toxic exposure levels In Section II, these models serve as hypotheses for targeted experimental investigations using in vitro root-organ (Chapter 5) and greenhouse culture systems (Chapters 6 and 7)

Note on publications from this Section

The contents in this Section were first published in the following proceedings

- Audet P, Charest C (2007) Heavy metal phytoremediation from a meta-analytical perspective *Environ Pollut* 147 231-237
- Audet P, Charest C (2007) Dynamics of arbuscular mycorrhizal symbiosis in heavy metal phytoremediation meta-analytical and conceptual perspectives *Environ Pollut* 147 609-614
- Audet P, Charest C (2008) Allocation plasticity & metal-partitioning meta-analytical perspectives in phytoremediation *Environ Pollut* 156 290-296

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Chapter 2

Trends and perspectives in metal phytoremediation

2.1 Objectives

When occurring at high soil exposure levels, metal nutrients (e.g. Cr, Cu, Fe, Mn, Ni, and Zn) and non-essential metals (e.g. Cd, Co, and Pb) represent persistent environmental stressors (Pilon-Smits 2005). Many plant species have shown a remarkable ability to take up and sequester metal pollutants in the objective of restoring ecosystem function, a process known as phytoremediation. Still, for this metal phytoextraction process to be effective, plants must tolerate significant environmental and physiological challenges such as cellular oxidative stress, fluctuations in soil pH, and soil nutrient imbalances (Chaney et al. 1997, Meagher 2000, Pilon-Smits 2005, Salt et al. 1995, 1998). In the objective of quantitatively assessing trends in the current body of soil metal remediation literature, I conducted a meta-analysis to assess the effectiveness of metal phytoextraction by determining the relationships between plant growth, metal uptake, and soil metal exposure for a broad number of metal nutrients and non-essential metals among different plant species. In doing so, I have detected key plant physiological tendencies which have been somewhat underestimated by conventional qualitative reviews.

2.2 Methods

(a) Meta-analysis

Based on the methods of meta-analysis by Hedges and Olkin (1985) and Lipsey and Wilson (2001), I calculated the strength of correlation between metrics of plant metal uptake (tissue concentration and total content), biomass production (dry mass), and soil metal exposure (concentration) using combined data extracted from multiple peer-reviewed studies. After a thorough scientific literature review, 36 articles were selected according to the criteria that they dealt with herbaceous plants and measured biomass and metal uptake. The selection criteria also required that studies consist in both greenhouse or field experiments having the soil mineral composition described and the data presented in tables to ensure the highest accuracy in data reporting. All of the metals (*e.g.* As, Cd, Co, Cr, Cu, Fe, Mn, Pb, U, and Zn) with their soil concentration ranges, and the 50 different plant species included in our study are appended (2.6 Supplementary data). The key variables included the soil (mg kg^{-1} dry soil) and plant metal concentrations (mg kg^{-1} dry mass), total plant metal content (mg plant^{-1}), and plant dry mass (g) for shoot and (or) root organs.

(b) Metrics

Together, plant metal content ($\text{Metal}_{\text{plant}}$) and concentration ($[\text{Metal}]_{\text{plant}}$) were selected to estimate metal uptake, whereas the specific extraction yield percentage ($\text{SEY}_{\%}$) and bioconcentration factor (BCF) were selected as relative indexes of metal uptake in relation to metal exposure. The $\text{SEY}_{\%}$, representing the percent ratio of metal content

relative to soil metal exposure ($[Metal]_{soil}$) (adapted from Audet & Charest 2006), is defined as:

$$\left(\frac{Metal_{plant}}{[Metal]_{soil}} \right) \times 100\% \quad (1)$$

The BCF, representing the ratio of metal concentration to soil metal exposure (Dowdy and McKone 1997), is defined as:

$$\frac{[Metal]_{plant}}{[Metal]_{soil}} \quad (2)$$

Typically, the $SEY\%$ and BCF are used as indicators of contaminant biomagnification from incremental trophic levels (Newman & Unger 2003). Likewise for plants, the BCF can be used as an estimate of trophic transfer efficiency, whereby values greater than 1 could be indicative of hyperaccumulation (Zhang et al. 2002). Lastly, the metal tolerance index (TI) was selected to estimate relative plant growth in relation to metal exposure.

The TI, representing a ratio between the dry masses (DM) of metal exposed versus non-exposed control plants (Wilkins 1957, 1978), is defined as:

$$\frac{DM_{exp}}{DM_{control}} \quad (3)$$

Here, TI values lower than 1 indicates a relative decrease in biomass. Values equal or greater than 1 indicate no difference or relative increase compared to control, which could suggest a growth dilution effect.

(c) Statistical analyses

According to Zar (1999), the Pearson product-moment correlation test was used to determine correlations between each indicator of plant growth, metal uptake, and soil

metal exposure. Each correlation coefficient (r) was determined by fitting a general linear model to the data, adding statistical terms to account for different sources of variance (e.g. metal species, plant species, reference source, plant organ type, and experimental environment), and then solving for the coefficient of determination (r^2) and correlation coefficient (r). This procedure enabled me to estimate the added statistical variance attributed to combining data from multiple studies. When sufficient degrees of freedom were available for a robust statistical analysis, the correlations were also calculated for each individual metal (e.g. Zn, Cd, Pb, Cu, Ni, Cr, Co, Mn, and Fe) to determine the metal-specific correlations. The Fisher correlation comparison test was used to compare coefficient values. The data were Log-transformed as required to improve the linearity of the correlations and to meet the assumptions of the parametric statistical analyses, meanwhile residual values of SEY% and BCF were used to avoid any bias associated with autocorrelation. All of the Fisher statistics (F), coefficients of determination (r^2), degrees of freedom (df), and p-value estimates were calculated using S-Plus® 7.0 (Insightful Corp., Seattle, WA).

2.3 Results

The plant metal content (Fig. 2.1a) and concentration (Fig. 2.1b) are shown having positive correlations ($r = 0.53$ and 0.50 , respectively) in relation to increasing soil metal concentration – coefficient values are summarized in Table 2.1. These correlation coefficients were considerably improved once fitted to account for variance attributed to metal species ($r_M = 0.65$ and 0.61), reference source ($r_r = 0.75$ and 0.73), and plant species ($r_{sp} = 0.76$ and 0.82); whereas the study type (*e.g.* field or greenhouse) did not account for much of the variability. The metal-specific coefficients (Table 2.2) also shared these same tendencies, with the exception of Cr, Fe, and Mn which showed no significant trends likely as a result of a low statistical power due to small sample size. Unlike the direct estimates of plant metal uptake, the relative estimates of SEY% (Fig. 2.2a) and BCF (Fig. 2.2b) indicated negative correlations ($r = -0.54$ and -0.52 , respectively) in relation to increasing soil metal concentrations – as summarized in Table 2.1. Again, these correlation coefficients were improved once fitted to account for the metal species ($r_M = -0.64$ and -0.61), reference source ($r_r = -0.75$ and -0.80), and plant species ($r_{sp} = -0.74$ and -0.81), but not the study type. The metal-specific correlation coefficients of SEY% were also mostly all negative (Table 2.3), except for Pb, Ni, Cr, and Mn indicating non-significant correlations and Co showing a weakly positive relationship. Likewise, the metal-specific BCF values for Cr and Fe showed no correlations, while Ni, Co, and Mn showed weakly positive correlations with soil-metal concentration. Lastly, the TI index showed negative correlation values in relation to the soil metal concentration (Fig. 2.3a), plant metal content (Fig. 2.3b), and plant metal

concentration (Fig. 2.3c) – as summarized in Table 2.4. The strength of all correlation coefficients was greatly improved once fitted for the plant species ($r_{sp} = -0.49, -0.44,$ and -0.49) and reference source ($r_r = -0.55, -0.49,$ and -0.54).

Table 2.1 Correlation coefficient values (r) for the plant metal content (M_{plant}), plant metal concentration ($[M_{\text{plant}}]$), residual Specific Extraction Yield % ($\text{SEY}_{\%}$), and residual Bioconcentration Factor (BCF) in relation with the soil metal concentration ($[M_{\text{soil}}]$).

Variables	df	r	r_M	r_{sp}	r_r	r_t	r_{st}
Metal Content – M_{plant}	508	0.53 ^c	0.65 ^b	0.75 ^a	0.76 ^a	0.56 ^{bc}	0.55 ^c
Metal Concentration – $[M_{\text{plant}}]$	761	0.50 ^d	0.61 ^c	0.73 ^b	0.82 ^a	0.59 ^c	0.51 ^d
Extraction Yield – $\text{SEY}_{\%}$	508	-0.54 ^c	0.64 ^b	-0.75 ^a	0.74 ^a	-0.55 ^c	-0.54 ^c
Bioconcentration Factor – BCF	761	-0.52 ^c	0.61 ^b	-0.80 ^a	0.81 ^a	-0.52 ^c	0.59 ^c

The r values for raw and fitted coefficients (metal species, r_M , reference, r_r , species, r_s , plant tissue type, r_t , study type, r_{st}) and degrees of freedom (df) are shown. All p values < 0.01 . Different letters within each row indicate significant differences between correlation coefficients according to Fisher's comparison test at $p < 0.05$.

Table 2.2 Metal specific correlation coefficient values (r) for the plant metal content (M_{plant}) and concentration ($[M_{\text{plant}}]$) in relation with the soil metal concentration ($[M_{\text{soil}}]$).

Metal Species	Plant Metal Content – M_{plant}						Plant Metal Concentration – $[M_{\text{plant}}]$					
	df	r	r_r	r_{sp}	r_t	r_{st}	df	r	r_r	r_{sp}	r_t	r_{st}
Zn	186	0.29 ^c	0.73 ^a	0.59 ^b	0.39 ^c	0.33 ^c	202	0.24 ^c	0.90 ^a	0.84 ^a	0.25 ^c	0.71 ^b
Cd	156	0.71 ^b	0.85 ^a	0.85 ^a	0.71 ^b	0.75 ^b	211	0.56 ^b	0.90 ^a	0.86 ^a	0.57 ^b	0.67 ^b
Pb	80	0.40 ^b	0.44 ^b	0.76 ^a	0.55 ^b	0.56 ^b	142	0.05 ^d	0.85 ^a	0.77 ^b	0.52 ^c	0.44 ^c
Cu	44	0.34 ^b	0.57 ^b	0.80 ^a	0.60 ^{ab}	0.48 ^b	64	0.39 ^b	0.73 ^a	0.61 ^b	0.55 ^{ab}	0.43 ^b
Ni	36	0.89 ^a	0.90 ^a	0.89 ^a	0.89 ^a	0.79 ^a	64	0.78 ^b	0.81 ^b	0.89 ^{ab}	0.79 ^b	0.78 ^b
Cr	20	0.43 ^{ns}	0.48 ^{ns}	0.46 ^{ns}	0.52 ^{ns}	0.46 ^{ns}	28	0.35 ^{ns}	0.51 ^a	0.46 ^{ns}	0.76 ^a	0.46 ^{ns}
Co	10	0.99					10	0.99 ^a				
Mn	2	0.52 ^{ns}	0.99 ^{ns}		0.53 ^{ns}		20	0.91 ^a	0.89 ^a	0.89 ^a	0.91 ^a	0.90 ^a
Fe							4	-0.06 ^{ns}	0.77 ^{ns}	0.77 ^{ns}	-0.06 ^{ns}	

The r values for raw and fitted coefficients (metal species, r_M , reference, r_r ; species, r_s ; plant tissue type, r_t , study type, r_{st}) and degrees of freedom (df) are shown. All p -values < 0.01 . Different letters within each row indicate significant differences between correlation coefficients according to Fisher's comparison test at $p < 0.05$. Empty coefficient fields indicate that the correlation model could not be fitted with variability terms since the data offered too few degrees of freedom (e.g. taken from either one plant species, reference source, plant tissue type, or study type only).

Table 2.3 Metal specific correlation coefficient values (r) for the residual Specific Extraction Yield % (SEY%) and residual Bioconcentration Factor (BCF) in relation with the soil metal concentration ($[M_{soil}]$).

Metal Species	Specific Extraction Yield – SEY%						Bioconcentration Factor – BCF					
	df	r	r _r	r _{sp}	r _t	r _{st}	df	r	r _r	r _{sp}	r _t	r _{st}
Zn	186	-0.73 ^c	-0.89 ^a	-0.82 ^b	-0.75 ^{bc}	-0.73 ^c	202	-0.73 ^c	-0.96 ^a	-0.92 ^a	-0.73 ^c	-0.83 ^b
Cd	156	-0.55 ^b	-0.79 ^a	-0.79 ^a	-0.56 ^b	-0.62 ^b	211	-0.63 ^b	-0.91 ^a	-0.88 ^a	-0.64 ^b	-0.72 ^b
Pb	80	-0.18 ^{ns}	-0.61 ^{ab}	-0.67 ^a	-0.38 ^b	-0.56 ^{ab}	142	-0.21 ^c	-0.79 ^a	-0.68 ^b	-0.23 ^c	-0.23 ^c
Cu	44	-0.55 ^b	-0.81 ^{ab}	-0.84 ^a	-0.69 ^{ab}	-0.61 ^b	64	-0.68 ^b	-0.84 ^a	-0.77 ^b	-0.74 ^{ab}	-0.69 ^b
Ni	36	0.74 ^{ns}	0.77 ^a	0.76 ^a	0.75 ^a	0.75 ^a	64	0.34 ^b	0.48 ^b	0.73 ^a	0.39 ^b	0.36 ^b
Cr	20	0.11 ^{ns}	-0.79 ^a	-0.21 ^{ns}	-0.33 ^b	-0.22 ^{ns}	28	-0.17 ^{ns}	-0.42 ^a	-0.36 ^{ns}	-0.73 ^a	-0.35 ^{ns}
Co	10	0.98					10	0.98				
Mn	2	-0.94 ^{ns}	-0.94 ^{ns}		-0.94 ^{ns}		20	0.70 ^a	0.71 ^a	0.71 ^a	0.86 ^a	0.70 ^a
Fe							4	-0.50 ^{ns}	-0.83 ^{ns}	-0.83 ^{ns}	-0.50 ^{ns}	

The r values for raw and fitted coefficients (metal species, r_M, reference, r_r, species, r_s, plant tissue type, r_t, study type, r_{st}) and degrees of freedom (df) are shown. All p-values < 0.01. Different letters within each row indicate significant differences between correlation coefficients according to Fisher's comparison test at p < 0.05. Empty coefficient fields indicate that the correlation model could not be fitted with variability terms since the data offered too few degrees of freedom (e.g. taken from either one plant species, reference source, plant tissue type, or study type only).

Table 2.4 Correlation coefficient values (r) for the soil metal concentration ($[M_{\text{soil}}]$), plant metal content (M_{plant}), and plant metal concentration ($[M_{\text{plant}}]$) in relation with the tolerance index (TI).

Correlation comparison	df	r	r_M	r_{sp}	r_r	r_t	r_{st}
Soil Metal Conc. – $[M_{\text{soil}}]$	236	-0.21 ^b	-0.29 ^b	-0.49 ^a	-0.55 ^a	-0.27 ^b	-0.24 ^b
Plant Metal Content – M_{plant}	236	-0.12 ^{ns}	-0.23 ^{ns}	-0.44 ^a	-0.39 ^a	-0.21 ^{ns}	-0.15 ^{ns}
Plant Metal Conc. – $[M_{\text{plant}}]$	220	-0.19 ^b	-0.32 ^b	-0.49 ^a	-0.54 ^a	-0.24 ^b	-0.20 ^b

The r values for raw and fitted coefficients (metal species, r_M ; reference, r_r ; species, r_s ; plant tissue type, r_t ; study type, r_{st}) and degrees of freedom (df) are shown. All p -values < 0.01 . Different letters within each row indicate significant differences between correlation coefficients according to Fisher's comparison test at $p < 0.05$.

Figure 2.1 Plant metal content (mg DM^{-1}) (a) and metal concentration ($\text{mg kg}^{-1} \text{DM}$) (b) in relation with soil metal concentration ($\text{mg kg}^{-1} \text{dry soil}$).

Figure 2.1

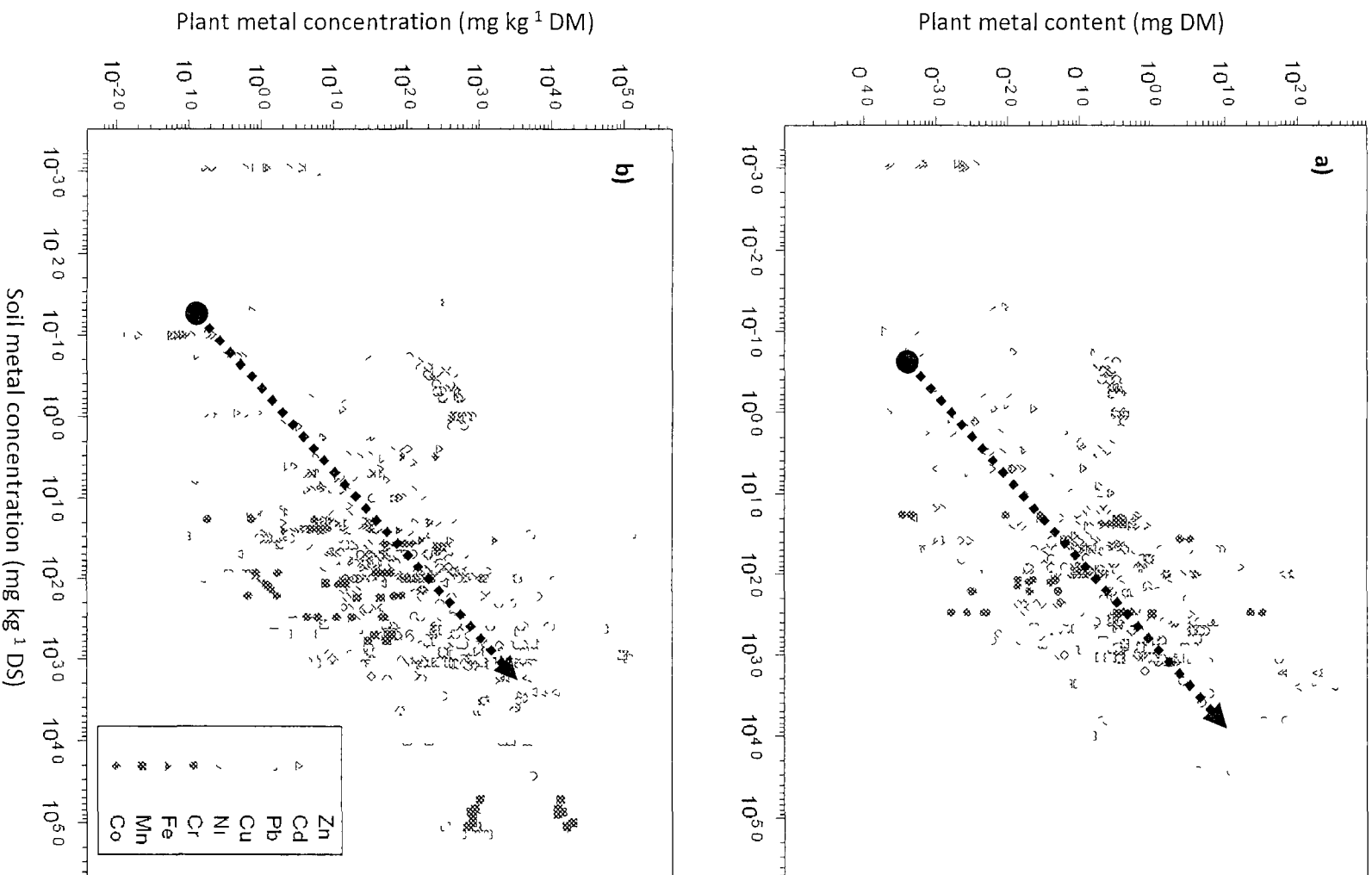


Figure 2.2 Log-residual values of Specific Extraction Yield % (SEY%) (a) and Bioconcentration Factor (BCF) (b) in relation with soil metal concentration (mg kg^{-1} dry soil).

Figure 2.2

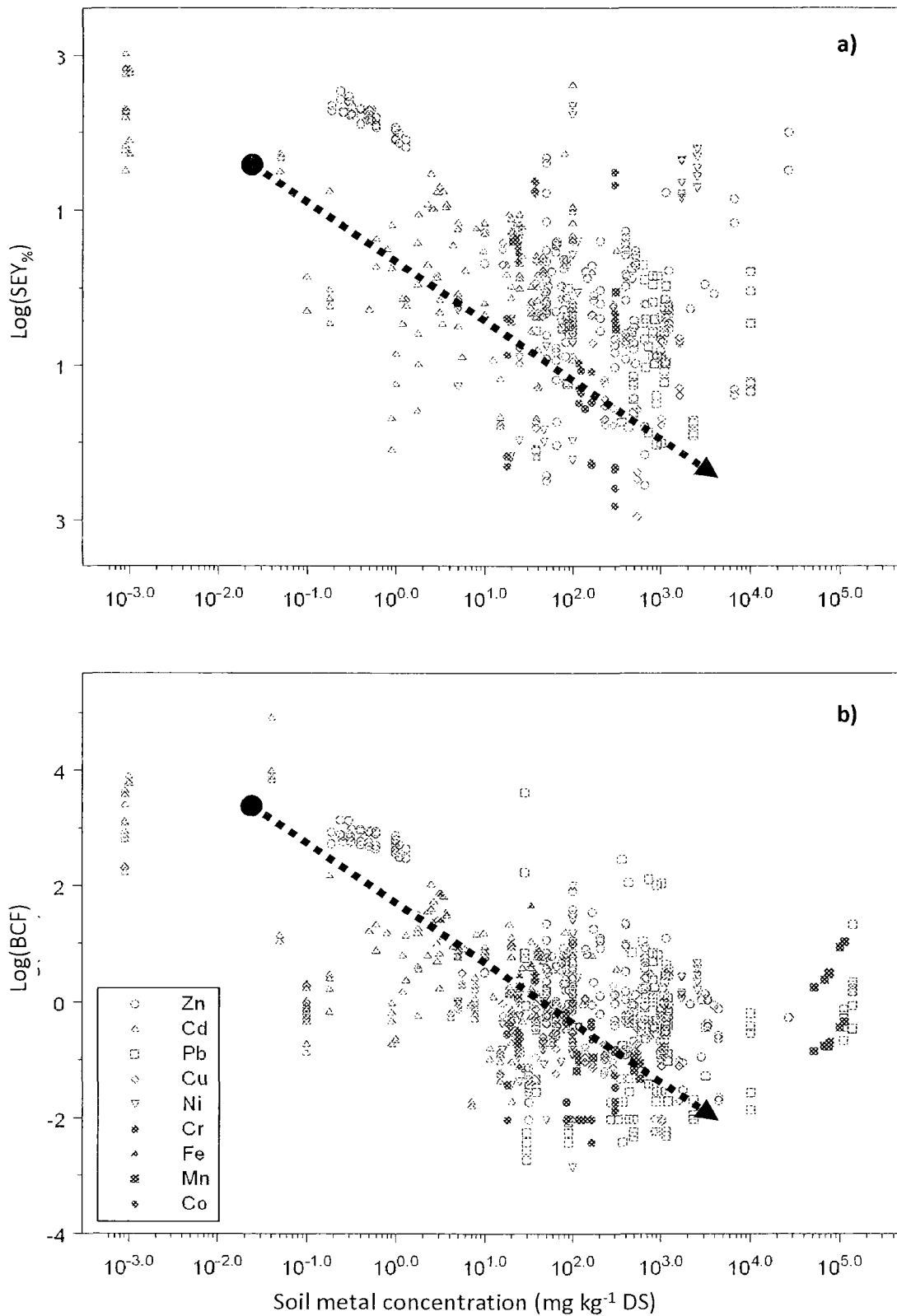
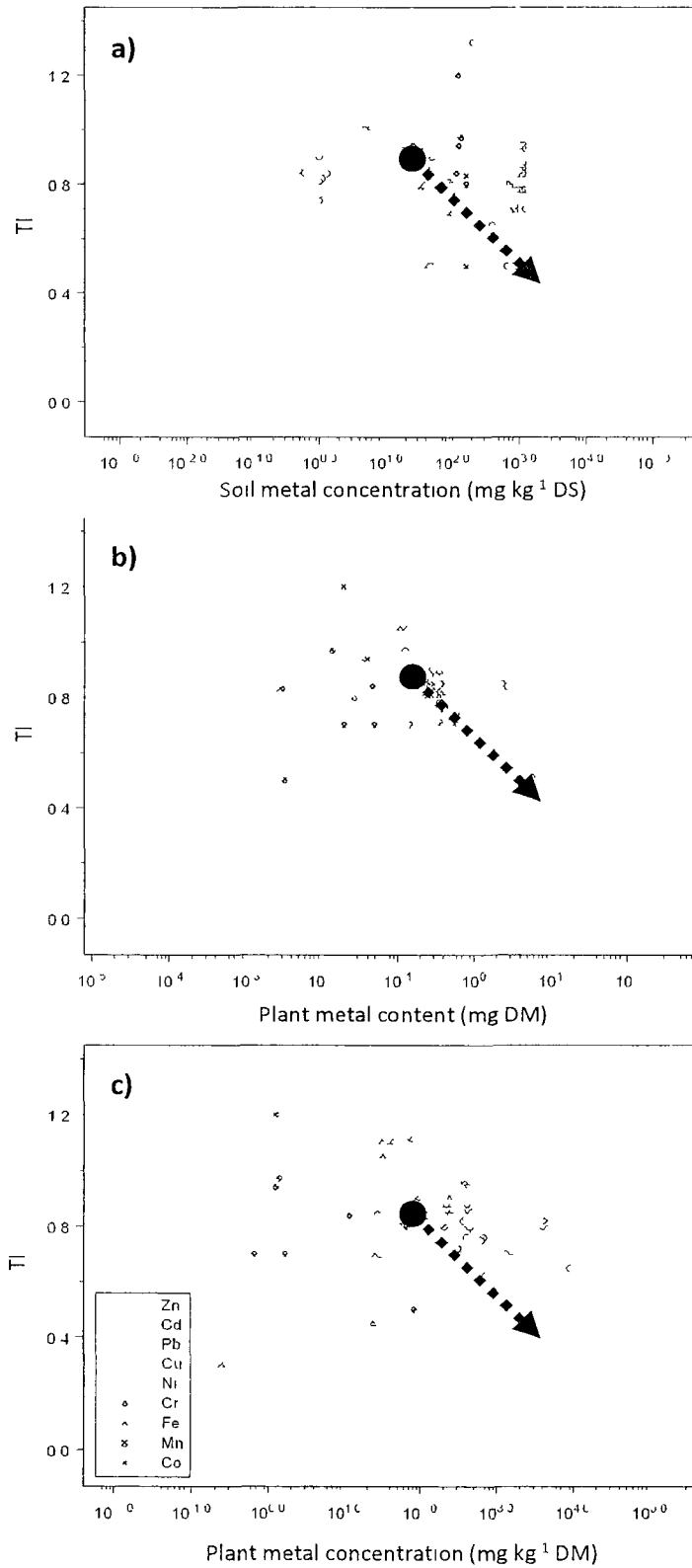


Figure 2.3 Tolerance Index (TI) in relation with soil metal concentration (mg kg^{-1} dry soil) (a), plant metal content (mg DM^{-1}) (b), and plant metal concentration (mg kg^{-1} DM) (c). The reference line refers to a TI value of 1.

Figure 2.3



2.4 Discussion

Initially, the data indicated that the plant metal uptake was strongly correlated with soil metal exposure; this tendency was also detected among each individual metal species with few exceptions. Of the selected studies for analysis, metal hyperaccumulator species (otherwise known as *metallophytes*) emerged as having taken up the highest metal concentrations and total metal contents reaching uptake values of 325 mg DM^{-1} and $125\,000 \text{ mg kg}^{-1} \text{ DM}$, despite being subjected to soil metal exposure levels three to five orders of magnitude greater than control soils (Li et al. 2003; Chen et al. 2003a). Although previous reviews of the phytoremediation literature have interpreted these findings as representing a high potential for phytoremediation purposes (Chaney et al. 1997; Meagher 2000; Pilon-Smits 2005; Salt et al. 1995, 1998), the indicators of relative plant metal uptake (*i.e.* SEY% and BCF) show tendencies to the contrary, whereby the effectiveness of phytoextraction and the production of biomass decline sharply in relation to increasing metal exposure. When combined with other environmental factors such as fluctuations in soil-pH and nutrient bioavailability (Foy et al. 1978; Marschner 1986), such a decline reflects the overall metal toxicity burden that can disrupt cellular processes and alter functional interactions with beneficial soil microbes (Giller et al. 1998; Hayman & Tavares 1985; Leyval et al. 1997; McGrath et al. 1995). In this case, it is plausible that the plant's resource allocation likely shifts more toward phytochelatin and metal sequestration metabolism as opposed to biomass production to overcome such an impending environmental stress and conserve essential functions (Cobbett 2000; Maier et al. 2003; Wang et al. 2005), then resulting in a fundamental

compromise between the plant's growth capacity and its intrinsic stress resistance. For this reason, it is likely that incidences of successful metal phytoextraction may only be limited to better adapted species, such as hyperaccumulator and metallophytic species (typically members of the Brassicaceae family, such as *Thlaspi* and *Brassica* sp.), which have developed highly effective sequestration and phytochelation strategies (Xue et al. 2004; Yanai et al. 2006). Otherwise, the meta-analytical findings indicate that plant species suffer a significant growth decline under such metal toxicity conditions.

As for the metal-specific tendencies, it is shown that the plant metal uptake tendencies and growth responses were generally the same between the metal species as described above, despite a few exceptions. These exceptions (*e.g.* Cr, Mn, and Fe) highlight some limitations of the meta-analytical approach inherent to having a small sample size or narrow data distribution which then decreases the inferential power of analysis. Accordingly, some metal-specific coefficient values reported here may not be considered representative of any significant biological trends until more data are available. Likewise, it is also acknowledged that some metals behave differently depending on localized edaphic conditions (Walker et al. 2003; Apak 2002), such that certain metal species can be more bioavailable than others and thereby preferentially taken-up depending on the soil's composition and inherent physicochemical characteristics (*e.g.* redox potential, pH – Galli et al. 1994; McBride 1994). For this reason, the purpose of fitting statistical terms (for instance, metal species, organ type, or study type) to the correlation coefficients has enabled to isolate some of the intrinsic variability associated with having combined data from multiple studies, thereby

strengthening the robustness of the analysis. Not surprisingly, the metal species, reference source, and plant species type accounted for the highest proportions of variance. As previously suggested, this finding is likely indicative of differences in metal speciation due to soil composition, the possible addition of chelating agents depending on the experimental design of the selected studies (Blaylock et al. 1997; Chen et al. 2003b; Cui et al. 2004; Jiang and Yang 2004), or even the plant species' life history strategy (*e.g.* hyperaccumulators v. non-hyperaccumulators – Delorme et al. 2001; Marchiol et al. 2004; Shen et al. 2002).

Altogether, this Chapter emphasizes the value of using meta-analysis for quantifying trends in comparative plant physiology and environmental science. With this strategy, it has been highlighted that plants face various physiological and ecological challenges when subjected to soil metal conditions ranging from trace to toxic conditions, particularly the likelihood of a metal toxicity burden resulting in a compromise between the plant's relative growth capacity and its intrinsic stress resistance. In the following sections, the meta-analytical investigation will assess the role of the AM symbiosis in plant metal uptake and stress tolerance (Chapter 3) as well as the consequences of plant life history strategies toward resource allocation plasticity and plant metal partitioning by comparing different plant guilds (Chapter 4).

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2.6 Supplementary data

Table 2.5 Metal species and soil concentration ranges comprised in the meta-analysis

Species	Concentration range (mg kg ⁻¹ dry soil)	Reference
Zn	0.2 - 27.413	Audet & Charest 2006, Ayoub et al 2003, Bi et al 2003, Barazani et al 2002, Bi et al 2003, Chen et al 2003a, 2004, Cui et al 2004, Delorme et al 2001, Freeman et al 2005, Gildon & Tinker 1983, Hammer & Keller 2002, Kubota & Takenaka 2003, Li & Christie 2001, Marchiol et al 2004a, b, Mereno et al 2002, Shen et al 2002, Wang et al 2005, Weissenhorn et al 1995, Wu et al 2004, Xia 2004, Yanai et al 2006
Cd	0.001 - 770	Ayoub et al 2003, Barazani et al 2002, Chen et al 2003b, 2004, Citterio et al 2003, Delorme et al 2001, Gildon & Tinker 1983, Hammer & Keller 2002, Hutchinson et al 2000, Kubota & Takenaka 2003, Marchiol et al 2004a, b, Pichtel et al 2000, Riverra Becerral et al 2002, Shen et al 2002, Wang et al 2005, Weissenhorn et al 1995, Wu et al 2004, Xia 2004, Yanai et al 2006
Pb	24 - 140.500	Barazani et al 2002, Blaylock et al 1997, Chen et al 2003b, Cui et al 2004, Huang et al 1997, Kubota & Takenaka 2003, Marchiol et al 2004a, b, Mereno et al 2002, McGrath et al 1997, Pichtel et al 2000, Rydlova & Vosatka 2003, Shen et al 2002, Vivas et al 2003, Wang et al 2005, Weissenhorn et al 1995, Wu et al 2004, Xiong et al 1997
Cu	5 - 1.631	Barazani et al 2002, Gildon & Tinker 1983, Hammer & Keller 2002, Jiang & Wang 2004, Kubota & Takenaka 2003, Marchiol et al 2004a, b, Mereno et al 2002, McGrath et al 1997, Shen et al 2002, Wang et al 2005, Weissenhorn et al 1995, Wu et al 2004, Yanai et al 2006
Ni	5 - 2.570	Barazani et al 2002, Citterio et al 2003, 2005, Freeman et al 2005, Gildon & Tinker 1983, Marchiol et al 2004a, b, McGrath et al 1997, Li et al 2003
Cr	18 - 300	Citterio et al 2003, 2005, Marchiol et al 2004a, b
Fe	3 - 34	Barazani et al 2002, Kubota & Takenaka 2003
Co	24 - 37	Li et al 2003
Mn	21 - 11.4000	Barazani et al 2002, Kubota & Takenaka 2003, McGrath et al 1997, Weissenhorn et al 1995, Xue et al 2004

Table 2.6 Plants species comprised in the meta-analysis

Species	Reference	Species	Reference
<i>Achillea millefolium</i>	Pichtel et al 2000	<i>Pennisetum glaucum x P Purpureum</i>	Xia 2004
<i>Aesculus glabra</i>	Pichtel et al. 2000	<i>Phytolacca acinosa</i>	Xue et al 2004
<i>Agrostemma githago</i>	Pichtel et al 2000	<i>Pisum sativum</i>	Huang et al 1997, Riverra-Becerril et al 2002
<i>Agrostis capillaris</i>	Rydlova & Vosatka 2003	<i>P. pratense</i>	Bi et al 2003, Chen et al 2003a, Li & Christie 2001
<i>Allium cepa</i>	Gildon & Tinker 1983	<i>Platanus occidentalis</i>	Pichtel et al 2000
<i>Alyssum corsicum</i>	Li et al 2003	<i>Potentilla norvegica</i>	Pichtel et al 2000
<i>Ambrosia artimissifolia</i>	Pichtel et al 2000	<i>Raphanus sativus</i>	Chen et al 2003b, Marchiol et al 2004a, b
<i>Anfropogon virginicus</i>	Pichtel et al 2000	<i>Rosa multiflora</i>	Pichtel et al 2000
<i>Arabis gemmifera</i>	Kubota & Takenaka 2003	<i>Sonchus oleraceus</i>	Xiong 1997
<i>Brassica carinata</i>	Marchiol et al 2004a	<i>Stenotaphrum secundatum</i>	Xia 2004
<i>B. juncea</i>	Blaylock et al 1997, Cui et al 2004, Marchiol et al 2004a, b, Su & Wong 2004, Wu et al 2004	<i>Taraxacum officinale</i>	Pichtel et al 2000
<i>B. napus</i>	Marchiol et al 2004a, b	<i>Thlaspi arvense</i>	Freeman et al 2005
<i>B. napa</i>	Shen et al 2002	<i>T. caerulescens</i>	Ayoub et al 2003, Delorme et al 2001, Freeman et al 2005, Hammer & Keller 2002, Hutchinson et al 2000, McGrath et al 1997, Yanai et al 2006
<i>B. pekinensis</i>	Mereno et al 2002	<i>T. ochroleucum</i>	McGrath et al 1997
<i>Cannabis sativa</i>	Citterio et al 2003, 2005	<i>T. officinale</i>	Ayoub et al 2003
<i>Elsholtzia splendens</i>	Jiang & Yang 2004, Wang et al 2005	<i>T. oxyceras</i>	Freeman et al 2005
<i>Glechoma hederacea</i>	Pichtel et al 2000	<i>T. perfoliatum</i>	Freeman et al 2005
<i>Hordeum vulgare</i>	Ayoub et al 2003	<i>T. pratense</i>	Delorme et al 2001
<i>Lepidium heterophyllum</i>	Hutchinson et al 2000	<i>T. rosulare</i>	Freeman et al 2005
<i>Medicago lupulina</i>	Pichtel et al 2000	<i>Trifolium pratense</i>	Vivas et al 2003
<i>Mullen verbascum</i>	Pichtel et al 2000	<i>Triticum aestivum</i>	Huang et al 1997, Shen et al 2002, Cui et al 2004
<i>Nicotiana glauca</i>	Barazani et al 2002	<i>Vetiveria zizanioides</i>	Xia 2004
<i>N. rustica</i>	Audet & Charest 2006	<i>Vigna radiata</i>	Shen et al 2002
<i>Paspalum notanum</i>	Xia 2004	<i>Zea mays</i>	Chen et al 2004, Weissenhorn et al 1995

Meta-references

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Chapter 3

AM symbiosis and plant metal uptake

3.1 Objectives

In addition to the widely recognized view that AM fungi enhance plant stress-tolerance when subjected to drought or nutrient deficient conditions (Augé 2001; Barea et al. 2002, Charest et al. 1997; Subramanian & Charest 1998), recent reports acknowledge a significant role when challenged by metal toxicity and related environmental conditions (Audet and Charest 2006; Christie et al. 2004, Leyval et al. 1997). In this regard, two antithetical hypotheses have been proposed (Chapter 1 2) as to the role of AM symbiosis under these conditions in which the mycorrhizosphere (*i.e.* combined roots and extraradical hyphae) is credited for either (1) increasing metal uptake to supplement the plant nutrient status (Davies et al. 2001, 2002; Díaz et al. 1996; Hovsepian and Greipsson 2004), or (2) decreasing metal uptake as a result of AM-induced biosorption (otherwise known as *metal binding*) processes which can reduce metal bioavailability (Audet & Charest 2006; Chen et al. 2004; Joner et al. 2000; Weissenhorn et al. 1995). Building from the methods and notions presented in Chapter 2, I investigated both of these hypotheses to determine a role for AM fungi in plant metal uptake and ultimately present an integrative conceptual model depicting plant stress tolerance and the inherent role of AM symbiosis.

3.2 Methods

(a) Meta-analysis

Based on the methods of Hedges & Olkin (1985) and Lipsey & Wilson (2001), I assessed the effects of AM fungi on plant metal uptake, biomass production (dry mass), and root colonization levels by calculating the strength of correlation for these parameters in relation with increasing soil metal exposure using data extracted from multiple peer reviewed studies. After a thorough scientific literature review, 20 articles were selected according to the criteria that they had dealt with herbaceous plants grown with or without AM fungi under greenhouse experimental conditions. The selection criteria also required that the soil mineral composition be described and the data presented in tables to ensure the highest accuracy in data reporting. Overall, these studies all represented potted plants grown until maturity (ranging between 6 to 12 weeks depending on the species) and subjected to at least 4 weeks of metal-exposure. Key variables included soil metal concentration (mg kg^{-1} dry soil), plant metal concentration (mg kg^{-1} dry mass) and (or) content (mg plant^{-1}), and plant dry mass (g) among shoots and (or) roots. The data for AM root colonization was extracted from studies having used the standardized percent (%) colonized root length method of Giovannetti & Mosse (1980). All of the metals (*e.g.* As, Cd, Co, Cr, Cu, Fe, Mn, Pb, U, and Zn) and their soil concentration ranges are appended (3.6 Supplementary Data), as well as the plant species and AM fungal species included in the study.

(b) Metrics

The plant metal concentration ($[Metal]$) and content ($Metal$) of shoots and roots were used to estimate plant metal uptake, while the shoot and root dry masses (DM) were used to estimate plant growth. With these estimates, I calculated the % AM effect-size within the meta-data which served to assess the effect of AM fungi on the selected plant physiological parameters relative to the non-AM control plants (modified from Plenchette et al. 1983). The AM effect-size equations for (1) plant metal uptake and (2) biomass production are defined as:

$$\left(\frac{[Metal]_{AM} - [Metal]_{nonAM}}{[Metal]_{nonAM}} \right) \times 100\% \quad (1)$$

$$\left(\frac{DM_{AM} - DM_{nonAM}}{DM_{nonAM}} \right) \times 100\% \quad (2)$$

(c) Statistical analyses

According to Zar (1999), the Pearson product-moment correlation test was used to determine the strength and significance of relationships between % AM effect-sizes toward plant metal uptake, biomass production, or AM root colonization in relation to increasing soil metal concentrations. The data were Log-transformed as required to improve the linearity of the correlations and to meet the assumptions of the parametric analyses. The coefficient values for all the parameters were determined for two separate concentration intervals representing the low (10^{-3} to 1 mg kg^{-1} dry soil) and high (1 to 10^4 mg kg^{-1} dry soil) metal exposure levels according to the criteria that the low soil metal concentration interval would refer to the reported control-type (non-contaminated) soil, whereas the high concentration interval refers to the treatment-

type (metal spiked) soils. In this regard, the statistical power of analyses for the low soil metal concentration interval was notably lower than that of the high concentration interval due to the fewer available data. All of the Fisher statistics (F), coefficients of determination (r^2), degrees of freedom (df), and p-value estimates were calculated using S-Plus® 7.0 (Insightful 2005).

Table 3.1 Correlation coefficient values (*r*) for AM effect-size % on plant metal content, plant metal concentration, and biomass, as well as AM root colonization in relation with the soil metal concentration.

Parameter	Soil metal concentration					
	low concentration interval			high concentration interval		
	(10 ⁻³ - 1 mg kg ⁻¹ dry soil)			(1 - 10 ⁴ mg kg ⁻¹ dry soil)		
	<i>r</i>	<i>df</i>	<i>p</i>	<i>r</i>	<i>df</i>	<i>p</i>
Plant metal concentration	0.83	22	<10 ⁻⁷	-0.38	177	<10 ⁻⁴
Plant metal content	0.83	14	<10 ⁻⁴	-0.25	131	<10 ⁻³
Plant biomass	0.24	30	0.19	0.24	130	<0.01
AM root colonization	0.43	21	<0.05	-0.1	172	0.21

Figure 3.1 AM effect-size % on plant metal concentration (a) and plant metal content (b) in relation to increasing soil metal concentration. The vertical reference line separates the low (10^{-3} to 1 mg kg^{-1} dry soil) and high (1 to 10^4 mg kg^{-1} dry soil) soil metal concentration intervals.

Figure 3.1

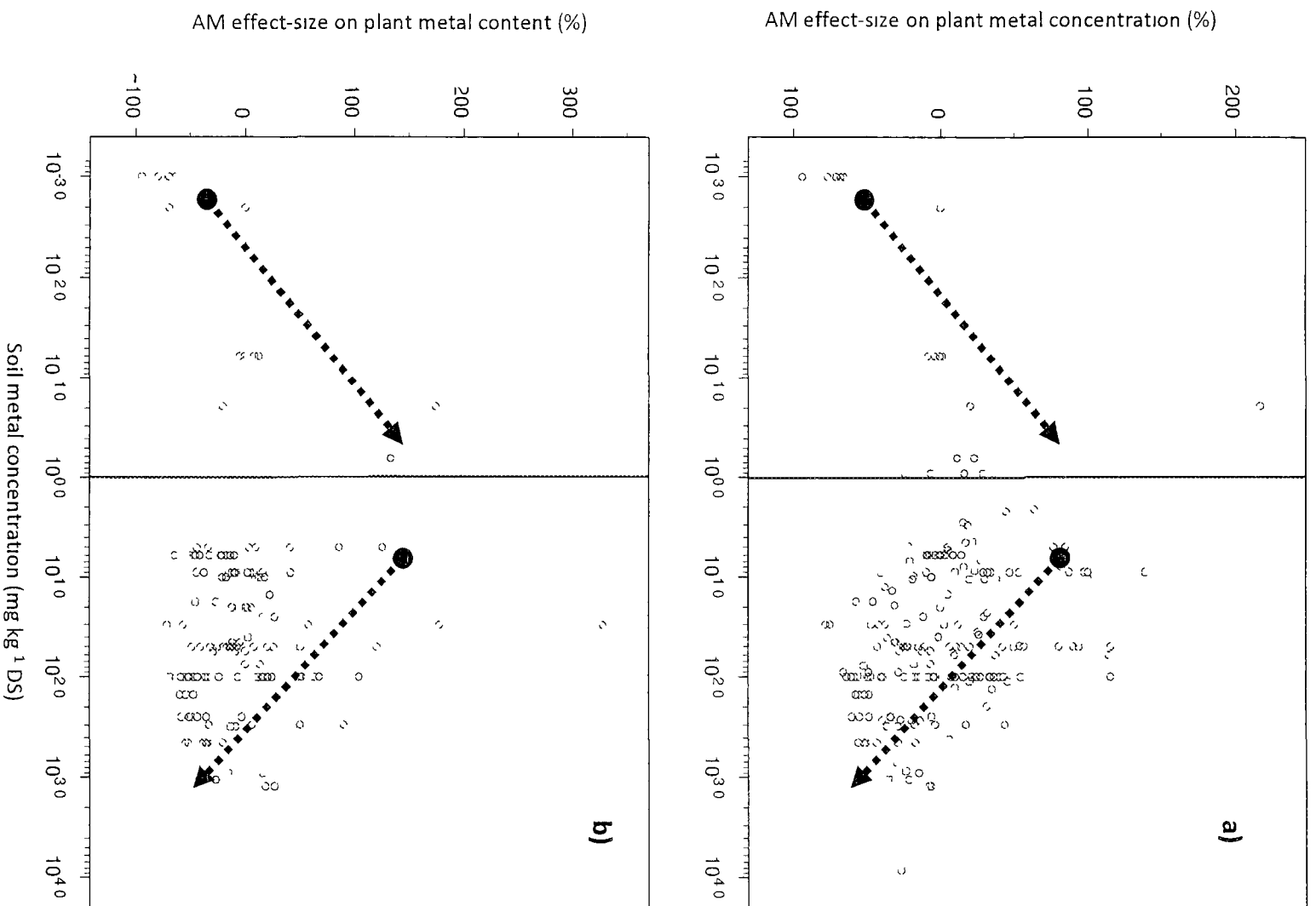


Figure 3.2 AM effect-size % on plant growth in relation to increasing soil metal concentration. The vertical reference line separates the low (10^{-3} to 1 mg kg^{-1} dry soil) and high (1 to 10^4 mg kg^{-1} dry soil) soil metal concentration intervals.

Figure 3.2

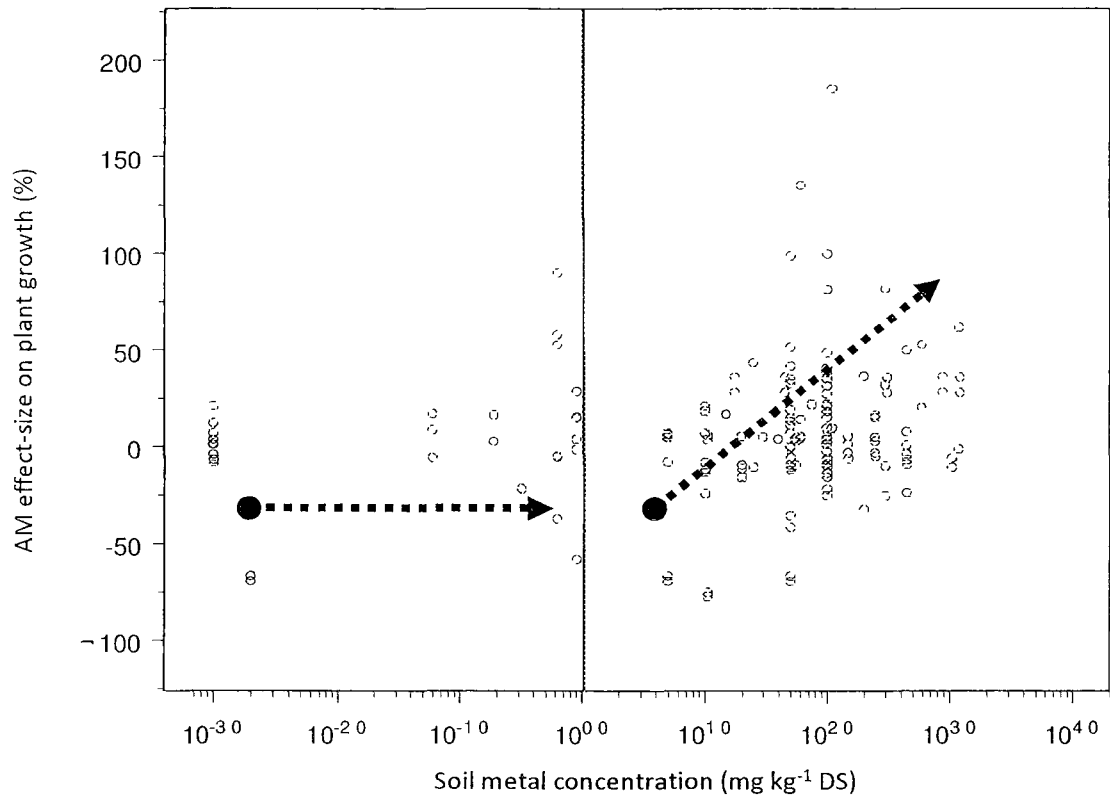
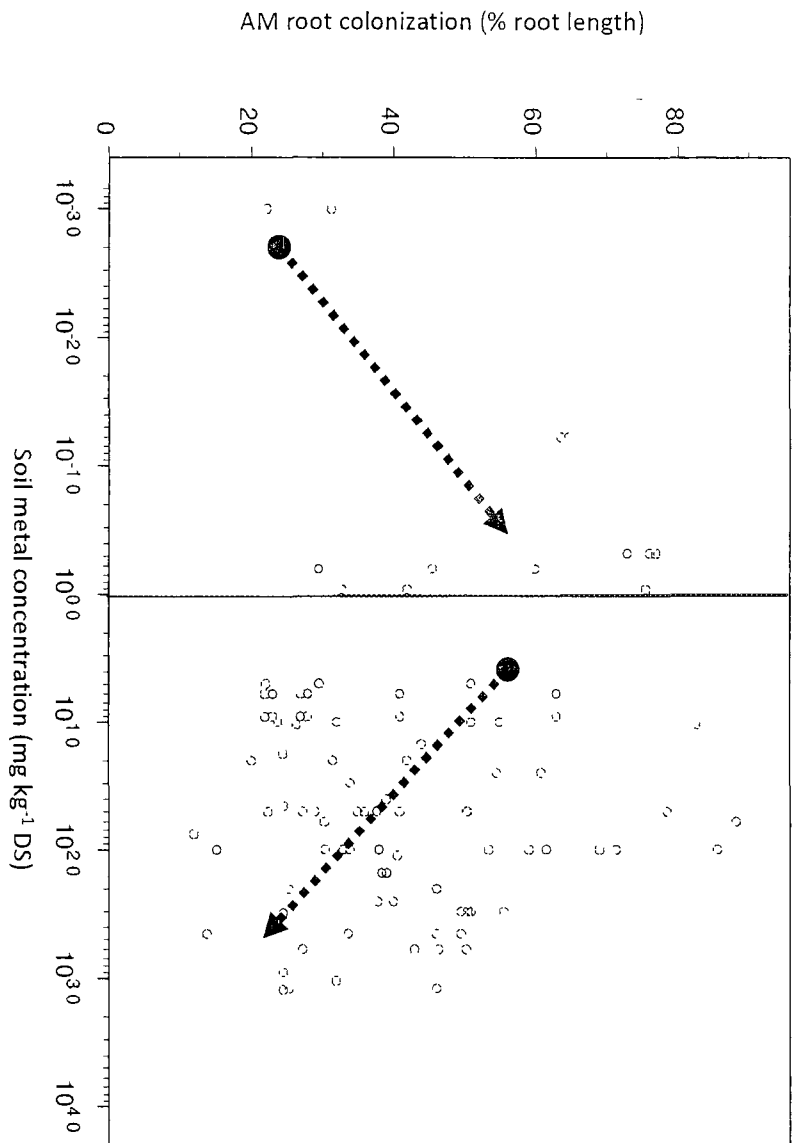


Figure 3.3 AM root colonization (% colonized root length) in relation to increasing soil metal concentration. The vertical reference line separates the low (10^{-3} to 1 mg kg^{-1} dry soil) and high (1 to 10^4 mg kg^{-1} dry soil) soil metal concentration intervals.

Figure 3.3



- 3.4** Conceptual model of plant metal uptake integrating the role of AM symbiosis. Designated are zones referring to the 'enhanced uptake' and 'metal biosorption' hypotheses. AM plants show greater metal uptake than non-AM plants at low metal exposure levels, but lower uptake at high soil-HM level.

Figure 3.4

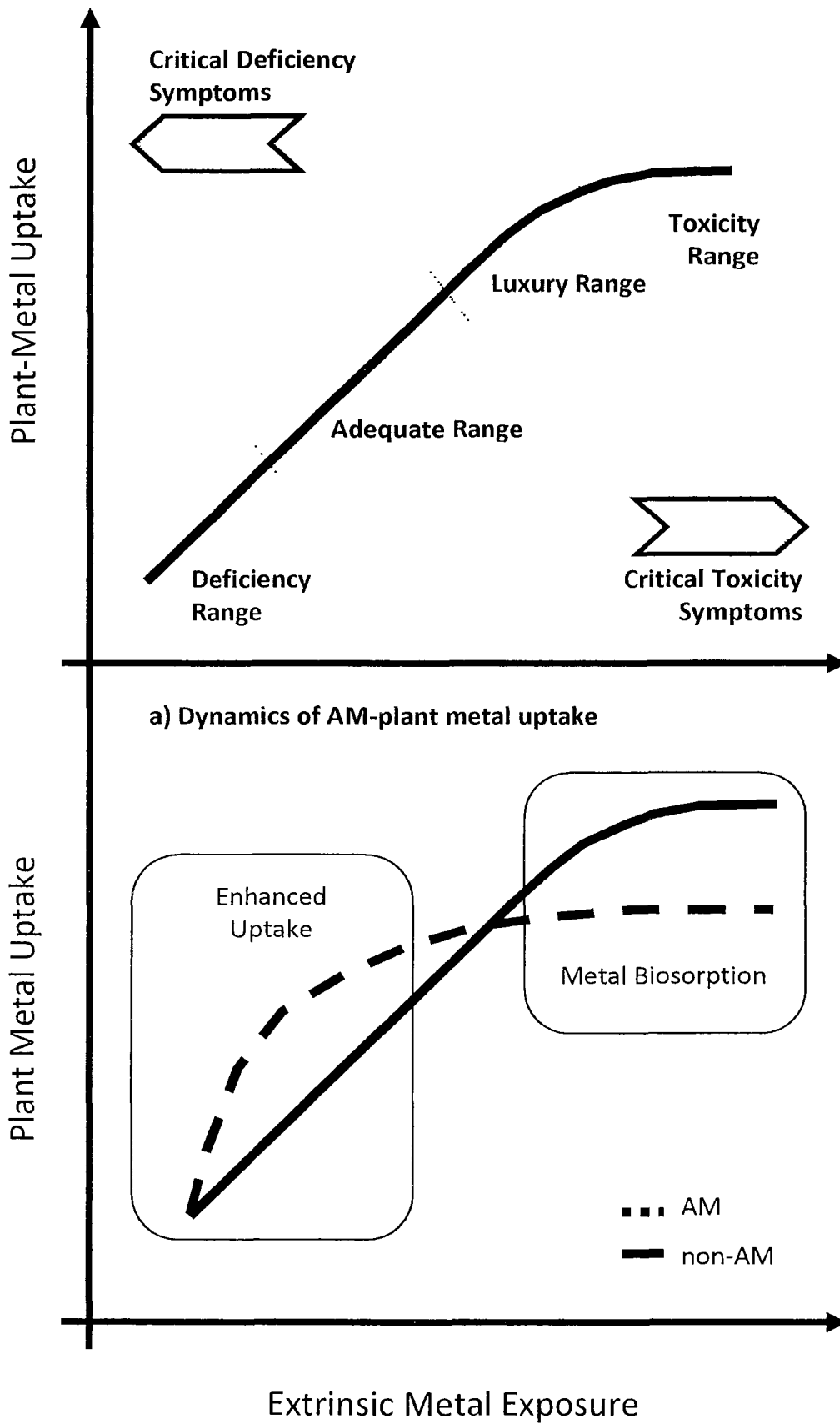
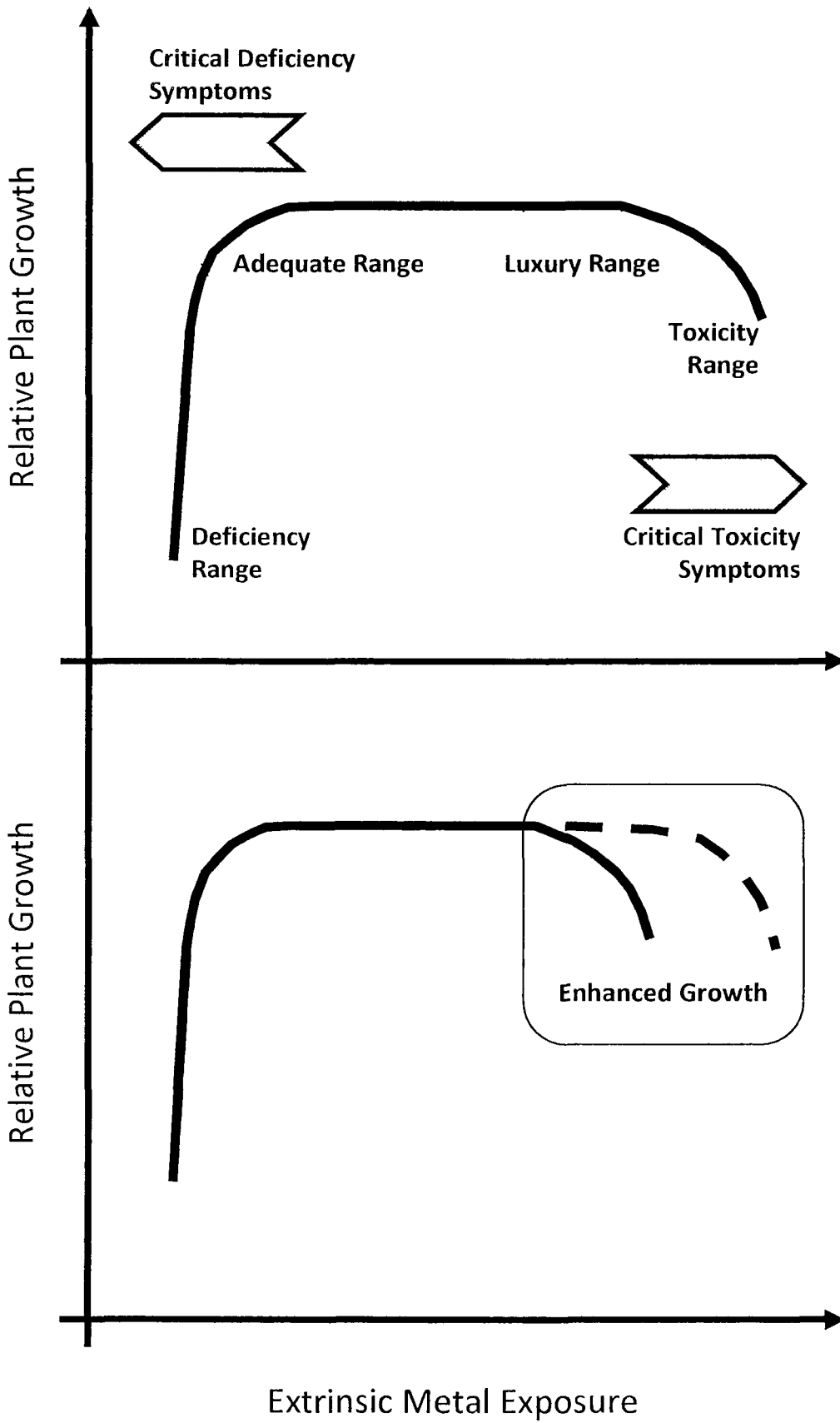


Figure 3.5 Conceptual model of relative plant growth integrating the role of AM symbiosis. Designated are zones referring to the 'metal biosorption' hypotheses resulting in enhanced AM-plant growth at high soil-HM level.

Figure 3.5



3.4 Discussion

The meta-analytical findings presented here have revealed that the AM fungi play a dynamic role in plant metal uptake and plant growth when subjected to increasing soil metal exposure levels, especially within the context of the hypotheses presented in Chapter 1.2. First, as predicted by the 'enhanced uptake' hypothesis, it was determined that AM colonized plants show greater metal uptake than non-AM plants at low soil metal levels. This relative increase in metal uptake could be attributed to the fact that the mycorrhizosphere has a more expansive uptake capacity than the rhizosphere alone which can enhance the uptake and translocation of metals from extraradical hyphae to plant roots, and then shoots (Burleigh et al. 2003, González-Guerrero et al. 2005, Rosewarne et al. 1999). As such, the mycorrhizosphere can provide greater access to soil resources which benefit the plants' health status (particularly when subjected to environmentally stressful conditions) by supplementing their nutrient requirements. On the other hand, as predicted by the 'metal biosorption' hypothesis, the AM effect-size response shifted with increasing soil metal exposure indicating lower AM than non-AM plant metal uptake. Correspondingly, the AM plants also had a nearly 2-fold increase in biomass under these increasingly high soil metal conditions. This shift in 'AM effect' toward decreasing plant metal uptake (rather than increasing it) is attributed to the AM-induced metal biosorption processes which can reduce metal bioavailability in the mycorrhizosphere. In this case, the extraradical hyphae have previously been shown to hold a high affinity for binding metal ions directly to their negatively charged surface constituents (*e.g.* carboxyls, hydroxides, oxy-hydroxides, sulfhydryls), in addition to their

exudation of metal chelators which can precipitate metal-ligands in the soil solution as well as their sequestration of metal ions in their fungal tissues (Apak 2002; Gadd 1993; Gonzalez-Chavez et al. 2002). Further to these mechanisms, the proliferation of AM-roots and extraradical hyphae is also recognized for increasing the soil's retention capacity and stabilizing its matrix structure compared to non-AM roots (Augé et al. 2001; Bearden & Peterson 2000; Miller & Jastrow 1990). Altogether, these characteristics could contribute to buffering the soil environment and stabilizing its physicochemical properties in relation to environmental stress. Consequently, these effects can benefit AM-plant growth by reducing soil metal bioavailability and limiting metal uptake to delay the onset of metal phytotoxicity.

In view of these new observations, a conceptual model of plant metal uptake is presented based on the notions of Kirk (2002) and Marschner (1995) which now accommodates both the 'enhanced uptake' and 'metal biosorption' hypotheses to provide an integrative perspective as to the role of AM symbiosis in plant stress tolerance (Fig. 3.4 and 3.5). Notably, this model suggests that the mycorrhizosphere can enhance metal uptake under certain environmental conditions, but then reduce metal uptake under others. For this reason, AM plant metal uptake is represented by a hyperbolic curve compared with the more linear uptake of non-AM plants. Here, it is suggested that plants may invest in mycorrhizal 'enhanced uptake' to supplement their essential nutrient requirements under resource deficiency conditions (*i.e.* low soil metal concentrations), but then benefit from 'metal biosorption' processes to buffer the soil environment when faced with potentially toxic metal exposure (*i.e.* high soil metal

concentrations – Audet & Charest 2006; Chen et al. 2004; Joner et al., 2000; Weissenhorn et al. 1995). In this latter case, the process of AM-induced metal biosorption is considered to be an extrinsic stress-avoidance strategy which could complement the intrinsic stress resistance processes of phytochelation and internal sequestration pathways (Cobbett 2000; Maier et al. 2003). In this model, this enhanced stress tolerance is expressed as an increase in relative plant growth among AM than non-AM plants, especially within the toxicity range. Notably, a critical aspect of the model presumes that the AM fungi and the mycorrhizosphere should be both prolific and intrinsically tolerant under such environmentally stressful conditions. In this respect, the meta-analytical findings showed that AM root colonization levels tended to be significantly higher for the low soil metal concentration interval than high interval, which suggests the existence of a metal toxicity burden which could deleteriously affect the host plant's investment in mycorrhizal symbiosis. Under experimental conditions, such a toxicity burden has been manifested as decreases in AM spore germination and extraradical hyphal development (Del Val et al. 1999; Leyval et al. 1997; Pawlowska & Charvat 2004; Weissenhorn et al. 1995). Nevertheless, of the studies included in this meta-analysis, many AM-plants expressed high levels of root colonization (up to 80%) even within the high soil metal concentration interval implying that the symbiosis was active despite soil metal conditions nearly 3 orders of magnitude greater than control-type (non-contaminated) soils. By recognizing that increasing soil metal concentrations can severely challenge plant growth and development (Chapter 2), the AM fungi should also ultimately be burdened in spite of any beneficial mycorrhizospheric effects toward

buffering the proximal soil growth environment. As such, a discrepancy exists regarding using the % length of root colonization as an estimate of mycorrhizal investment, particularly since this parameter does not provide insight into expansiveness of the mycorrhizosphere or its 'enhanced uptake' and 'metal biosorption' capacity. And so, a more comprehensive experimental investigation would be required to more effectively assess these hypotheses, as provided in Section III – Experimental Analysis of this thesis.

3.5 References

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3.6 Supplementary data

Table 3.2 Arbuscular mycorrhizal (AM) and plant species included in the meta-analysis

AM species	Plant species	Soil HM	References
<i>Glomus caledonium</i> (Nicol & Gerd) Gerdemann & Trappe	<i>Zea mays</i> L	Zn	Chen et al 2004b
	<i>Pteris vittata</i> L	As, U	Chen et al 2006
<i>Glomus intraradices</i> Schenck & Smith	<i>Nicotiana rustica</i> L	Zn	Audet and Charest 2006
	<i>Nicotiana tabacum</i> L	Cd	Janouskova et al 2005
	<i>Pisum sativum</i> L	Cd	Rivera-Becerril et al 2002
	<i>Pteris vittata</i> L	As, U	Chen et al 2006
<i>Glomus mosseae</i> (Nicol & Gerd) Gerdemann & Trappe	<i>Allium cepa</i> L	Zn, Co	Gildon and Tinker 1983
	<i>Cannabis sativa</i> L	Cd , Cr, Ni	Citterio et al 2005
	<i>Pteris vittata</i> L	As, U	Chen et al 2006
	<i>Trifolium pratense</i> L	Cd, Pb, Zn	Bi et al 2003, Chen et al 2003, Li and Christie 2001, Vivas et al 2003a
	<i>Trifolium repens</i> L	Cd, Zn	Vivas et al 2003b, Zhu et al 2001
	<i>Trifolium subterraneum</i> L	Cd	Joner and Leyval 1997
	<i>Zea mays</i> L	Cd, Cu, Mn, Pb, Zn	Chen et al 2004a, Weissenhorn et al 1995
<i>Glomus sp</i> ^a	<i>Cynodon dactylon</i> (L) Pers	As	Leung et al 2006
	<i>Glycine max</i> L	Cd, Cu, Fe, Mn, Zn	Heggo et al 1990
	<i>Lolium perenne</i> <i>multiflorum</i> (Lam) Parnell	Cd	Yu et al 2005
	<i>Pteris vittata</i> L	As, U	Leung et al 2006

^a Consortium of *Glomus sp*

Table 3.3 Metal species and soil concentration ranges included in the meta-analysis

Metal species	Concentration range (mg kg⁻¹ dry soil)	References
As	1 - 106	Chen et al 2006, Leung et al 2006
Cd	0.001 - 8.371	Chen et al 2004a, Citterio et al 2005, Heggo et al 1990, Janouskova et al 2005, Joner and Leyval 1997, Rivera-Becerril et al 2002, Vivas et al 2003b, Weissenhorn et al 1995, Yu et al 2005
Co	5 - 75	Gildon and Tinker 1983
Cr	50 - 300	Citterio et al 2005
Cu	0.91 - 45	Heggo et al 1990, Weissenhorn et al 1995
Fe	7.9 - 77.4	Heggo et al 1990
Mn	2.2 - 310	Heggo et al 1990, Weissenhorn et al 1995
Ni	5 - 100	Citterio et al 2005
Pb	30 - 895	Vivas et al 2003a, Weissenhorn et al 1995
U	106	Chen et al 2006
Zn	0.19 - 1.220	Audet and Charest 2006, Bi et al 2003, Chen et al 2003, 2004b, Gildon and Tinker 1983, Heggo et al 1990, Li and Christie 2001, Weissenhorn et al 1995, Zhu et al 2001

Meta-references

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Chapter 4

Resource allocation plasticity and plant metal partitioning

4.1 Objectives

As discussed in Chapters 2 and 3, soil metal exposure gives rise to a number of environmental challenges resulting in a fundamental compromise between plant growth and stress tolerance. Besides investing in mycorrhizal symbiosis to circumvent these challenges, it is postulated that plants may also adjust their relative biomass allocation and metal distribution to different organ systems (*i.e.* roots *v.* shoots) under these conditions in referring to the processes of allocation plasticity and metal partitioning (Bell & Lechowicz 1994, Gedroc et al. 1996, Wilson 1988*a*, 1988*b*). Accordingly, plants have previously been categorized with respect to their specific allocation plasticity and metal-partitioning strategy to then distinguish between 'slow-grower' and 'fast-grower' species (Grime 1979, Chapin 1980). In the present context of AM plant association extending from the previous meta-analytical findings, trends in allocation plasticity and metal partitioning are investigated here among different plant guilds when subjected to soil metal concentrations ranging from low (trace) to high (toxic) levels. More specifically, these notions and hypotheses are assessed in relation to four different plant families selected for representing distinctive growth strategies and having differential investments in AM symbiosis (*i.e.* mycotrophic *v.* non-mycotrophic life history strategies) the Brassicaceae, Fabaceae, Poaceae, and Solanaceae.

4.2 Methods

(a) Meta-analysis

Based on methods by Hedges & Olkin (1985) and Lipsey & Wilson (2001), trends in allocation plasticity and metal partitioning were assessed by comparing the relative allocation of biomass and relative distribution of metals to either root or shoot organs in relation to increasing soil metal exposure levels among four different plant families: the Brassicaceae, Fabaceae, Poaceae, and Solanaceae. These families were chosen since many of their species have previously been studied in the interests of phytoremediation and provided sufficient degrees of freedom for a robust statistical analysis. Following a thorough scientific literature review, 19 studies were selected according to the criteria that they dealt with herbaceous plants and provided measures of plant biomass and metal uptake for both roots and shoots. The selection criteria also required that the studies consist of greenhouse experiments having the soil mineral composition described and the data presented in tables to ensure the highest accuracy in data reporting. Overall, these studies all represented potted plants grown until maturity (ranging between 6 to 12 weeks depending on the species) and subjected to at least 4 weeks of metal-exposure. Key variables included plant organ dry mass (g), plant metal concentration (mg kg^{-1} dry mass) and (or) content (mg per organ or $\text{mg per whole plant dry mass}$), and total extractable soil metal concentration (mg kg^{-1} dry soil). All of the metals (e.g. Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) with their respective soil concentration ranges are appended (Supplementary Data) as well as the plant species analyzed in the study.

(b) Metrics

Indexes of allocation plasticity and metal partitioning were used to assess the relative allocation of dry mass (DM) and distribution of metal concentrations ([Metal]) to roots versus shoots. The equations for allocation plasticity (1) and plant metal partitioning (2) are defined as:

$$\frac{DM_{root}}{DM_{shoot}} \quad (1)$$

$$\frac{[Metal]_{root}}{[Metal]_{shoot}} \quad (2)$$

In this case, values ≥ 1 indicated a greater or equal allocation to roots than shoots, whereas values < 1 indicated a greater allocation to shoots than roots.

(c) Statistical analyses

Based on methods by Zar (1999), polynomial equations were calculated using a stepwise regression procedure to obtain the highest fitted statistical model. The residual-fit spread was also examined to ensure that the data meet normal distribution and homoscedasticity assumptions, meanwhile Cook's distances were used calculated to test for outliers (data not shown). All the data were Log-transformed to enhance the fit of the models to meet the assumptions of parametric analyses. Notably, statistically relevant models were determined for each plant family with the exception of the Solanaceae which had a low data resolution due to small sample size and narrow data distribution. The polynomial equations $[f(x)]$, coefficients of determination $[r^2]$, degrees of freedom $[df]$, and p-value estimates were determined using S-Plus 8.0 (Insightful Corp., Seattle, WA).

4.3 Results

All of the plotted data are grouped by plant family and fitted with two polynomial smoothing curves derived from the regression analyses. The upper solid line is fitted to the data representing the highest significant polynomial equations (*e.g.* 3rd degree polynomial), whereas the dashed line is fitted to the linear equations (*e.g.* 1st degree polynomial). The index of biomass partitioning is shown plotted as a function of plant metal content (Fig. 4.1*a*) and soil metal concentration (Fig. 4.1*b*). Here, the highest polynomial line is representative of the Fabaceae and Poaceae showing a shift in biomass partitioning from roots to shoots (*e.g.* shift in the proportional allocation to shoots) and back to roots as plant and soil metal levels increase. By contrast, the Brassicaceae did not show this trend such that the proportional biomass allocation remained relatively constant for this interval as represented by the non-significant linear equation (dashed line). Findings for the Solanaceae were not considered representative of any significant trends due to a small sample size and narrow data distribution. The index of plant metal partitioning is shown plotted as a function of plant metal content (Fig. 4.2*a*) and soil metal concentration (Fig. 4.2*b*). Here, the highest polynomial curve is most representative of the Fabaceae, Poaceae, and Brassicaceae. As above, this trend indicates an increasing shift in plant metal concentration from roots to shoots (*e.g.* shift in the proportional distribution of metals to shoots) as the soil metal concentration increased. However, this significant relationship was not found for the Brassicaceae in relation to the plant metal content; a tendency that may be attributed to a lower data

resolution and narrow distribution, as with the Solanaceae. All of the associated regression statistics are summarized in Tables 4.1 and 4.2.

Based on these findings, a conceptual model of allocation plasticity (Fig. 4.3a) and plant-metal partitioning (Fig. 4.3b) is described comparing 'fast-grower' and 'slow-grower' plant growth strategies. Typifying an inversed parabolic curve, the 'fast-growers' are characterized by a shift in biomass partitioning to roots under low metal exposure levels, then shoots at intermediate levels, and again roots at high levels. Likewise, the 'slow growers' follow a similar tendency albeit less prominently. As for the plant metal partitioning, both growth strategies partition metals more to roots relative to shoots with increasing metal exposure, but this tendency is more prominent among 'slow' than 'fast-growers'.

Table 4.1 Summary of polynomial equations for biomass partitioning (root shoot¹) as a function of plant metal content (mg dry mass⁻¹) and soil metal concentration (mg kg⁻¹ dry soil)

Parameter	Family	Biomass partitioning			
		$f(x)$	r^2	df	p
Plant metal content	Brassicaceae	$0.01x^2 - 2.5$	0.18	15	0.22
	Fabaceae	$0.4x + 0.2x^2 - 0.02x^3 - 1.4$	0.58	20	$>10^{-4}$
	Poaceae	$0.1x + 0.05x^2 - 0.02x^3 + 0.5$	0.32	25	>0.05
	Solanaceae	$-0.01x - 0.9$	0	6	0.79
Soil metal concentration	Brassicaceae	$0.1x - 2.5$	0.11	24	0.09
	Fabaceae	$3.27x + 0.5x^2 - 0.4x^3 - 6.4$	0.47	20	$>10^{-3}$
	Poaceae	$0.3x - 0.7$	0.44	28	0.05
	Solanaceae	$0.01x - 0.9$	0.78	6	0.78

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

Table 4.2 Summary of polynomial equations for plant metal concentration partitioning (root shoot⁻¹) as a function of plant metal content (mg dry mass⁻¹) and soil metal concentration (mg kg⁻¹ dry soil).

Parameter	Family	Plant-HM concentration partitioning			
		$f(x)$	r^2	df	p
Plant-HM Content	Brassicaceae	$-0.2x+0.03x^2-0.1$	0.34	19	>0.01
	Fabaceae	$x0.8+0.1x^2+1.7$	0.74	21	>10 ⁻⁶
	Poaceae	$0.1x-0.2x^2+2.6$	0.42	8	0.1
	Solanaceae	$-0.28x+1.1$	0.49	6	0.05
Soil-HM Concentration	Brassicaceae	$0.4x+0.1x^2-0.1x^3+0.3$	0.1	95	>0.05
	Fabaceae	$2.6x+1.3x^2+0.01x^3-0.x^4+1.5$	0.43	19	>0.05
	Poaceae	$0.4x+0.2x^2+0.1x^3-0.01x^4+1.5$	0.41	25	>10 ⁻³
	Solanaceae	$-0.2x+0.5$	0.1	20	0.3

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

Figure 4.1 Biomass partitioning (root shoot^{-1}) as a function of plant metal content (mg dry mass^{-1}) (a) and soil metal concentration (mg kg^{-1} dry soil) (b). The upper solid line is fitted to data representing the highest significant polynomials, whereas the dashed line is fitted to the data representing the non-significant equations.

Figure 4.1

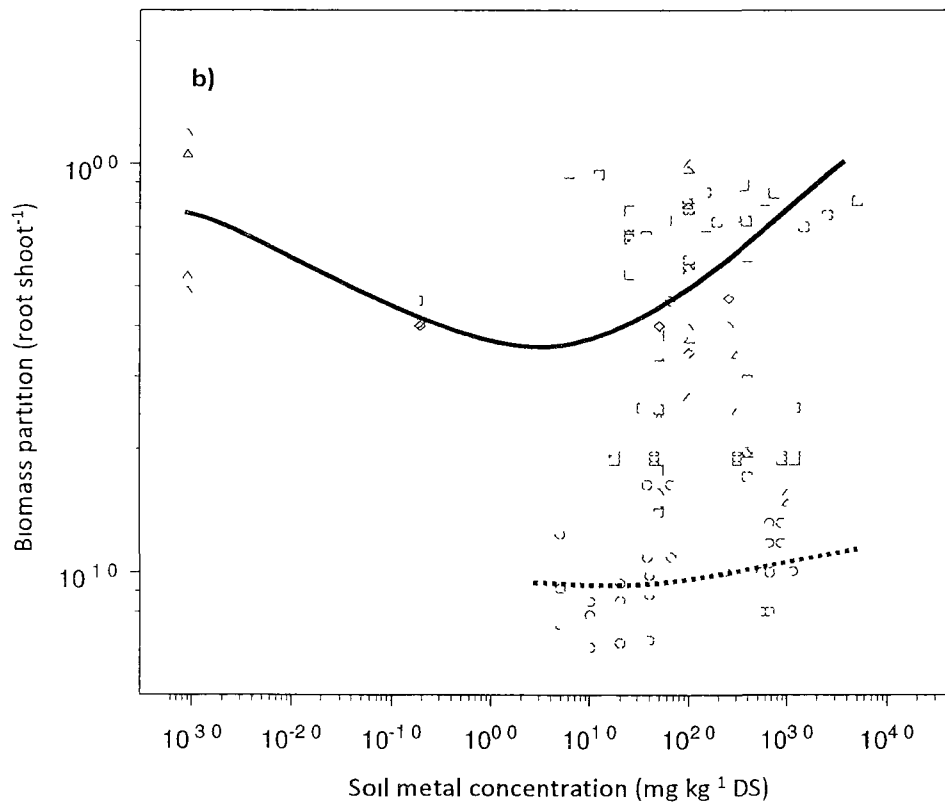
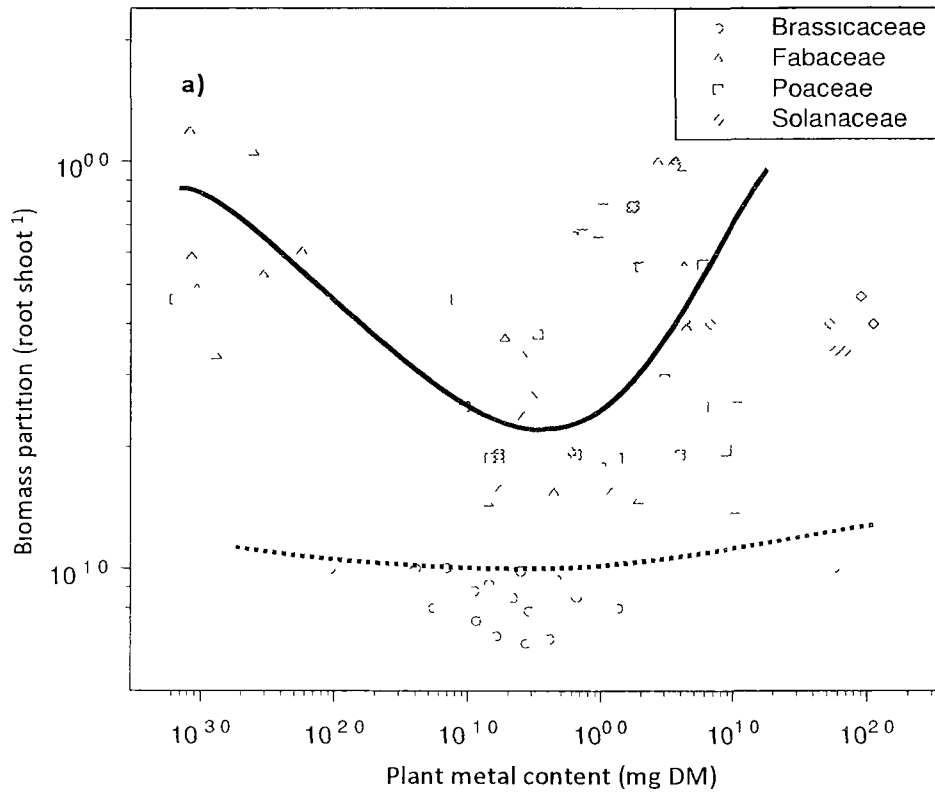


Figure 4.2 Plant metal concentration partitioning (root shoot⁻¹) as a function of plant metal content (mg dry mass⁻¹) (a) and soil metal concentration (mg kg⁻¹ dry soil) (b). The upper solid line is fitted to data representing the highest significant polynomials, whereas the dashed line is fitted to the data representing the non-significant equations.

Figure 4.2

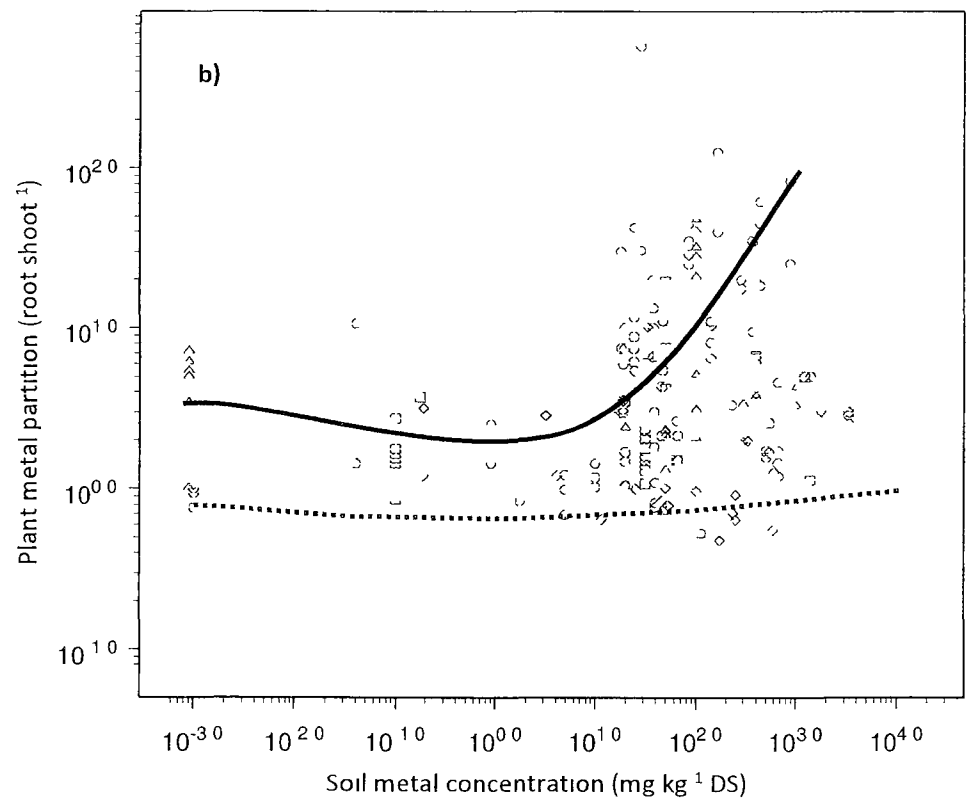
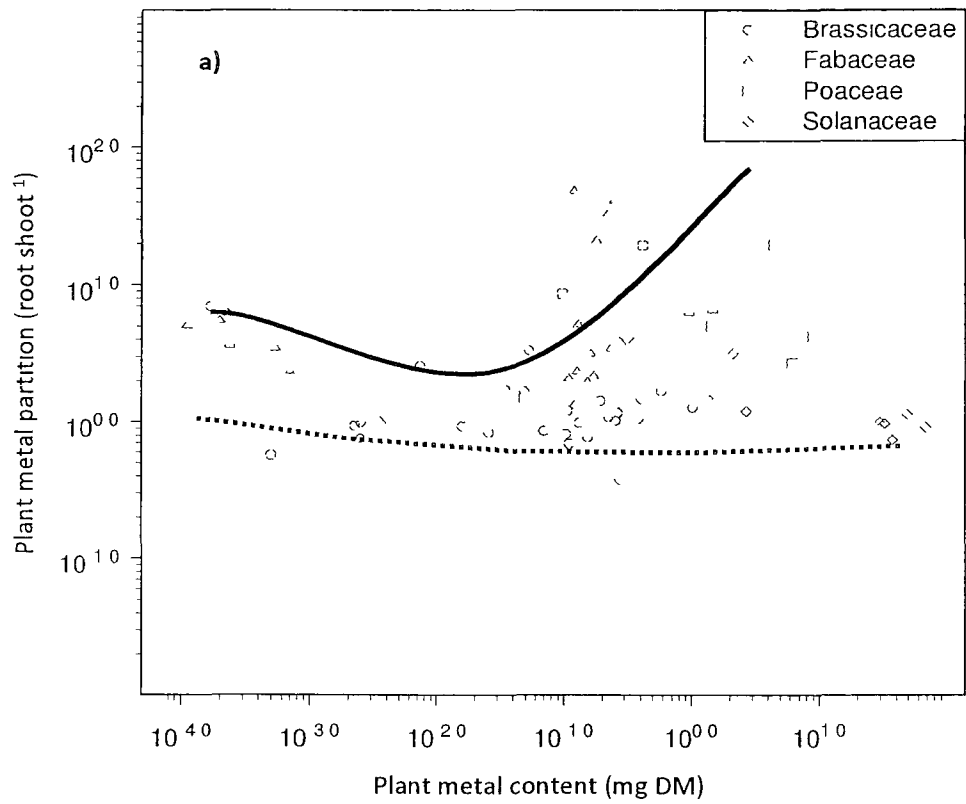
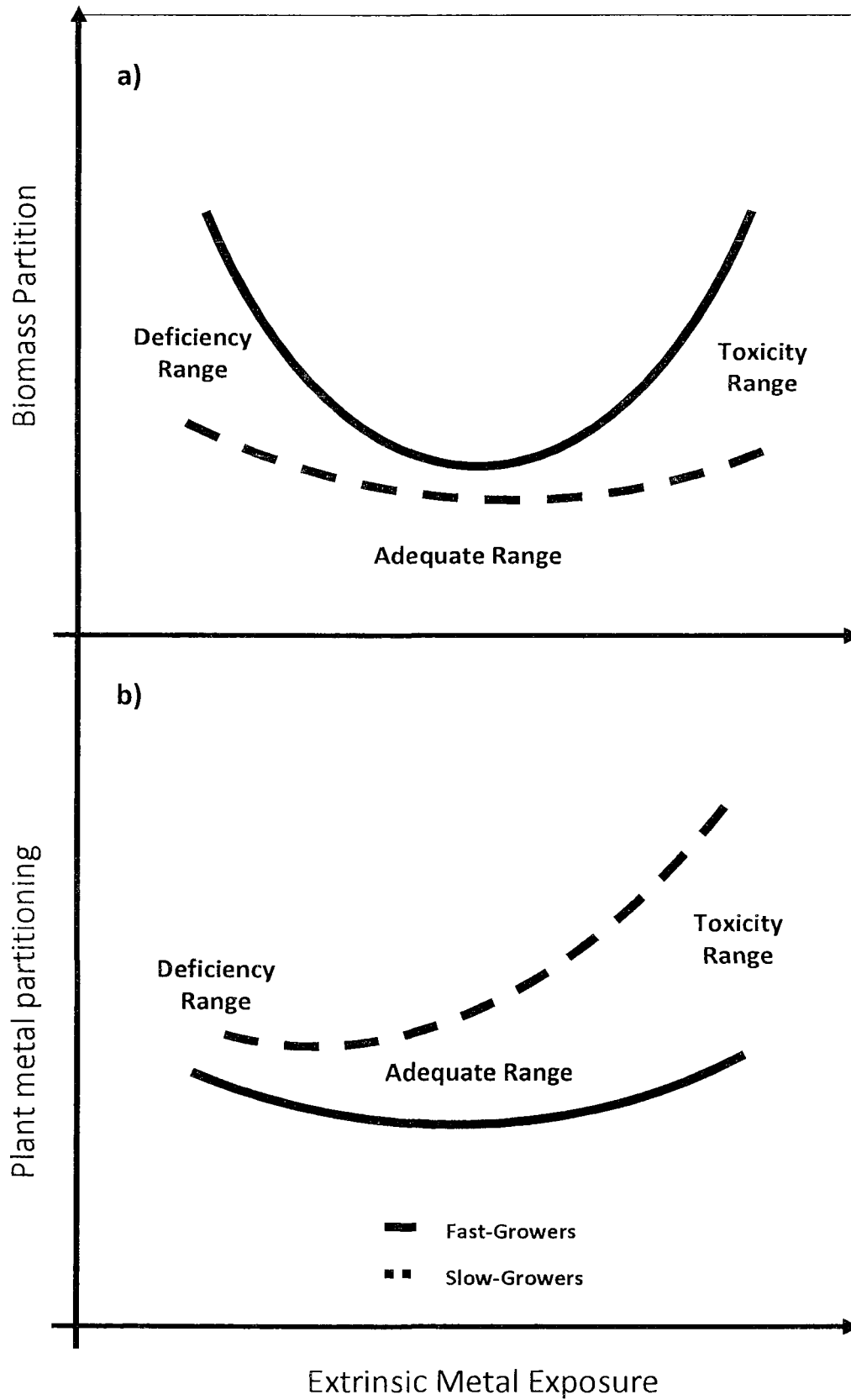


Figure 4.3 Conceptual model of allocation plasticity (a) and plant-metal partitioning (b) for 'fast-grower' (solid line) and 'slow-grower' (dotted line) types. Designated are three growth zones representing the deficiency, adequate, and toxicity ranges.

Figure 4.3



4.4 Discussion

The meta-analytical findings presented here have revealed a key relationship regarding plant allocation plasticity in which plants may modify their relative allocation of biomass from roots to shoots depending on soil metal exposure levels. In this regard, the greater proportional allocation of plant resources towards root development could contribute in overcoming challenges commonly associated with both nutrient deficiency and metal toxicity, such as metal imbalances and mutual antagonisms, by enhancing the roots' surface area and uptake capacity (Horst et al. 1990; Wilson 1988*b*). More subtly, the rhizosphere may also buffer the proximal soil-environment through the increased exudation of mucilage consisting of organic acids (*e.g.* polyuronic acids) which can impact the key soil characteristics, such as pH and redox potential, and contribute in mobilizing mineral nutrients (Marschner 1995; Mench et al. 1988; Neumann & Römheld 2000; Ray et al. 1988). While root exudation has a general function of protecting the root apical zones from desiccation and facilitating ion uptake, it is also known to contribute in promoting the development of beneficial microbial communities (*e.g.* mycorrhizal fungi and other soil microorganisms – St-Arnaud & Elsen 2005; Yergeau et al. 2006). Consistent with past hypotheses (Chapter 3), these soil microorganisms can contribute significantly toward buffering growth conditions via 'metal-binding' and soil aggregation processes (Joner et al. 2000; Mullen et al. 1989; Morel et al. 1986, 1991). Still, the patterns of allocation plasticity reported here are not the same between all plant families: for instance, the Fabaceae and Poaceae expressed a much higher degree of allocation plasticity than the Brassicaceae which showed no specific pattern of

biomass allocation. As discussed more thoroughly below, such differences between plant family guilds could be associated to their respective life history strategies. As for relationships tested among the Solanaceae, these findings were not considered to be representative of any biological trend due to a small sample size and narrow data distribution. Similarly to the trend of shifting biomass allocation, the plant's metal partitioning is also shown to differ in relation to increasing soil metal concentration such that roots tend to have proportionally higher metal concentrations in their tissues under potentially toxic exposure levels relative to shoots—a trend observed more prominently among the Brassicaceae than the Fabaceae and Poaceae. In plant cells, metal ions often cause oxidative stress through binding to enzymes and prosthetic groups, thus disrupting essential metabolic functions (Baccouch et al. 1998, Cho & Seo 2005, Schützendubel & Polle 2002). Plants may express some metabolic plasticity in regulating metal distribution more to roots in order to reduce oxidative stress in photosynthetic shoot organs. This perspective could provide a nuance to the 'metal defense hypothesis' which postulates that plants mobilize and (or) hyper-accumulate metals in shoots to deter insect herbivores (Behmer et al. 2005, Davis & Boyd, 2000, Pollard & Baker 1997). However, it is recognized that more detailed investigations would be needed to verify these respective aspects of plant metal-partitioning.

From the findings above, a model of allocation plasticity and plant metal partitioning is proposed here based upon the notions of 'slow' and 'fast growers' plant characteristics and life history strategies proposed by Grime (1979) and Chapin (1980). Owing to their relatively high growth rates, the 'fast-growers' tend to have a cup shaped

pattern of biomass allocation indicating a high degree of allocation plasticity, contrary to the more subdued and saucer-shaped allocation plasticity profile of 'slow-growers'. As discussed previously, the ability to adjust the relative biomass to roots could improve access to limiting resources and promote more favorable conditions within the proximal soil-environment due to the activity of roots and beneficial soil microbial. By contrast, plants having 'slow-grower' characteristic could show a higher level of metabolic plasticity due to their active modulation metal partitioning between roots and shoots and owing to their potentially more effective stress-resistance metabolisms (*e.g.* phytochelatin and metallothionein production). From the present meta-analytical findings, the Brassicaceae are considered as mostly expressing the 'slow-grower' characteristics described above which could enable them to tolerate potentially toxic metal conditions and contribute to their status as hyperaccumulators, as evidenced by many *Thlaspi* and *Brassica* species (Freeman et al. 2004; Marchiol et al. 2004; Peer et al. 2003). On the other hand, the Fabaceae and Poaceae are considered as mostly expressing 'fast-grower' characteristics enabling their adaptation to contaminated environments through the modulation of their biomass allocation from roots to shoots, as in the case of *Trifolium* and *Lolium* species (Arienzo et al. 2004; Bidar et al. 2007). Notably, these plant families differ regarding their investment in mycorrhizal symbiosis, especially under environmentally stressful conditions. Typically mycotrophic plant families (Smith & Read 1997), such as the Fabaceae, Poaceae and Solanaceae, could invest in mycorrhizal symbiosis as an extrinsic stress tolerance strategy to complement their intrinsic resistance mechanisms; whereas, the typically non-mycotrophic families,

such as the Brassicaceae, may rely more on phytochelatin production and sequestration pathways (Cobbett 2000; Cobbett & Goldsbrough 2002).

In conclusion, the meta-analytical findings presented here indicate that metal exposure gives rise to various stress tolerance strategies relating to the relative investment in allocation plasticity and metal partitioning. Notably, this investment may correspond with the life history strategies of different plant guilds relating to their investment in symbiotic associations; a tendency which could be indicative of the compromise existing between intrinsic versus extrinsic stress tolerance strategies and adaptations used by plants to circumvent metal stress. By integrating these notions into a conceptual framework, a new perspective of plant adaption to environmental stress has been proposed which may provide a testable basis for further experimental investigations.

4.5 References

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4.6 Supplementary data

Table 4.3 Plant families and species comprised in the meta-analysis

Family	Species	Reference
Brassicaceae	<i>Brassica carinata</i>	Marchiol et al. 2004b
	<i>Brassica napus</i>	Marchiol et al. 2004a, 2004b
	<i>Brassica juncea</i>	Blaylock et al. 1997; Marchiol et al. 2004b; Su and Wong 2004
	<i>Raphanus sativus</i>	Marchiol et al. 2004a, 2004b
	<i>Thlaspi arvense</i>	Hammer and Keller 2002
	<i>Thlaspi caerulescens</i>	Ayoub et al. 2003; Hammer and Keller 2002; Mcgrath et al. 1997
	<i>Thlaspi ochroleucum</i>	Mcgrath et al. 1997
Fabaceae	<i>Pisum sativum</i>	Rivera-Becerril et al. 2002; Zhu et al. 2001
	<i>Trifolium pratense</i>	Chen et al. 2003; Bi et al., 2003; Vivas et al. 2003
	<i>Trifolium repense</i>	Li and Christie 2001; Zhu et al. 2001
Poaceae	<i>Andropogon virginicus</i>	Pichtel et al. 2000
	<i>Hordeum vulgare</i>	Ayoub et al. 2003
	<i>Lolium perenne</i>	Li and Christie 2001; Zhu et al. 2001
	<i>Triticum aestivum</i>	Athar and Ahmad 2002
	<i>Zea mays</i>	Chen et al. 2004a, 2004b
Solanaceae	<i>Nicotiana glauca</i>	Barazani et al. 2004
	<i>Nicotiana rustica</i>	Audet and Charest 2006

Table 4.4 Metal species and soil concentration ranges included in the meta-analysis

Metal species	Concentration range (mg kg ⁻¹ dry soil)	References
Cd	0.001-100	Athar and Ahmad 2002, Ayoub et al. 2003; Barazani et al. 2004; Chen et al 2004a; Hammer and Keller 2002; Marchiol et al. 2004a; McGrath et al 1997, Pichtel et al 2000; Rivera-Becerril et al 2002, Su and Wong 2004, Vivas et al 2003b.
Cr	20-165	Athar and Ahmad 2002, Barazani et al 2004, Marchiol et al 2004a, 2004b, McGrath et al 1997
Cu	5-1470	Athar and Ahmad 2002, Barazani et al. 2004; Hammer and Keller 2002, Marchiol et al 2004a, 2004b, McGrath et al 1997
Mn	110-590	Athar and Ahmad 2002, Barazani et al 2004, Weissenhorn et al 1995
Ni	15-600	Athar and Ahmad 2002; Barazani et al 2004; Marchiol et al 2004a, 2004b, McGrath et al 1997
Pb	25-1400	Athar and Ahmad 2002, Barazani et al 2004; Marchiol et al 2004a, 2004b, McGrath et al 1997; Pichtel et al 2000; Vivas et al 2003a
Zn	0.1-10230	Athar and Ahmad 2002, Audet and Charest 2006, Ayoub et al 2003, Barazani et al. 2004; Bi et al 2003, Chen et al 2003 2004b; Hammer and Keller 2002, Li and Christie 2001, Marchiol et al 2004a, 2004b; McGrath et al 1997, Su and Wong 2004; Zhu et al 2001

Meta-references

Athar R, Ahmad M (2002) Heavy metal toxicity effect on plant growth and metal uptake by wheat, and on free living *Azotobacter* Water Air Soil Pollut 138 165-180

Audet P, Charest C (2006) Effects of AM colonization on 'wild tobacco' plants grown in zinc-contaminated soil Mycorrhiza 16 277-283

Ayoub AS, McGraw BA, Shand CA, Midwood AJ (2003) Phytoavailability of Cd and Zn in soil estimated by stable isotope exchange and chemical extraction Plant Soil 252 291-300

Barazani O, Sathiyamoorthy P, Manadhar U, Vulkan R, Golan Goldhirsh A (2004) Heavy metal accumulation by *Nicotiana glauca* Graham in a solid waste disposal site Chemosphere 54 867-872

Bi YL, Li XL, Christie P (2003) Influences of early stages of arbuscular mycorrhiza on uptake of zinc and phosphorus by red clover from a low phosphorus soil amended with zinc and phosphorus Chemosphere 50 831-837

Blaylock MJ, Salt DE, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley BD, Raskin I (1997) Enhanced accumulation of Pb by soil applied chelating agents Environ Sci Tech 31 860-865

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Li X, Christie P (2001) Changes in soil solution Zn and pH and uptake of Zn by arbuscular mycorrhizal red clover in Zn contaminated soil Chemosphere 42 201-207

Marchiol L, Assolari S, Sacco P, Zerbi G (2004a) Phytoextraction of heavy metals by canola (*Brassica napus*) and radish (*Raphanus sativus*) grown on multicontaminated soil Environ Pollut 132 21-27

Marchiol L, Sacco P, Assolari S, Zerbi G (2004b) Reclamation of polluted soil phytoremediation potential of crop-related *Brassica* species Water Air Soil Pollut 158 345-356

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Section III

Experimental

Analysis

Introduction to *in vitro* root-organ and whole-plant greenhouse culture

The study of the AM fungi has fore mostly benefited from three breakthroughs in the field of *in vitro* cultivation (1) the isolation of monoxenic AM fungal species (Mosse and Hepper 1975), (2) the sub cultivation of these fungal isolates on artificial culture media (Strullu and Romand 1986, 1987), and, (3) the integration of the Ri T DNA transformed carrot roots into *in vitro* culture (Becard and Fortin 1988). This transformation was achieved through the insertion of the Ri T-DNA plasmid from the ubiquitous soil bacterium *Agrobacterium rhizogenes* into carrot tissues which are then induced to morphologically develop as roots only – a condition known as ‘hairy roots’ (Giri and Narasu 2000). With the optimization of this system (St-Arnaud et al 1995, 1996), these roots have shown greater AM intraradical colonization and sustain higher extraradical hyphal development than non transformed root organ culture types, which has led to their preferential use in the study of plant and fungal physiology (Fortin et al 2002). In fact, the Ri T-DNA root-organ culture system has been widely used in the conservation of AM fungal germplasm, the determination of their taxonomy, the characterization of their life cycle, and the identification of metabolic processes unique to plant-fungus interactions (Declerck et al 2005, Fortin et al 2001). In this regard, the AM fungus *Glomus intraradices* (investigated in Chapters 5, 6, and 7) is among the best studied species in plant physiology and mycology. For these reasons, I selected the root organ culture system as a relevant experimental system to investigate the dynamic role of AM symbiosis in root metal uptake and stress-tolerance in concordance with the conceptual models presented in Section II.

Moreover, studies involving pot grown plants represent an underpinning of comparative plant physiology. In the present context, I designed two compartmental pot growth studies in the objective of bridging the notions presented in the meta-analytical and *in vitro* culture study components to expand our depiction of the AM symbiosis toward its impact on the soil environment (Chapters 6 and 7). Besides determining trends in AM plant metal uptake, metal partitioning and stress tolerance within the whole-plant system, these greenhouse culture systems have enabled me to discern between the rhizosphere (roots only), mycorrhizosphere (AM roots and extraradical hyphae), and hyphosphere (extraradical hyphae only) environments, and compare their respective zones of influence pertaining to the bioavailability of soil metals and the modulation of soil pH. In doing so, I have expanded our previous conceptual models to depict the impact of AM plant interactions on the soil environment. As such, I consider the revised model to be a relevant tool for environmental remediation practices by determining the fate of metal contaminants in relation with the activities of plants and soil micro organisms.

Note on publications from this Section

The contents in this Section were first published in the following proceedings

- Audet P, Charest C (2009) Contribution of AM symbiosis to *in vitro* root metal uptake From trace to toxic metal conditions Botany 87 913-921
- Audet P, Charest C (2010) Determining the impact of the AM-mycorrhizosphere on 'dwarf' sunflower Zn uptake and soil Zn bioavailability Journal of Botany doi:10.1155/2010/268450
- Audet P, Charest C (2010) Assessing the AM-mycorrhizosphere's stratum of influence plant metal uptake and soil metal bioavailability in a compartmental growth environment Applied and Environmental Soil Science doi:10.1155/2010/812157

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Chapter 5

***In vitro* root-organ culture: AM symbiosis and plant metal uptake**

5.1 Objectives

In Chapters 3 and 4, a conceptual model of plant growth and metal uptake was proposed integrating the highly influential role of the AM symbiosis. The model (Fig. 5.1a and 5.1b) suggests that the AM fungi hold a dual role in plant metal uptake by (1) increasing metal nutrient uptake at low (trace) exposure levels to supplement the plant nutritional status, but then (2) lessening uptake at high exposure levels through metal biosorption processes to alleviate plant metal toxicity and any subsequent nutrient imbalances. As such, I consider that AM fungi could hold a key role for phytoremediation purposes by enhancing metal phytostabilization processes, rather than metal phytoextraction. In this Chapter, an *in vitro* carrot root-organ system in dual-culture with an AM fungus is presented to test the previous hypothetical models using the essential micronutrient zinc (Zn) as a typical metal contaminant which is known to reach environmentally toxic levels due to extensive fertilizer application, metal galvanization, and rubber vulcanization (Barceloux 1999; Chaney 1993; Cavagnaro 2008). With this experimental design, the goal was to compare the patterns of AM versus non-AM root metal uptake, to discern the relative contribution of extraradical hyphal uptake and translocation to host roots, and to determine any potential effects metal toxicity on root mass production, AM root colonization, and fungal spore development.

Figure 5.1 Conceptual model of AM-plant metal uptake (a) and relative plant growth (b) in relation to increasing extrinsic metal concentrations. Designated are zones referring to the 'enhanced uptake' and 'metal biosorption' hypotheses. AM plants show greater metal uptake than non-AM plants at low metal exposure levels, but lower uptake at high metal exposure level. Accordingly, AM plants have greater metal stress tolerance compared to non-AM plants as expressed by an increased relative growth status.

Figure 5.1

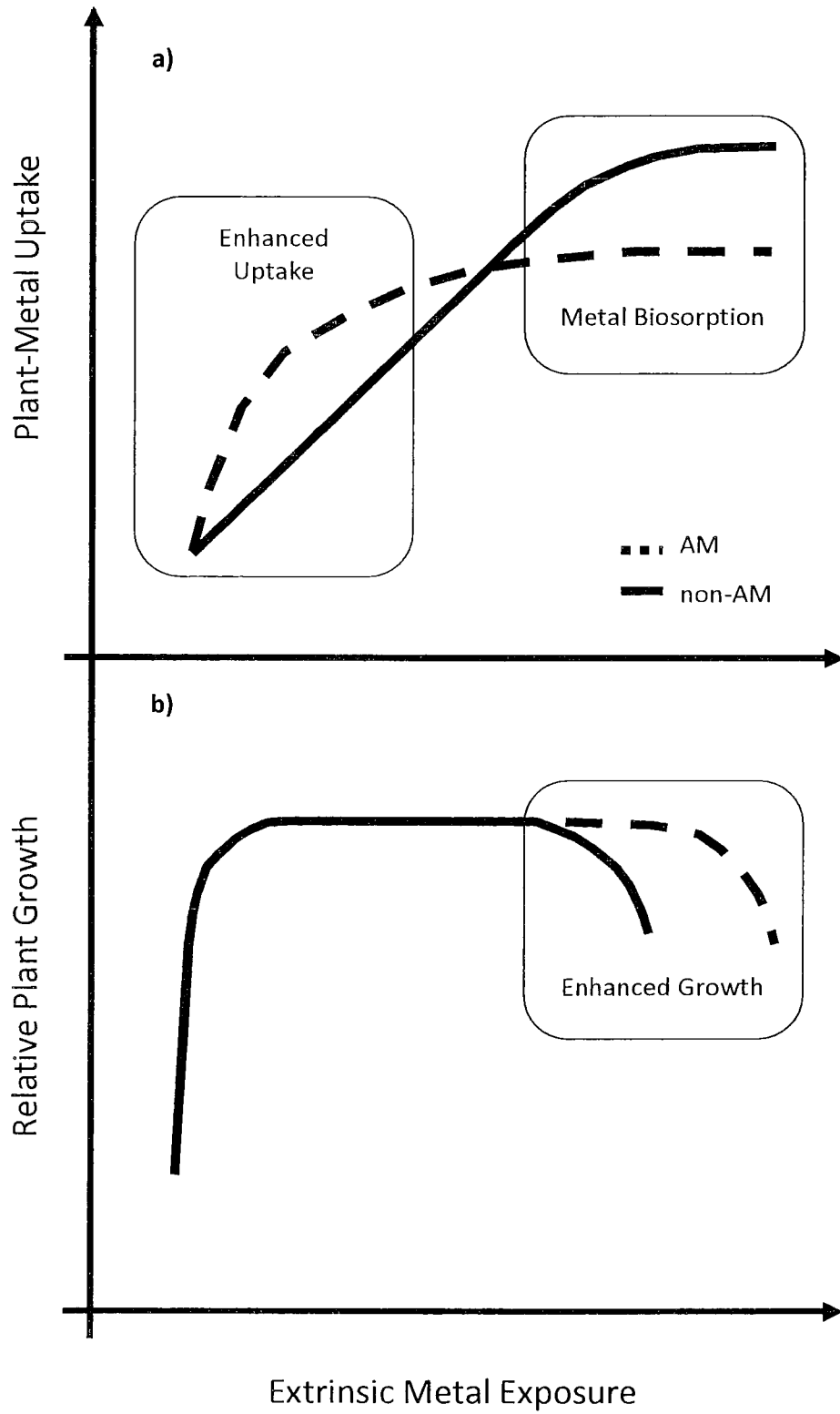


Figure 5.2 Top view schematization of the non-AM (a) and AM-colonized (b) Ri T-DNA carrot root-organs in one compartment plates with the latter showing extraradical hyphae and fungal spores. Also shown are top-view (c) and side-view (d) of AM roots in two compartment plates showing roots and hyphae in the root-hyphal compartment (RHC) and hyphae and spores only in the hyphal compartment (HC).

Figure 5.2

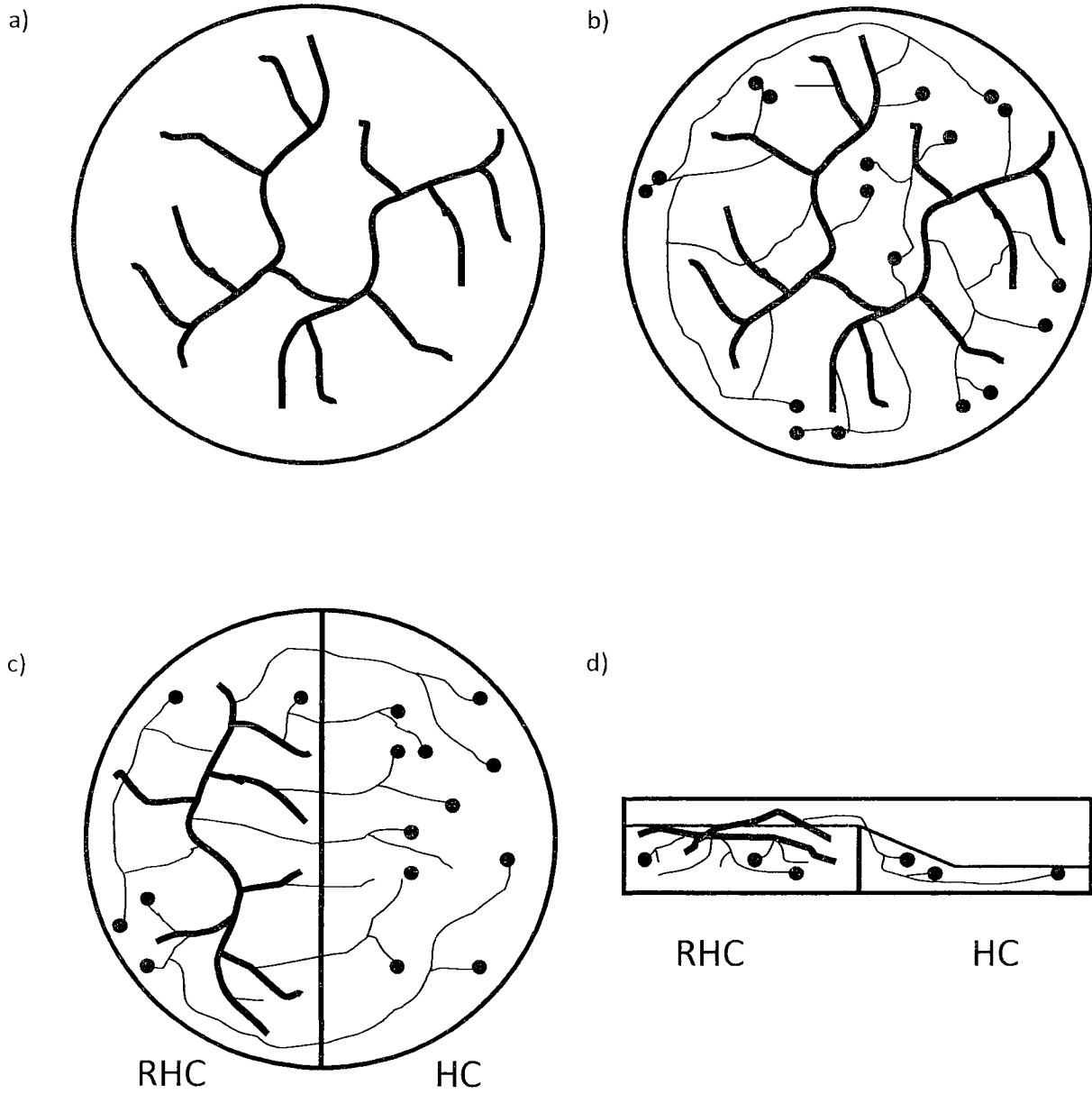
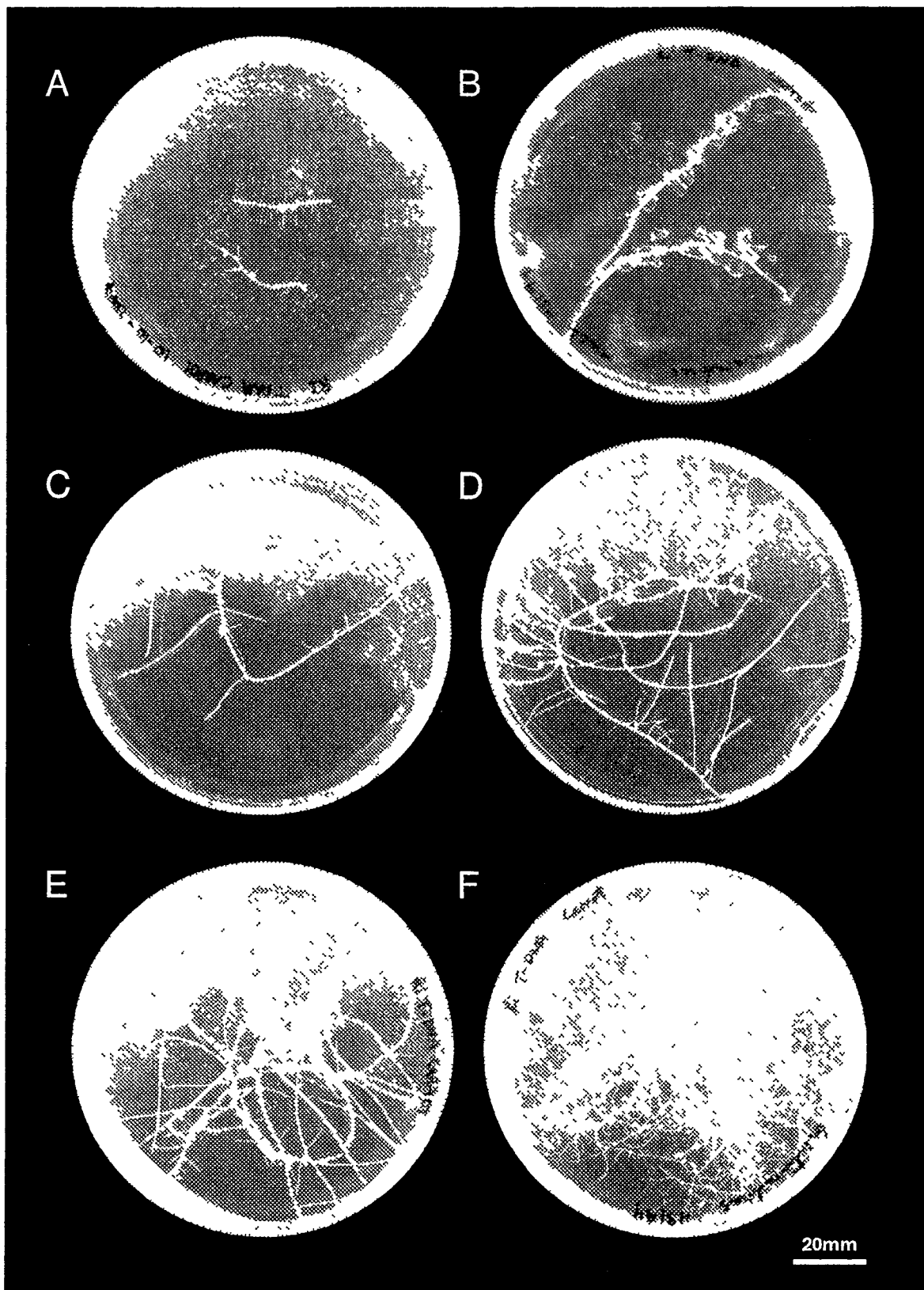


Figure 5.3 Developmental stages of the Ri T-DNA carrot root-organs from weeks 1 to 8.

Figure 5.3



5.2 Methods

In two successive experiments, Ri T-DNA transformed carrot roots (*Daucus carota* L.) were inoculated (or not) with propagules of the AM fungus *G. intraradices* (*Glomus intraradices* Schenck & Smith – isolate DAOM 181602) and grown in a low mineral medium either in one- or two-compartment Petri plates (100 mm x 15 mm) according to the methods of St-Arnaud et al. (1995, 1996). The medium contained sucrose as the primary source of carbon for roots and was amended with one of four Zn concentrations (0, 0.1, 0.5, and 1.0 mM Zn) representing trace to toxic metal conditions. Here, the 0-Zn control treatment contained a baseline concentration of 9 μ M Zn.

(a) Experimental design

In the first experiment, which was designed to determine the overall patterns of AM and non-AM root-organ metal uptake, one-compartment plates (Fig. 5.2a and 5.2b) were filled with 25 mL of the medium solution (pH 5.5) containing one of the four Zn concentrations and solidified with 0.4 % (m/v) Gelrite® gellan gum (Sigma-Aldrich™, Canada). Each plate consisted of two 3 cm roots aseptically excised from a culture collection, transferred onto the medium, and inoculated (AM) or not (non-AM control) with ~50 fungal propagules of *G. intraradices* consisting of spores and hyphae which were verified by microscopic examination. The plates were then sealed, inverted, and incubated (124 L Incubator, Conviron®, Canada) in darkness at 25 °C for eight weeks allowing for the growth and colonization of roots, proliferation of fungal hyphae, and production of spores (Fig. 5.3). To ensure an adequate biomass for

further analyses, five plates were pooled per replicate and seven replicates per treatment were prepared (1 root-organ x 2 AM^{+/} x 4 Zn^[0, 0.1, 0.5, 1.0]) for a total of 56 pooled replicates (280 total plates) for the entire experiment.

In the second experiment (Fig. 5.2c and 5.2d), two-compartment plates (100 mm x 15 mm) divided by a median partition were filled with 20 mL of the medium containing 0 mM Zn in the root-hyphal compartment (RHC) and 20 mL of the medium containing one of the four Zn concentrations in the hyphal compartment (HC). As described above, roots were transferred into the RHC, inoculated with fungal propagules, and incubated for eight weeks, non-AM roots serving as a control group were prepared in the same manner. To promote the crossing and proliferation of extraradical hyphae from the RHC to the HC, only the RHC medium contained sucrose whereas the HC did not and the HC medium was generated on a 45° downward slope (Fortin et al 2002, Cranenbrouck et al 2005). Over the experimental period, only the extraradical hyphae were permitted to cross the dividing wall to proliferate throughout the HC. Accordingly, all roots were monitored regularly and trimmed as required to prevent them from crossing the partition. Five plates were pooled per replicate and seven replicates per treatment were prepared (1 root-organ x 1 AM x 4 Zn^[0.0, 0.1, 0.5, 1.0] + 1 non-AM Zn^[0]) for a total of 35 pooled replicates (175 total plates) for the entire experiment.

(b) Root physiological and chemical analyses

After an eight-week growth period, pooled replicates (n=4) of AM and non-AM roots from randomly selected treatment blocks were chosen to measure the dry mass and

metal concentrations for both the one- and two-compartment experiments. The roots were carefully removed from the growth medium, rinsed with dH₂O, frozen with liquid nitrogen, and lyophilized at -60 °C in a partial vacuum for 72 h (Unitrap 10-100 Virtis Inc., Gardiner, USA). Freeze-dried roots were then weighed, ground, and each sample (~20-120 mg) was transferred into acid-washed Teflon® bombs (Nalgene® Oak Ridge, USA). Samples were dissolved in 2 mL of 16 M HNO₃, heated for 10 h in a 80 °C water bath, and diluted with 20 mL pure grade H₂O (EMD, Germany). In the same manner, ten samples of Apple Leaves (Standard Reference Material #1515) from the National Institute of Standards and Technology (NIST, USA) and five blanks consisting solely of HNO₃ and H₂O were also prepared to ensure the quality and accuracy of the metal analysis. All sample solutions were analyzed via Inductively Coupled Plasma Optical Emission Spectrometry (730-ES, Varian Inc., Canada) to determine the Zn concentrations. A mean of 97.6 ± 1.2 % of Zn was recovered from the analysis of the reference standards. The instrumental limit of detection calculated from the standard deviation of repeated measurements was 0.0033 µg g⁻¹, the HNO₃ dissolution method limit of detection calculated from procedural blanks was 0.0256 µg g⁻¹, and the instrumental limit of quantification within the dynamic range of the calibration curve was 0.0111 µg g⁻¹.

Further to assessing mean root dry mass, indexes of relative growth were determined using liberal (Wilkins 1978) and conservative (Wilson 1988) indicators of

metal toxicity. The Wilkins (1) and Wilson (2) tolerance indexes, calculated in relation to control treatments within each treatment block (n=4), are defined as:

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

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

Index values approaching 0 indicate decreasing Zn tolerance (or increasing toxicity) in relation to the control treatments.

(c) AM root colonization assay

Roots from the remaining pooled replicates (n=3) were carefully removed, rinsed, and stained with aniline blue 0.02% dye solution (6.78 mM aniline blue – 500 ml glycerol – 450 ml dH₂O – 50 ml 1 % HCl) according to Dalpé (1993). Fifty ~1-2 cm root segments per replicate were mounted on slides and examined at 100X and 400X magnification using a compound microscope (CX41, Olympus Inc., Canada). Mycorrhizal colonization was estimated according to Giovannetti and Mosse (1980) and Dalpé (1993) by determining the % frequency of colonization, % length of root colonization, and relative density (# mm⁻¹ root length) of fungal structures (e.g. vesicles). The formulas for the % frequency (3), % length of root colonization (4), and relative density (5) are defined as:

$$\frac{\#Segment_{AM}}{\#Segment_{total}} \times 100\% \quad (3)$$

$$\frac{RootLength_{AM}}{RootLength_{total}} \times 100\% \quad (4)$$

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

Together, these respective indicators provide insight into the relative distribution and intensity of the AM root colonization (Allen 2001).

Following the harvest of roots, the growth medium from the plate replicates was collectively pooled (5 *per* replicate – n=7) and analyzed for the determination of fungal spore production by excising the medium to fragment the hyphal network and dissolving it in 10 mM sodium citrate buffer, pH 6.0 (Doner and Bécard 1991). The dissolved medium solutions were then vacuum-filtered across a 20 µm nylon filter apparatus (Millipore™, USA) to isolate the spores which were later re-suspended in 50 mL dH₂O. Ten 1 mL sub-samples of the re-suspended spores were taken from each replicate and examined under the compound microscope using a Sedgewick-Rafter counting cell (Wilco®, USA) to extrapolate the total number of spores. For the two-compartment experiment, the growth medium from each compartment was analyzed separately to isolate and compare the total number in the RHC and HC, respectively.

(d) Statistical analyses

One- and two-way analyses of variance (ANOVA) with Bonferonni and Scheffé studentized range tests were performed for mean comparison analyses (Zar 1999). 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 parametric analyses. All of the

Fisher statistics (F), degrees of freedom (df), and p-value estimates were calculated using S-Plus 8.0 statistical software (Insightful Corp., Windows, Seattle, WA).

5.3 Results

In the one-compartment experiment, significant differences were observed when comparing the AM versus non-AM root Zn uptake as Zn concentrations in the medium increased (Fig 5 4a and 5 4b) Overall, AM root Zn concentrations were nearly two-fold greater ($31.7 \mu\text{g g}^{-1} \text{DM}$) than non-AM roots (17.9) at the low rates of exposure, but then two fold lower at the high rates (890 to 1919 in AM versus 1508 to 4160 in non-AM roots) These results were also found for the total Zn content of roots, albeit showing a high degree of statistical variance Meanwhile, both AM and non AM root growth were found to be deleteriously affected by metal uptake whereby dry mass declined by six fold (Fig 5 5) and noticeable stress (*e.g.* root browning) gradually appeared as Zn concentrations in the medium increased Despite these detrimental effects, AM root dry mass at low Zn treatments (0 and 0.1mM) tended to be 10 to 20 % greater than non-AM roots as corroborated by both indices of growth tolerance (Table 5 1), however these trends were not statistically significant As for AM root colonization (Table 5 2), the % frequency and % length of root colonization also decreased, most markedly between the 0.1 to 0.5mM Zn treatments The visual examination of AM roots indicated that the hyphal network was prolific among all the Zn treatments (data not shown), although the extraction of spores from the growth medium revealed a five- and then fifty fold decrease in spore production as Zn levels increased

In the two compartment experiment, the root Zn concentration was measured to determine the contribution of hyphal metal uptake and translocation from the HC

to the host roots (Fig. 5.6a and 5.6b). Here, two-fold higher Zn concentration and content were found among the AM versus non-AM roots at the 0 mM Zn control treatment ($69.5 \mu\text{g g}^{-1}$ DM & $6.5 \mu\text{g}$ in AM versus 23.8 & 2.1 in non-AM roots), followed by a linear increase in AM root Zn uptake with increasing Zn concentration in the HC medium (up to $455.0 \mu\text{g g}^{-1}$ DM and $53.6 \mu\text{g}$). Despite this increase, the root dry mass did not differ significantly between any of the AM or non-AM treatments which ranged between 91.0 and 101.2 mg (5.6 Supplementary data). Visual examination revealed that AM roots were well colonized and that the extraradical hyphae had crossed the median partition to proliferate throughout the HC (data not shown). However, the overall AM root colonization did not show any particular trends between the treatments (5.6 Supplementary data). Moreover, approximately six-fold greater number of spores were measured in the RHC than the HC (Fig. 5.7). In this case, the number of spores in the RHC did not differ between the Zn treatments, whereas a marked decline (from 1854 to 148) was found in the HC as Zn concentrations in this compartment increased.

Table 5.1 Indexes of root tolerance in one-compartment plates

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

[†]The 0 mM concentration indexes do not appear since they represent auto-correlation values of 1 due to the nature of the equation. Means (n=3) and SE (inside parentheses) are shown.

Shared letters in columns designate treatments that are not significantly different according to Bonferonni and Scheffe mean comparison 'ns' not significant

*** p<0.001

Table 5.2 AM root colonization and spore production in one-compartment plates

Zn (mM)	Root Colonization*				Spore Production†	
	Hyphae		Vesicles		Relative Density (no mm ⁻¹ root length)	Total (no)
	% Frequency	% Root Length	% Frequency	% Root Length		
0	91.5 ^a (4.9)	55.4 ^a (2.9)	34.9 ^a (1.4)	22.9 (1.8)	13.5 (0.2)	18475 ^a (2765)
0.1	91.5 ^a (0.7)	58.4 ^a (1.1)	32.6 ^a (3.9)	22.8 (1.5)	15.2 (1.1)	3450 ^b (1021)
0.5	77.2 ^b (5.3)	52.7 ^a (2.1)	17.1 ^{ab} (1.1)	25.2 (7.8)	15.1 (1.2)	575 ^c (175)
1.0	79.0 ^b (4.9)	37.8 ^b (4.2)	8.81 ^b (1.9)	12.5 (6.3)	8.8 (4.5)	383 ^c (122)
F-statistics	10.9*	8.3*	7.1*	1.2ns	1.65ns	38.2***

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 Scheffe mean comparison

ns' not significant

* p<0.05

*** p<0.001

Figure 5.4 Zn concentration (a) and Zn content (b) in non-AM (white bars) and AM roots (grey bars) from one-compartment plates. Pooled means ($n = 4$) and standard errors are shown. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffe' mean comparison ($p < 0.05$).

Figure 5.4

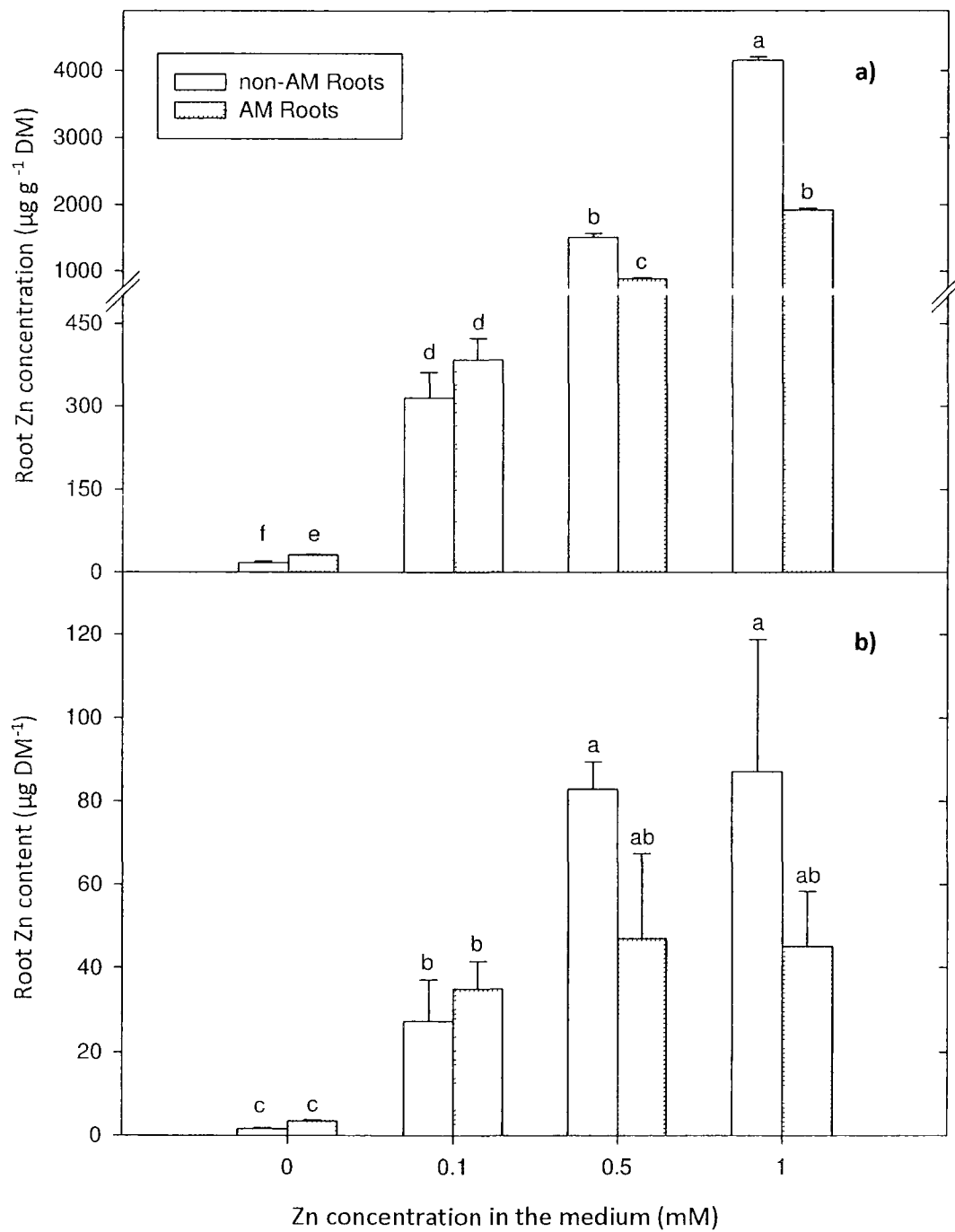


Figure 5.5 Dry mass of non-AM (white bars) and AM roots (grey bars) from one compartment plates. Pooled means ($n = 4$) and standard errors are shown. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffe' mean comparison ($p < 0.05$).

Figure 5.5

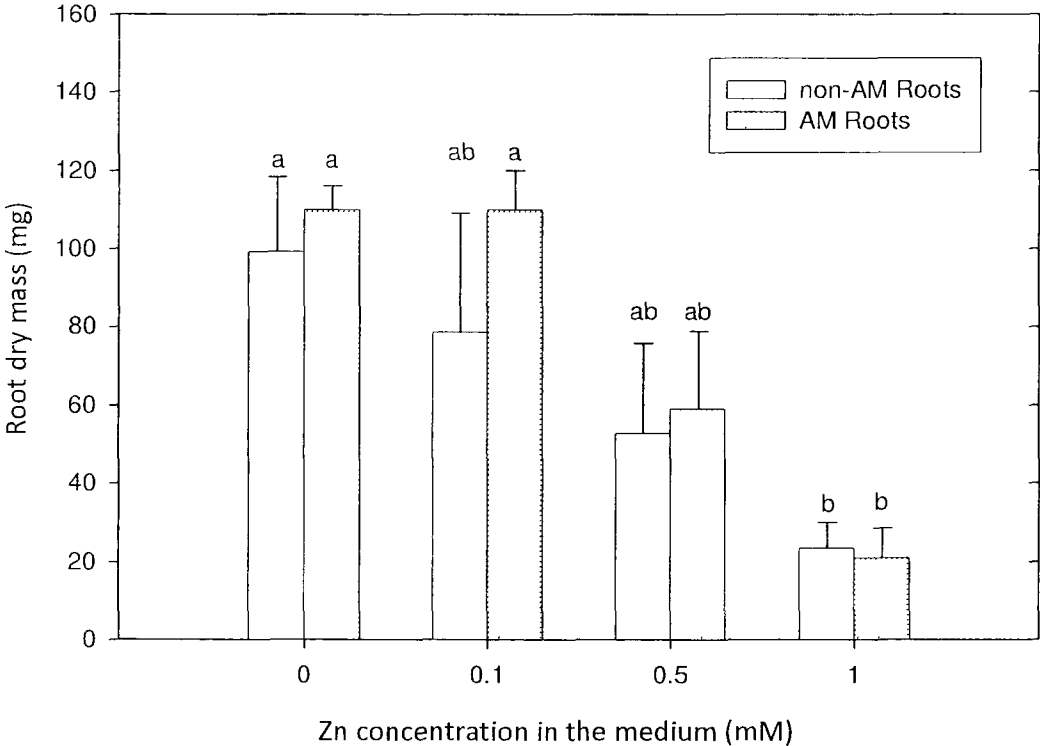


Figure 5.6 Zn concentration (a) and Zn content (b) in non-AM and AM roots translocated from two compartment plates. Pooled means ($n = 4$) and standard errors are shown. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffe' mean comparison ($p < 0.05$).

Figure 5.6

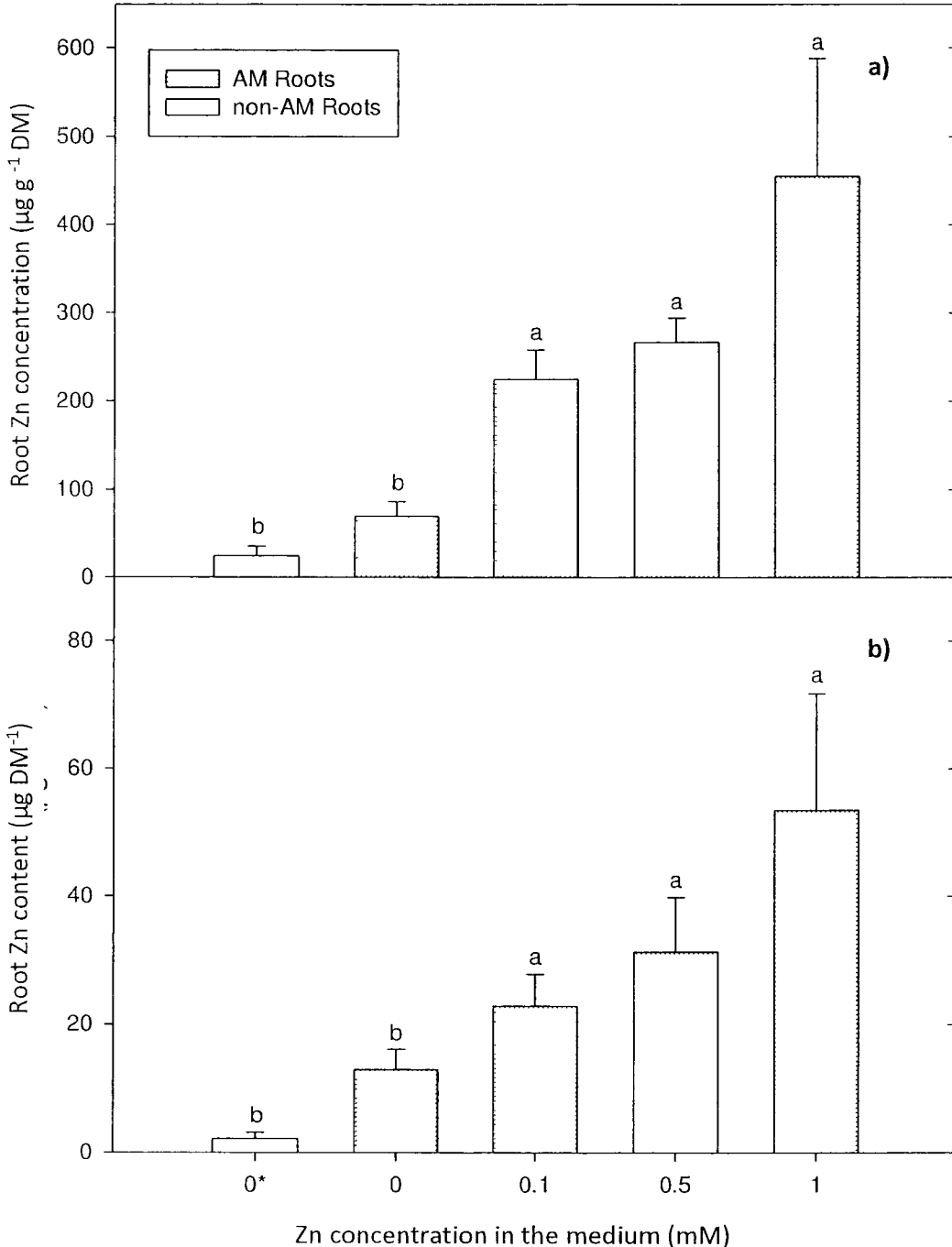


Figure 5.7 Number of spores extracted from the root-hyphal (RHC – white bars) and hyphal compartments (HC – grey bars) from two compartment plates. Pooled means (n = 7) and standard errors are shown. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffe' mean comparison ($p < 0.05$).

Figure 5.7

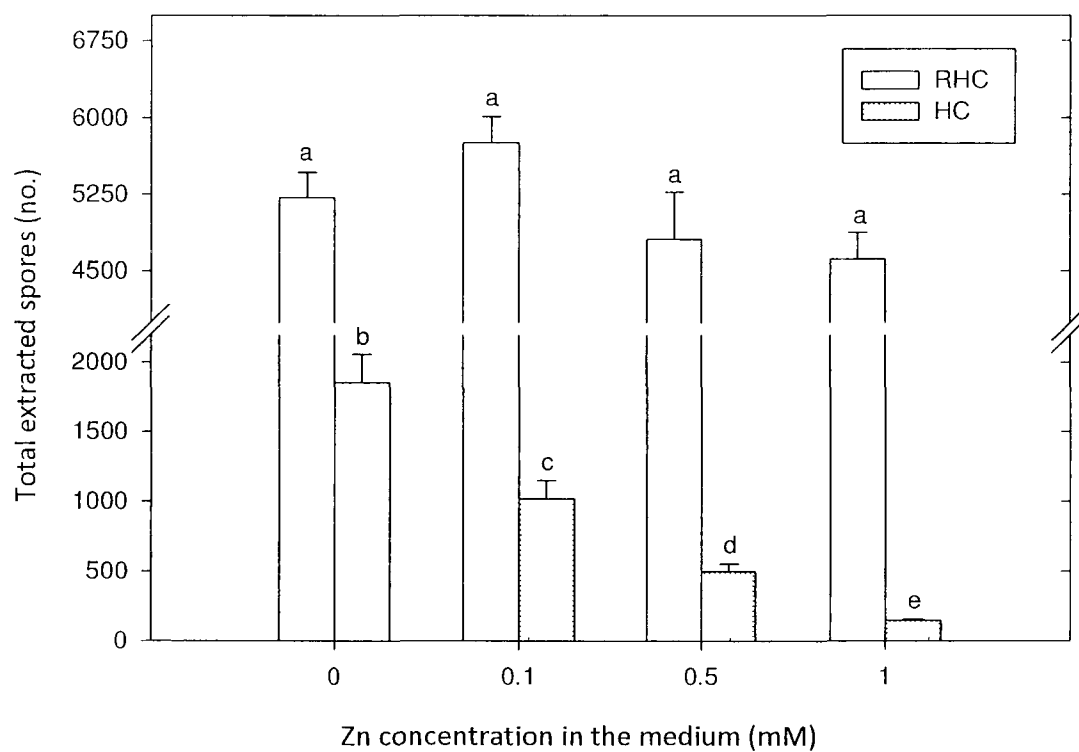
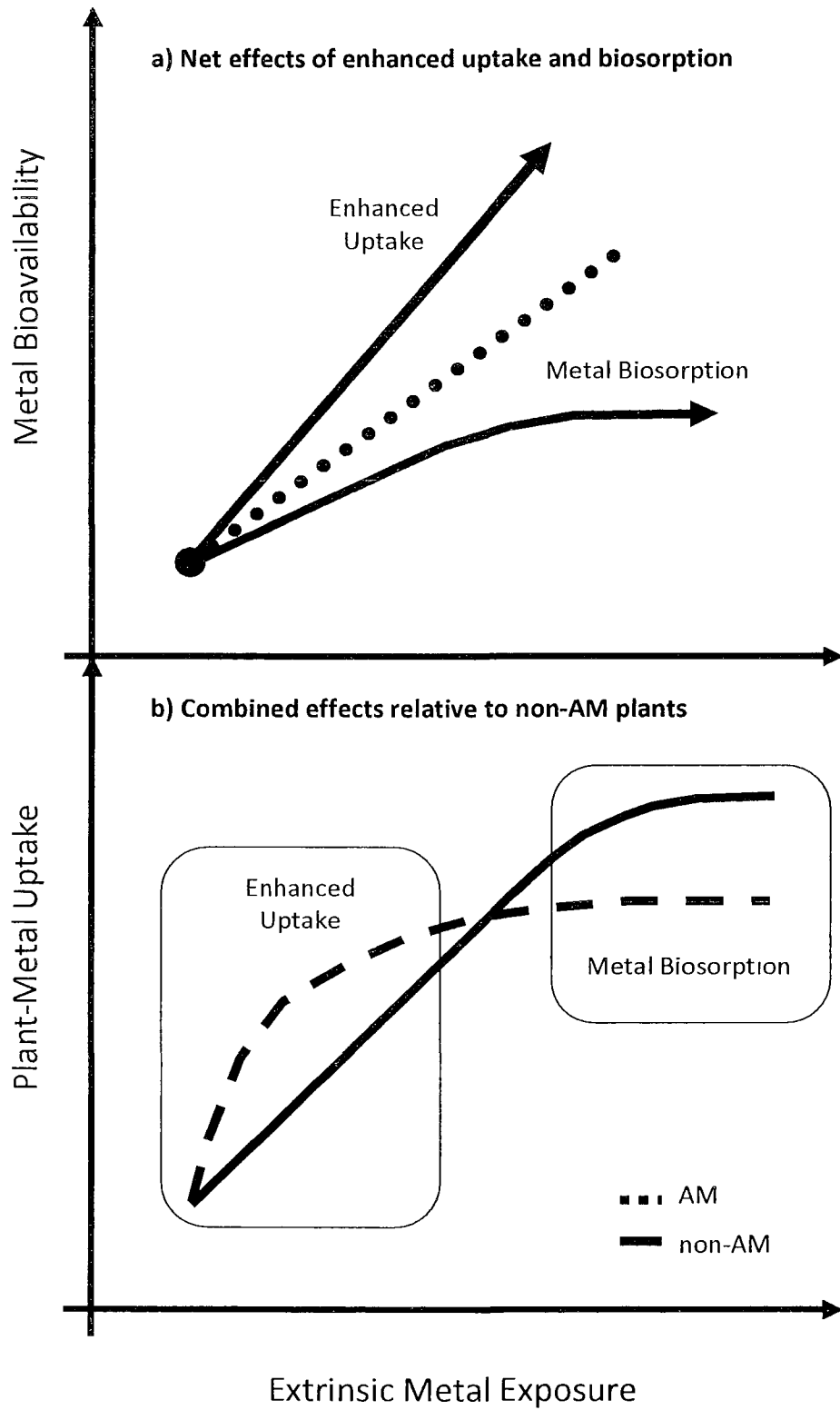


Figure 5.8 Revised conceptual model of AM-plant metal uptake showing the net individual effects (a) and combined effects (b) of AM-enhanced uptake and biosorption on metal bioavailability, and their combined effects on plant metal uptake.

Figure 5.8



5.4 Discussion

Consistent with the hypothetical model (Fig 5 1a and 5 1b), these overall findings demonstrate the dynamic impact of AM fungi (AMF) on root metal acquisition characterized by the dual processes of enhanced uptake and metal-binding. In the one compartment experiment, these processes contributed to a two-fold greater metal uptake in AM than non-AM roots at low Zn concentrations in the medium followed by a two-fold lower uptake at high concentrations. At low metal exposure, the extensive extraradical hyphae enhanced metal uptake by taking up and translocating metal ions to roots in exchange for photosynthates (Koide 1991, Smith and Smith 1990). Correspondingly, molecular analyses of *Medicago truncatula* and *Glomus intraradices* have shown that the regulation of specific ion transporters (e.g. ZIP protein family) can account for the active uptake of Zn and other microelements (Burleigh et al. 2003, Gonzalez-Guerrero et al. 2005). Plants benefit from this enhanced uptake mechanism by supplementing their elemental nutrient requirements to improve their health status, in our study, an effect that may have resulted in the 10 to 20% greater biomass (albeit not significant) among AM than non-AM roots at the low metal treatments. By contrast, as predicted, the impact of AM symbiosis can shift at high Zn treatments due to the binding properties of fungal tissues (Gonzalez –Guerrero et al. 2008) and their exudation of ligands, particularly owing to the immobilization of excess metal ions to carboxyl, hydroxide, oxyhydroxide, and sulfhydryl constituents (Gadd 1993, Galli et al. 1994, Gonzalez-Chavez et al. 2002). In this case, metals may be sequestered by the mycelium through

binding to the hyphal walls then diffusing into hyphal cells, or precipitated as metal-ligand complexes in the proximal growth environment (Joner et al. 2000; Jeffries et al. 2003). Consequently, these metal biosorption and sequestration processes are believed to decrease plant metal uptake by reducing the bioavailability of potentially toxic metals to then delay the onset of metal phytotoxicity (Chapter 3; Leyval et al. 1997; Audet and Charest 2006). In addition to these properties, mycorrhizospheric proliferation is also recognized for defining edaphic conditions by enhancing soil-moisture retention (Augé et al. 2001; Piotrowski et al. 2004), stabilizing the soil-structure matrix (Bearden 2001; Miller and Jastrow 1990), and enriching soil-microbial diversity (Filion et al. 1999; St-Arnaud and Elsen 2005). Taken as a whole, these characteristics contribute in supplementing plant nutrient uptake, but also lessening the uptake of toxic metals in complement to other intrinsic stress resistance mechanisms such as metallothionein and phytochelatin metabolism (Cobbett 2000; Cobbett and Goldsbrough 2002; Maier et al. 2003).

Whereas the one-compartment culture system has enabled the identification of general patterns of AM root metal uptake (characterized by increased uptake at low treatment levels and decreased uptake at high levels compared to non-AM roots), the two-compartment system has enabled to discern the contribution of mycorrhizal enhanced uptake and translocation to host roots. In this case, AM root colonization resulted in an over two-fold greater Zn uptake compared to non-AM roots followed by a linear increase in AM root metal uptake with increasing metal concentration in the hyphal compartment. Upon isolating this effect, it may be implied that AM-

enhanced uptake actively contributed in increasing metal uptake despite the contributions of metal biosorption processes which likely reduced the metal bioavailability in the hyphal compartment. Thus, it is probable that both of these fungal processes should occur simultaneously and (or) independently from one another. This scenario is supported, albeit conceptually, from the perspective that each phenomenon influences different aspects of root metal uptake: the first acting on the uptake capability of host roots, and the second on the bioavailability of metals in the mycorrhizosphere (Fig. 5.8*a* and 5.8*b*). The resulting pattern of AM root metal uptake may, therefore, change compared to non-AM roots depending on the prominence of these mycorrhizal-induced physico-chemical adaptations which then shape subsequent growth conditions (Hinsinger 2001*a*, 2001*b*; Lombi et al. 2001).

Beside the impact of AM symbiosis on root metal acquisition, it is recognized that many growth challenges are associated with conditions ranging from trace to toxic metal extremes which often result in a compromise between plant and fungal growth versus stress-tolerance (Chapter 2). As such, an approximate five-fold decrease in root dry mass and noticeable root browning symptomatic of metal phytotoxicity were observed at the highest extrinsic Zn concentrations. Unlike the model predictions, the growth status of AM roots did not significantly benefit from fungal metal-binding which was previously assumed to reduce metal toxicity through stress avoidance. This finding points out the probable existence of a critical phytotoxicity burden arising either directly due to cellular oxidative stress caused by metal uptake (Baccouch et al. 1998; Schützendübel and Polle 2002; Cho and Seo

2005) or indirectly due to elemental nutrient imbalances arising in the rhizosphere (McBride 1994; Kabata-Pendias 2007). Alternatively, the decrease in root dry mass could also be inherent to the culture system itself since there is no possibility of translocating metals to shoots, thereby potentially alleviating stress in roots. Nevertheless, decreases in AM root colonization frequency, length of root colonized, and fungal structure density were ultimately observed at the highest extrinsic metal concentrations in addition to a dramatic fifty-fold decrease in spore production. Here, it is suspected that the AMF are themselves burdened by high metal exposure as expressed by a severe depression in their proliferation (Pawlowska and Charvat 2004; Janoušková and Vosatková 2005). Despite these challenges, survey studies of metal-contaminated field sites attest to the remarkable resilience of plants and AMF when subjected to such environmentally stressful conditions, especially nutrient imbalances and fluctuations in pH (Del Var et al. 1999; Pawlowska et al. 2000).

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5.6 Supplementary data

Table 5.3 Root dry mass and AM colonization for the two-compartment experiment

Zn (mM)	Inoculation	Biomass (mg)	Root Colonization				
			Hyphae		Vesicles		
			% Frequency	% Root Length	% Frequency	% Root Length	Relative Density (# mm ⁻¹ root length)
0	non-AM	93.2 (1.5)	-	-	-	-	-
	AM	90.4 (3.9)	89.3 (3.4)	48.7 ^a (3.1)	46.7 ^a (0.3)	9.4 ^{ab} (2.4)	2.4 ^b (0.1)
0.1	AM	101.2 (7.5)	71.9 (4.9)	41.9 ^b (4.8)	26.8 ^{bc} (8.2)	9.6 ^a (1.6)	2.9 ^a (0.4)
0.5	AM	93.2 (1.2)	80.1 (3.3)	42.2 ^b (2.1)	21.9 ^c (10.1)	8.0 ^b (3.4)	1.8 ^c (0.5)
1	AM	91.0 (5.9)	75.8 (5.0)	47.0 ^{ab} (3.5)	34.5 ^{ab} (2.1)	7.2 ^b (2.5)	2.0 ^{bc} (0.2)
F-statistics							
Treatment		0.3ns	2.6ns	8.6***	15.6***	16.8***	22.6***

Means (n=3) and SE (inside parentheses) are shown.

Shared letters in columns designate treatments that are not significantly different according to Bonferonni and Scheffe mean comparison.

(-) non-applicable

'ns' not significant

*** p<0.001

Chapter 6

Greenhouse culture: Plant metal uptake and soil metal bioavailability in the AM-mycorrhizosphere

6.1 Objectives

In Chapters 3 and 4, a conceptual model derived from meta-analytical findings was proposed depicting the impact of the AM symbiosis on plant metal uptake from low to high metal exposure levels; a model that was further investigated in Chapter 5 using an *in vitro* carrot root-organ culture system. It was demonstrated that the AM fungi play a dual role in metal acquisition by (1) increasing nutrient uptake at low metal exposure levels, but then (2) lessening uptake at high levels through biosorption processes to delay the onset of metal toxicity (Fig. 6.1). Notably, in the *in vitro* study, it was also suggested that the 'enhanced uptake' and 'metal biosorption' processes likely occur independently in shaping plant metal uptake and subsequently influence a number of edaphic parameters. To expand on these notions, I believe that AM roots and extraradical hyphae hold an important role in increasing the soil's resiliency due to these same mechanisms. Accordingly, I present here the first of two compartmental pot growth systems designed to assess the contribution of non-AM roots (rhizosphere), AM roots and hyphae (mycorrhizosphere), or strictly extraradical hyphae (hyphosphere) on plant growth and metal uptake, soil metal bioavailability, and soil-pH. The compartmental pot system was selected to isolate each of these 'sphere environments' and isolate their impact on various plant physiological and edaphic factors in relation to increasing soil metal exposure levels.

Figure 6.1 Conceptual model of plant metal uptake (a) and relative plant growth (b) in relation to increasing extrinsic metal concentrations. Designated are zones referring to the 'enhanced uptake' and 'metal biosorption' hypotheses. AM plants show greater metal uptake than non-AM plants at low metal exposure levels, but lower uptake at high metal exposure level. Accordingly, AM plants have greater metal stress tolerance compared to non-AM plants as expressed by an increased relative growth status.

Figure 6.1

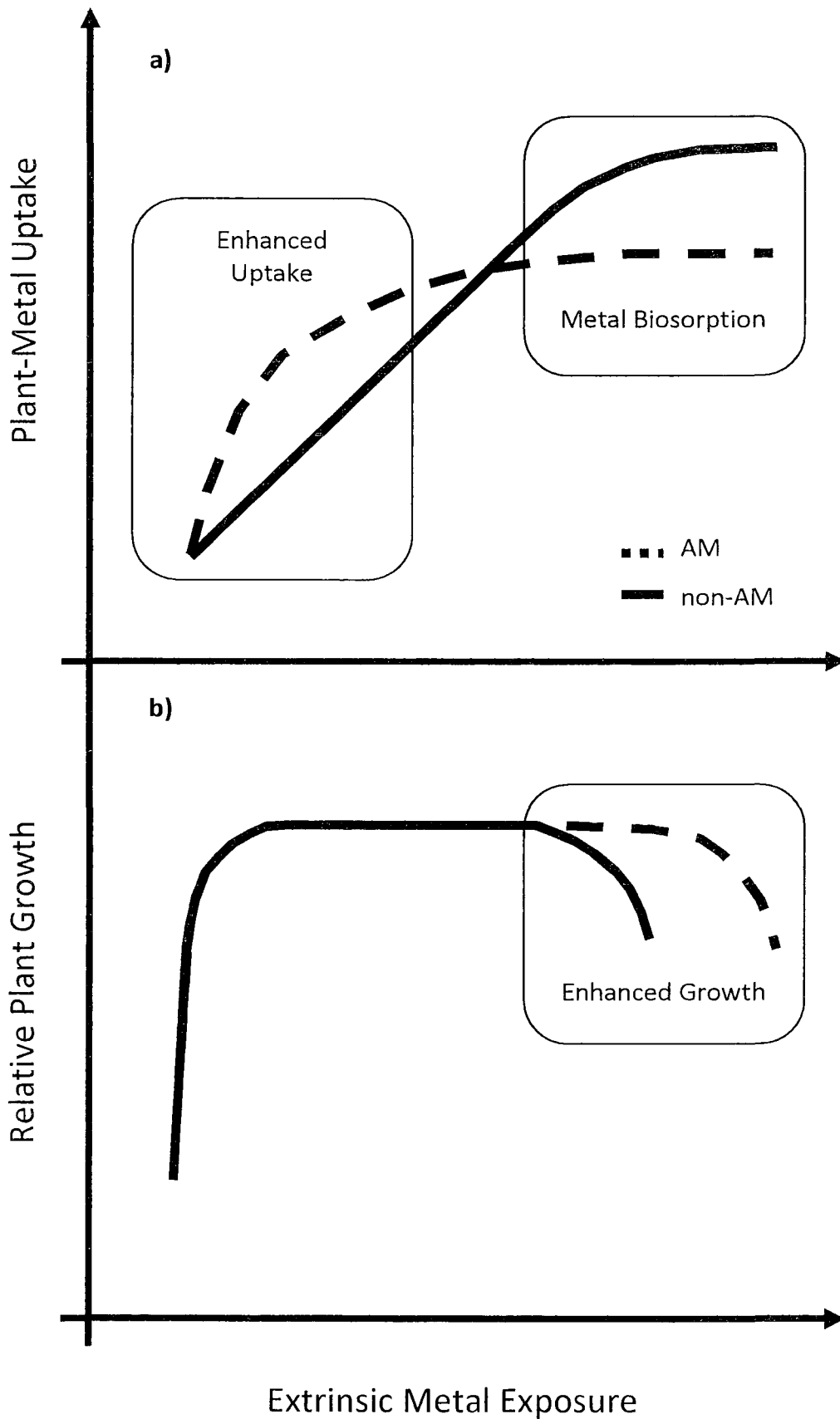


Figure 6.2 Schematization of the compartmental pot system (a) showing the proliferation of roots and (or) extraradical hyphae across the dividing filter. The inoculum/control substrate layer is also shown. Seeds were sown in the central compartment (CC), while the soil-Zn treatments were incorporated in the peripheral compartment (PC) represented as either (b) the rhizosphere (non-AM roots), (c) mycorrhizosphere (AM roots and hyphae), or (d) hyphosphere (extraradical hyphae) environments.

Figure 6.2

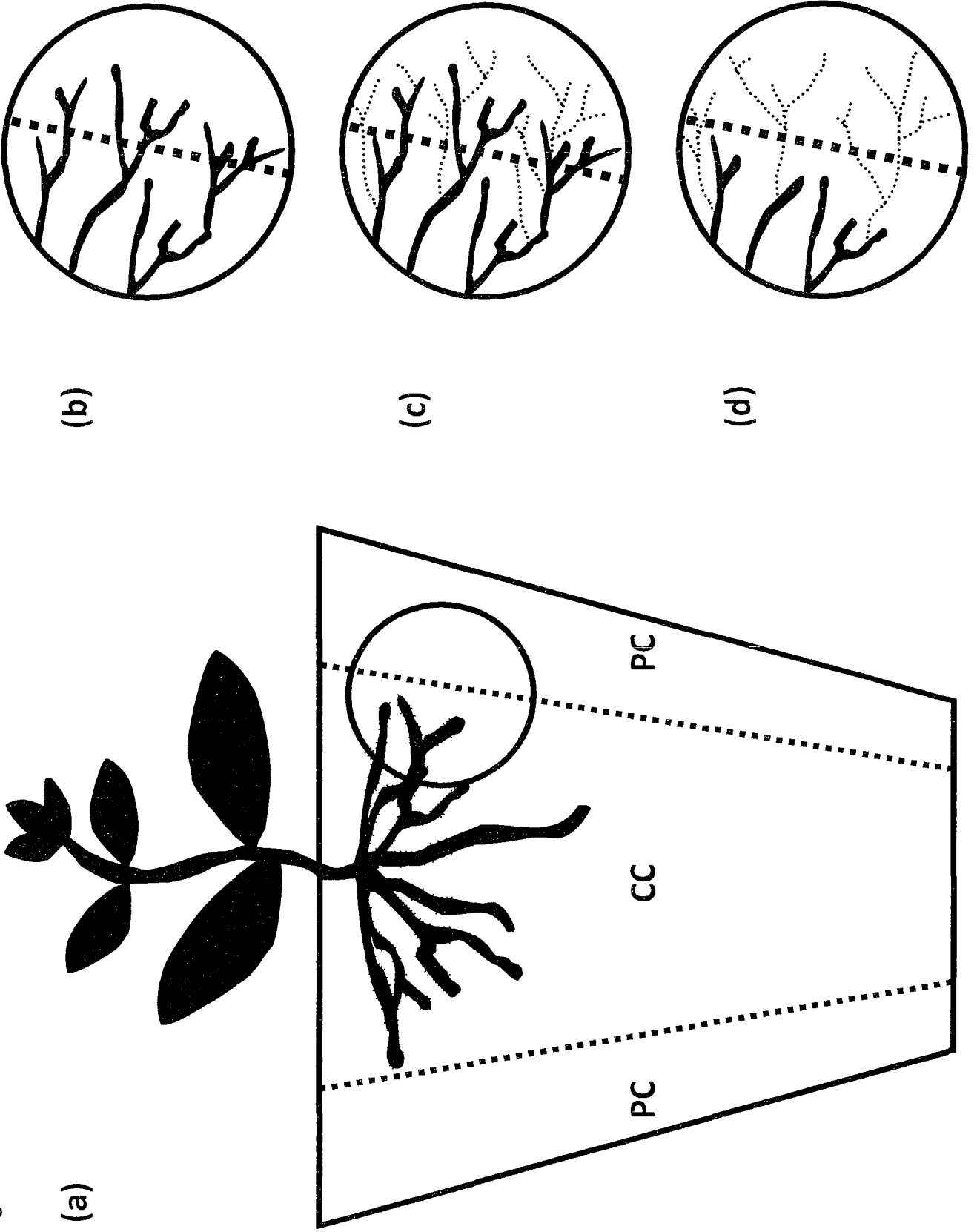


Table 6.1 Pre-experimental soil characteristics

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/100g [‡]	0.2 (0)
CEC Mg	meq/100g	1.8 (0.05)
CEC Ca	meq/100g	8.2 (1.26)
CEC Na	meq/100g	0.3 (0.03)
CEC Total	meq/100g	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)

*NaHCO₃ Extractable;

†NH₄ Acetate Extractable;

‡milli-equivalent per 100g

6.2 Materials and methods

(a) Experimental design

Based on preliminary growth studies (Chapter 8), dwarf sunflower (*Helianthus annuus* L var Pacino) and the AM fungus *Glomus intraradices* Schenck & Smith (isolate DAOM 181602) were selected as experimental organisms and, as previously (Chapter 5), the micronutrient zinc (Zn) as a typical metal contaminant. 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) inoculated (or not) with fungal propagules of *G. intraradices* (Myke® Pro Endo, Premier Tech, Riviere 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 dose containing 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 propagules. Each experimental pot system (Fig. 6.2a) was composed of a central compartment (7 L capacity) in which seeds were sown containing the pre-sterilized soil, and a surrounding peripheral compartment (7 L capacity) containing soil that was pre-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 (Audet and Charest 2006). 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 (Fig 6 2b – rhizosphere – 2000 μm), both AM roots and hyphae (Fig 6 2c – mycorrhizosphere – 2000 μm), or strictly extraradical hyphae (Fig 6 2d – 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 compartments were achieved by weighing appropriate volumes of pre autoclaved soil, adding specific Zn doses from a $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution, homogenizing the mixture with an industrial mixer, and air drying the soil. This soil pre-treatment 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 (Reid et al. 1998). The factorial design of the study [1 plant *sp.* x 3 sphere treatments (non AM rhizosphere, AM mycorrhizosphere, or AM hyphosphere) x 4 soil Zn concentrations (0, 50, 200, or 400 mg kg^{-1} DS) x 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 post-experimental 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 / 8 h (light / dark) photoperiod, an average light intensity of 364.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 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 (Smith et al. 1983): 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.

(b) 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 a 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. 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 $\mu g g^{-1}$.

(c) Soil chemical analyses

The pre-experimental (*i.e.* prior to Zn treatment) soil characteristics (Table 6.1) were determined from five 10 g soil samples dissolved in NaHCO₃ or NH₄ acetate solutions and analyzed using Atomic Absorption Spectrometry (Accutest Laboratories, Ottawa, ON, Canada). Further to these analyses, soil samples of all treatments were collected from both the central and peripheral compartments at harvest, and the un-seeded pots sampled prior to and after the experimental growth period. The soil-pH was analyzed directly from 5 g samples suspended in pure grade H₂O with a soil:solution ratio of 1:2.5 (*m/v*) according to Hendershot et al. (2008). To determine soil-Zn concentrations, 5 g soil from each treatment were suspended in 0.1 M NaNO₃ 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 following the method of Wenger et al. (2002). Five samples of Buffalo River sediment (Standard Reference Material #8704) from NIST along with five blanks consisting solely of 0.1 M NaNO₃ were also analyzed to ensure the quality and accuracy of the metal analysis. As with the plant tissue analysis, the instrumental limit of detection calculated from the procedural blanks and reference material was 0.0036 µg g⁻¹.

(d) AM 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 H₂O – 50 ml 1 % HCl) according to Dalpé (1993). 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 of 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 or arbuscules (Dalpé 1993; McGonigle et al. 1990). The formulas for the % frequency (1) and % length of root colonization (2) are defined as:

$$\frac{No\ Segments_{AM}}{No\ Segments_{Total}} \times 100\% \quad (1)$$

$$\frac{RootLength_{AM}}{RootLength_{Total}} \times 100\% \quad (2)$$

Together, these respective indicators provide insight into the relative distribution and intensity of the AM root colonization (Allen 2001).

(e) Statistical Analyses

One- and two-way ANOVA's 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 (Zar 1999). 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).

6.3 Results

Significant differences were observed in flower, shoot, and root Zn concentrations between the hyphosphere, mycorrhizosphere, and rhizosphere treatments with increasing soil-Zn addition (Figs. 6.3*a*, 6.3*b*, and 6.4*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 uptake than the mycorrhizosphere treatments, and 55 % to 75 % higher uptake than the hyphosphere treatments at both the 200 and 400 soil-Zn concentrations. As for plant growth (Table 6.2), 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 particular trends in relation to soil-Zn amendment. Still, as a potential 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 6.3). 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 non-mycorrhizal.

The soil-Zn concentrations were measured in the peripheral (Fig. 6.4a) and central compartments (Fig. 6.4b) and regression models calculated for each treatment (Table 6.4). 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 post-experimental 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 (Fig. 6.5a) and central compartments (Fig. 6.5b) and regression models calculated for each treatment (Table 6.5). 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 post-experimental 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), post-experimental 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).

Table 6.2 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)
F-values and levels of significance								
	Soil Zn (Zn)	0.63ns	1.31ns	3.63*	0.26ns	2.05ns	78.5***	1.41ns
	Sphere Treatments (S)	16.4***	0.14ns	3.33*	37.6***	1.42ns	63.2***	3.5*
	Zn x S	0.73ns	1.27ns	0.85ns	0.95ns	0.70ns	18.3***	0.54ns
	Block	1.28ns	2.04ns	0.85ns	0.11ns	0.58ns	0.35ns	1.41ns

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 Scheffe mean comparison tests

† Central Compartment ‡ Peripheral Compartment

ns non significant * p<0.05 ** p<0.01 *** p<0.001

Table 6.3 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}
	Zn x S	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 Scheffe mean comparison tests

'ns' non significant, * p<0.05

Table 6.4 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	$-(4.7 \times 10^{-4})x^2 + 0.89x - 1.46$	2573	0.99	1,14	<10 ⁻³	
				Hyphosphere	$-(4.7 \times 10^{-4})x^2 + 0.86x - 3.76$	618.8	0.98	2,13	<10 ⁻³	
				Mycorrhizosphere	$-(7.5 \times 10^{-4})x^2 + 0.97x - 8.48$	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	$(1.0 \times 10^{-7})x + 0.06$	0.10	0.01	1,14	0.75	
				Post-Experimental	Unseeded	$(1.0 \times 10^{-7})x + 0.04$	1.29	0.08	1,14	0.27
					Hyphosphere	$(1.0 \times 10^{-7})x + 0.03$	1.46	0.09	1,14	0.25
	Mycorrhizosphere				$(1.0 \times 10^{-7})x + 0.03$	1.52	0.10	1,14	0.24	
	Rhizosphere	$(1.0 \times 10^{-7})x + 0.07$	0.01		0.01	1,14	0.96			

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

Table 6.5 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	$(3.8e-6)x^2 - (2.8e-6)x + 5.78$	205.1	0.97	2,13	<10 ⁻³
				Hyphosphere	$(3.7e-6)x^2 - (2.6e-6)x + 5.71$	205.1	0.95	2,13	<10 ⁻³
				Mycorrhizosphere	$(2.8e-6)x^2 - 0.0021x + 5.63$	76.5	0.92	2,13	<10 ⁻³
		Rhizosphere		$(1.9e-6)x^2 - (1.6e-6)x + 5.49$	87.9	0.93	2,13	<10 ⁻³	
		Central	Pre-Experimental	Unseeded	$(1.0e-4)x + 6.34$	1.72	0.11	1,14	0.21
				Post-Experimental	Unseeded	$(1.0e-4)x + 5.78$	1.65	0.10	1,14
			Hyphosphere		$(3.7e-5)x + 5.59$	0.30	0.02	1,14	0.59
	Mycorrhizosphere		$(3.2e-5)x + 5.59$	0.24	0.02	1,14	0.64		
	Rhizosphere	$(2.8e-5)x + 5.57$	0.15	0.01	1,14	0.71			

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

Figure 6.3 Zn concentrations in flowers (a), shoots (b), and roots (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 Bonferonni and Scheffé mean comparison tests ($p < 0.05$).

Figure 6.3

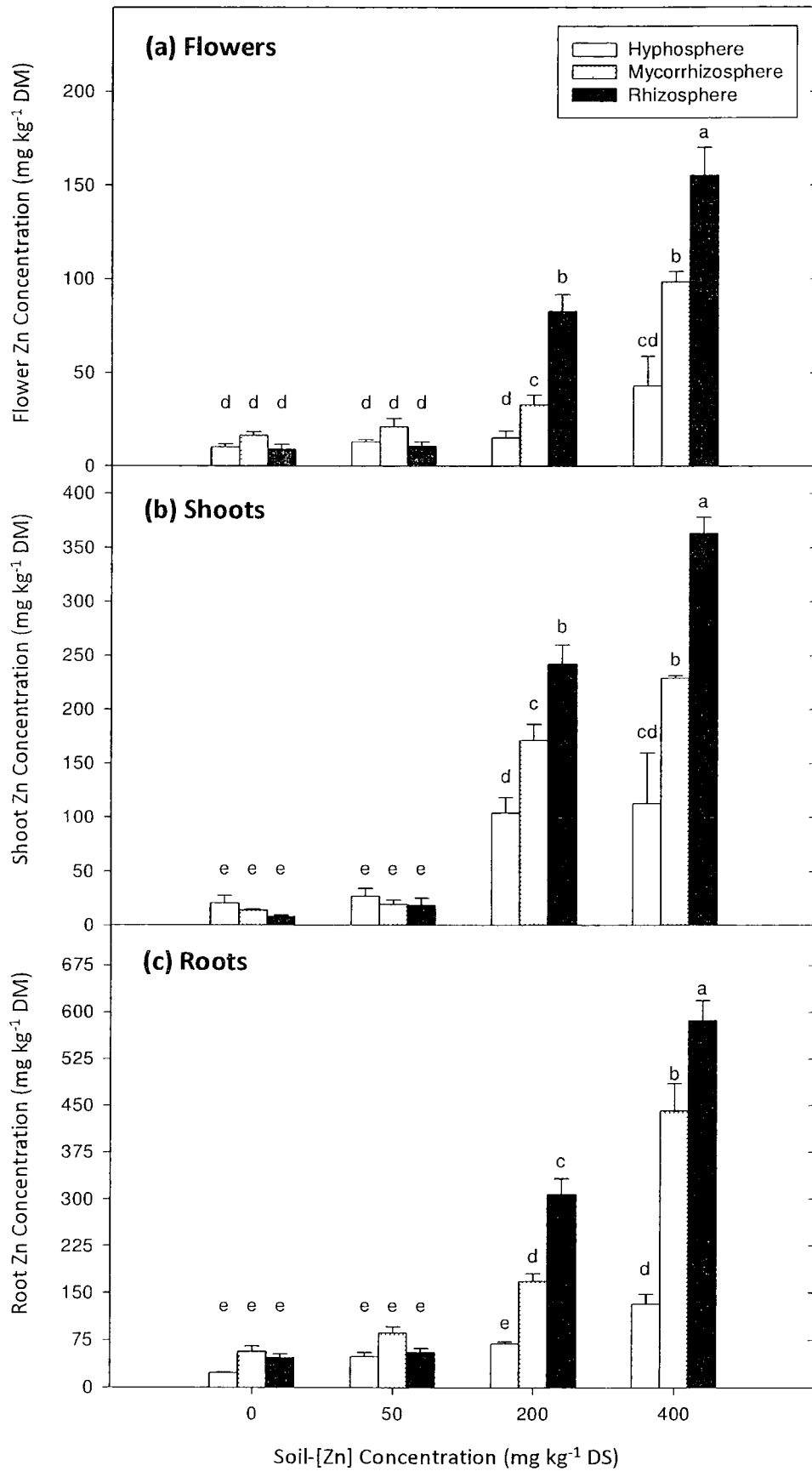


Figure 6.4 Soil-Zn concentrations in the peripheral (a) and central compartments (b). Means (n=4) and standard errors for the pre-experimental un-seeded (black diamond), post-experimental un-seeded (black square), hyphosphere (white square), mycorrhizosphere (white triangle), and rhizosphere (white circle) treatments are shown.

Figure 6.4

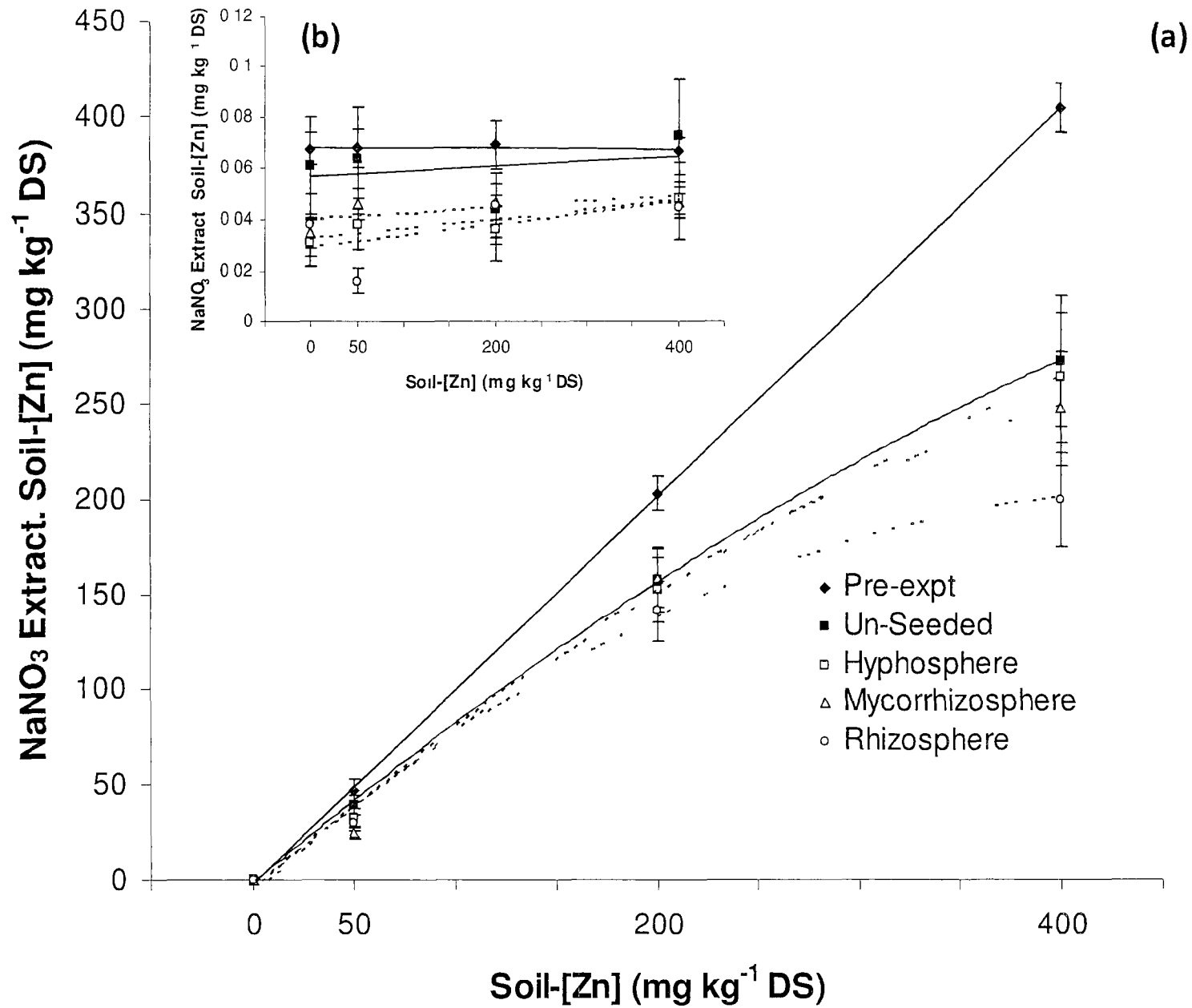
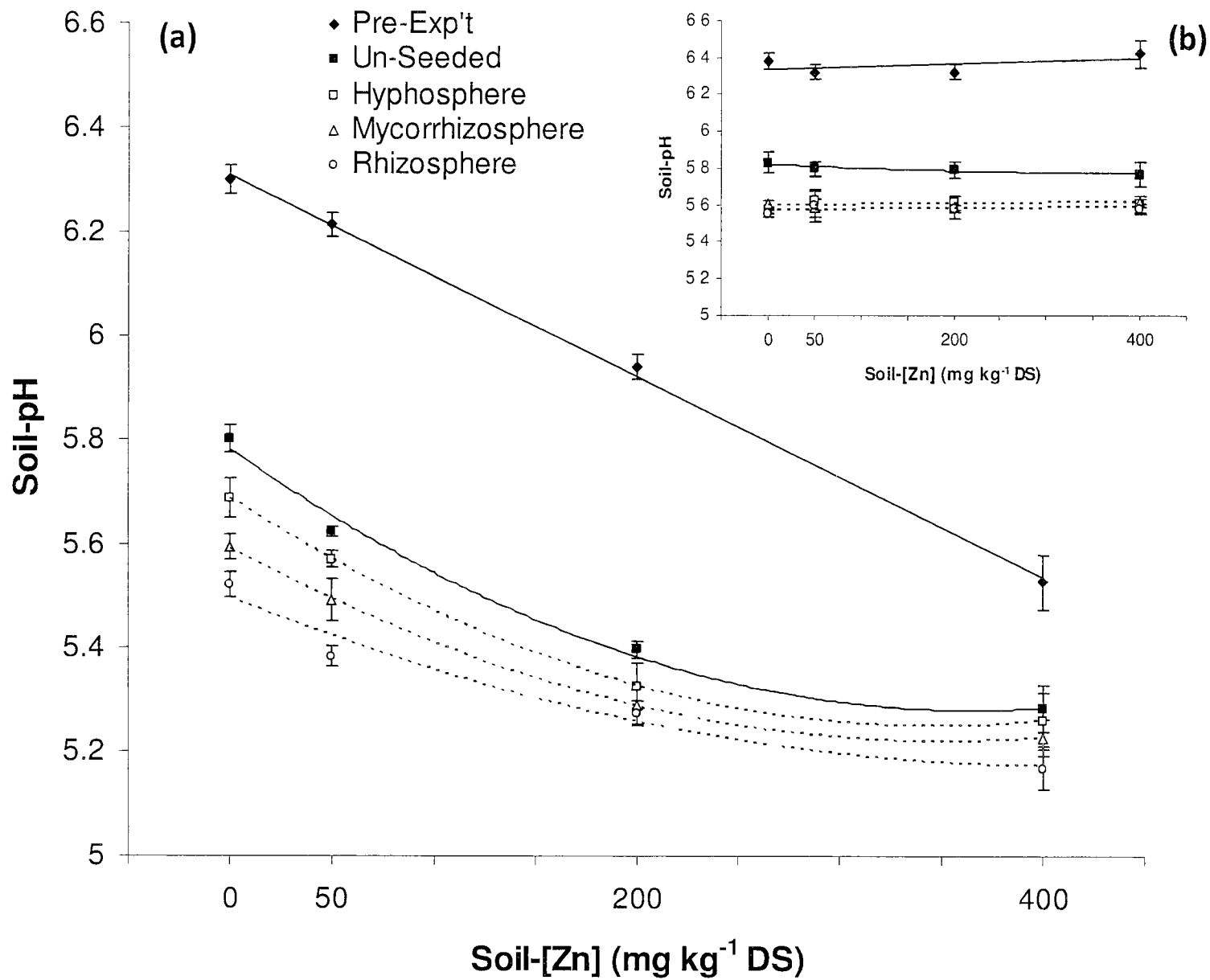


Figure 6.5 Soil-pH in the peripheral (a) and central compartments (b). Means (n=4) and standard errors for the pre-experimental un-seeded (black diamond), post-experimental un-seeded (black square), hyphosphere (white square), mycorrhizosphere (white triangle), and rhizosphere (white circle) treatments are shown.

Figure 6.5



6.4 Discussion

Consistent with the hypothetical model regarding the impact of AM symbiosis on plant growth and metal uptake (Chapters 3 and 4), 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 greenhouse studies that detected lower Cd, Zn, and Pb uptake and an improved growth status in various AM (*Glomus* sp.) versus non-AM plants (Bi et al. 2003; Li and Christie 2001; Rivera-Becerril et al. 2003; Zhu et al. 2001). As shown from the previous *in vitro* trials (Chapter 5), these AM metal uptake trends are primarily attributed with the AM-induced processes of soil metal biosorption, including hyphal metal-binding and metal-ligand precipitation (Gadd 1993; Gallie et al. 1994; Gonzalez-Chavez et al. 2002) that reduce metal bioavailability in the mycorrhizosphere to decrease plant metal uptake (Jeffries et al. 2003; Leyval et al. 1997; Meharg 2003). Correspondingly, further studies have shown that metals can be taken up and sequestered in fungal tissues instead of being transferred to roots (Joner et al. 2000; Chen et al. 2001; Gonzalez-Guerrero et al. 2007), thereby reducing cellular oxidative stress and delaying the onset of plant metal toxicity (Schutzendubel and Polle 2002). 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 metallothionein and phytochelatin metabolisms (Cobbett and Goldsbrough 2002). In addition to comparing the metal

uptake profiles of AM versus non-AM plants, the two-compartment pot system was specifically designed to determine the relative contributions of extraradical hyphae to host plant Zn uptake. In this regard, plants of the hyphosphere treatments had increasing Zn concentrations even at the high soil-Zn treatments. Also in agreement with the *in vitro* study (Chapter 5), 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 (Ker and Charest 2010; Declerck et al. 2003; Hovsepyan and Greipsson 2004; Davies et al. 2001). For this reason, it is believed 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. Accordingly, it is likely that their combined effects should shape the host plant's metal uptake profile.

Contrary to the conceptual model, no significant differences were detected between the AM and non-AM plant Zn uptake and no major symptoms of nutrient deficiency were observed among these plants when subjected to 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 (Marschner 1998; Smith and Gianinazzi-Pearson 1988). This can be attributed to the pre-experimental soil-Zn levels and fertilization regimes used in the 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, the general trends of slightly lower dry masses, fewer leaves, and shorter shoots were observed 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 (Fitter 1991). Corresponding with the considerable levels of AM root colonization reported here (*i.e.* 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 the compartmental pot systems, especially among the hyphosphere treatments. In this case, the 50 μm filter bags themselves may have limited the 'rootable' volume of the hyphosphere plants to then reduce their overall growth (Chapter 8). 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 (Li and Christie 2001). 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 $\text{H}^+/\text{H}_3\text{O}^+$ ions in the soil solution associated with the addition of Zn and water alter the soil's redox equilibrium and impact the solubility of metal nutrients Chuan et al. 1996; Martinez and Motto 2000; Ross 1994; Tack et al. 1996): a process referred to as *metal ageing* (Lock and Janssen 2003). Such inputs and metal ageing processes can detrimentally influence the soil's metal-binding capacity, resulting in an increased rate of metal leaching (Tack et al. 1996; Apack 2002; Bradl 2004). 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 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 (Bago et al. 1996; Eckhard et al. 1995; Gahoonia and Nielsen 1992; Li et al. 1991; Rufyikiri et al. 2004). In this regard, the extraradical hyphae can induce the moderate alkalisation of their growth substrate in relation to P and N uptake, whereas roots tend to acidify it. This could be relevant to the 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, it could be 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 (Miller and Jastrow 1990; Auge et al. 2001; Piotrowski et al. 2004), 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 the hypothetical model, it has been 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, it is also suggested that the mycorrhizosphere should play 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.

6.5 References

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Chapter 7

Greenhouse culture: Assessing the AM-mycorrhizosphere's stratum of influence

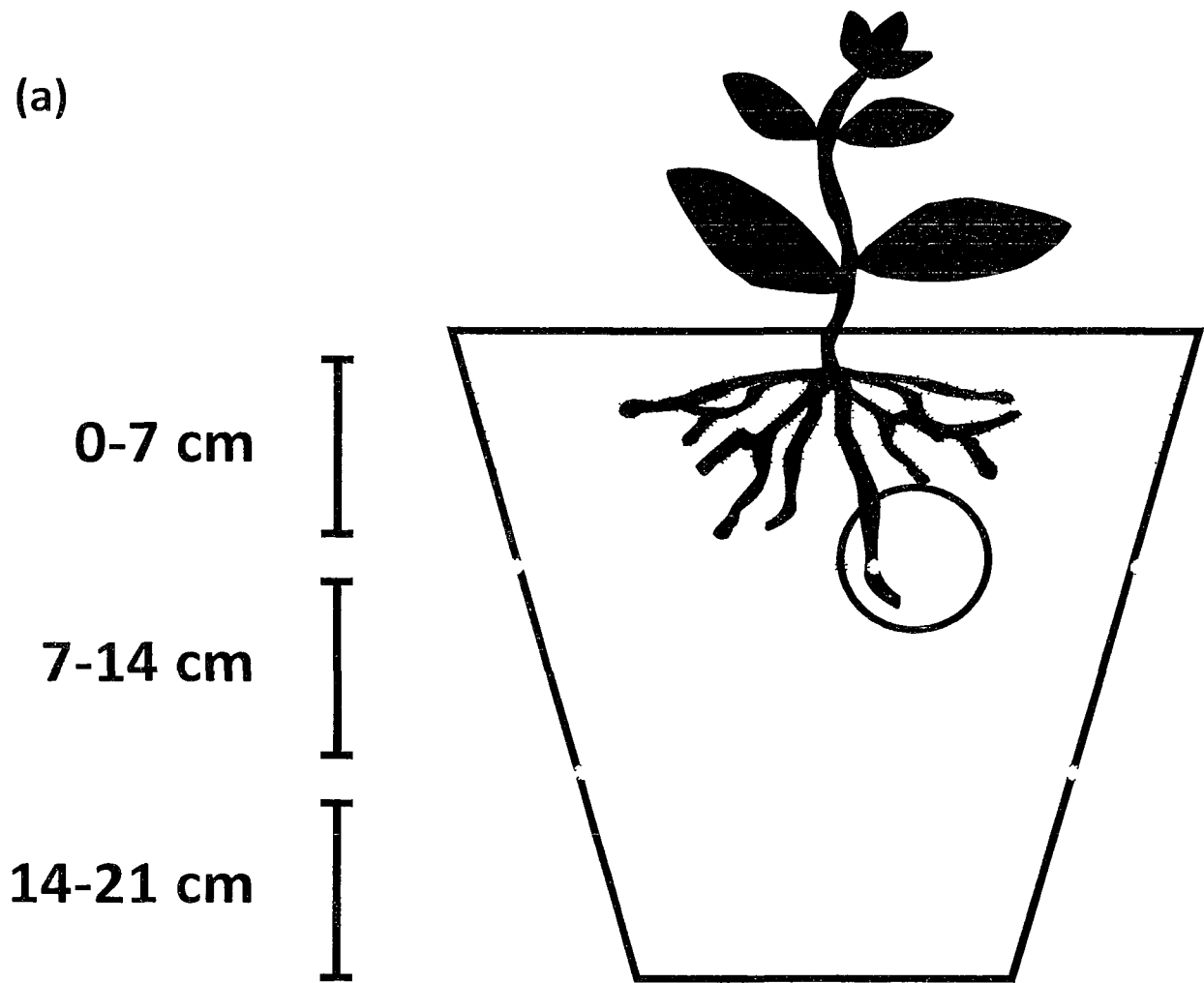
7.1 Objectives

In Chapter 6, three 'sphere environments' (*e.g.* the rhizo-, myco-, and hyphospheres) were isolated in a greenhouse growth study in order to compare their respective impacts on various plant physiological and edaphic factors in relation to increasing soil metal exposure levels. In this regard, it was hypothesized that certain mycorrhizospheric processes (*e.g.* enhanced uptake, metal biosorption, and soil-matrix stabilization) should enhance the soil's resiliency by reducing metal leaching and stabilizing the soil environment to then increase plant stress tolerance. For these reasons, it was considered that the AM symbiosis would have important implications for soil remediation management practices. To expand on these notions, a stratified compartmental growth system was designed in order to further assess the mycorrhizosphere's zone of influence toward plant physiological and edaphic parameters across successive soil depth strata. Notably, this second compartmental growth system is intended to complement notions present in the previous greenhouse experiment. As in Chapter 6, dwarf sunflower (*Helianthus annuus* L. var. Pacino) and the AM fungus *Glomus intraradices* Schenck & Smith (isolate DAOM-181602) were selected as experimental organisms and the micronutrient zinc (Zn) as a typical metal contaminant.

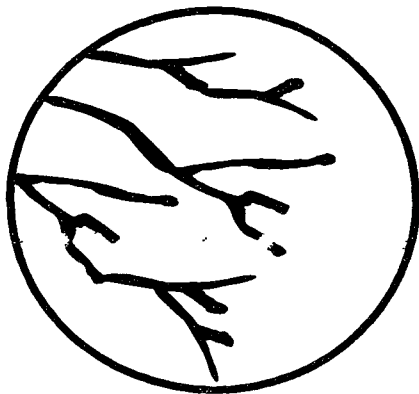
Figure 7.1 Schematization of the compartmental pot system (a) showing the proliferation of non-AM roots (b) or AM roots and extraradical hyphae (c) across the dividing filter. The inoculum/control substrate layer is shown in the top stratum compartment.

Figure 7.1

(a)



(b)



(c)



Table 7.1 Pre-experimental soil characteristics

Parameter	Unit	Value (SE)
P*	ppm	22 (1.01)
K [†]	ppm	95.2 (0.96)
Mg [†]	ppm	204 (7.42)
Organic Matter	%	4.2 (0.56)
Na [†]	ppm	67.3 (7.06)
Ca [†]	ppm	1546 (150)
CEC K	meq/100g [‡]	0.2 (0)
CEC Mg	meq/100g	1.8 (0.05)
CEC Ca	meq/100g	8.2 (1.26)
CEC Na	meq/100g	0.3 (0.03)
CEC Total	meq/100g	10.6 (1.36)
Base Saturation K	%	2.8 (0.35)
Base Saturation Mg	%	16.6 (1.90)
Base Saturation Ca	%	78.1 (1.67)
Base Saturation Na	%	2.5 (0.50)

*NaHCO₃ Extractable;
[†]NH₄ Acetate Extractable;
[‡]milli-equivalent per 100g

7.2 Materials and methods

(a) Experimental design

Dwarf sunflower plants were grown from seeds (McKenzie Seeds, Brandon, MB, Canada) for 10 weeks in three-compartment pot systems containing a previously autoclaved low mineral soil mixture (sand:potting soil, 1:1 v/v) and 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 added to the top compartment which contained 15 propagules g⁻¹ dry substrate (200 g inoculum dose having 2750-3000 total propagules per pot) for AM treatments, whereas the non-AM treatments received the same control substrate without propagules. Each experimental pot system (Fig. 7 1a) was composed of a three-compartment, vertically stratified pot (7 L total capacity) containing one of four pre-treated soil-Zn concentrations (0, 50, 200, 400 mg Zn kg⁻¹ dry soil) as determined from a previous study (Audet and Charest 2006). Each successive compartment (0-7 cm, 8-14 cm, and 15-21 cm) contained 2 L of soil and was separated by a 2000 µm pore-size nylon filter bag (Industrial Filter Manufacturing Ltd, Penetanguishene, ON, Canada) which permitted the proliferation of either non-AM roots (Fig. 7 1b) or both AM roots and extraradical hyphae (Fig. 7 1c) across each compartment. The soil-Zn treatments were achieved by weighing an appropriate volume of pre-sterilized soil, adding Zn from a ZnSO₄·7H₂O stock solution, homogenizing the mixture with an industrial mixer, and air-drying the soil. This soil pre-treatment 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.

(Reid et al. 1998). The factorial design of the study [1 plant *sp.* x 2 inoculates (non-AM rhizosphere; AM mycorrhizosphere) x 4 soil-Zn concentrations (0, 50, 200, or 400 mg kg⁻¹ DS) x 3 reps] provided a total of 24 plants, 1 plant *per* pot. Three replicates of un-seeded pots were also prepared for each of the soil-Zn treatments to compare the pre- and post-experimental soil-Zn bioavailability (NaNO₃ 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 / 8 h (light / dark) photoperiod, an average 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 un-seeded pots were watered daily avoiding any leaching and fertilized biweekly (200 ml *per* week) from weeks 4 to 10 using a low Zn and low phosphorus Long-Ashton nutrient solution, pH 4.4 (Smith et al. 1983): 2.0 mM K₂SO₄, 4.0 mM CaCl₂ anhydride, 1.5 mM MgSO₄·7H₂O, 1.5 mM NaH₂PO₄·H₂O, 5.0 μM NH₄NO₃, 0.01 mM MnSO₄·4H₂O, 1.0 μM CuSO₄·5H₂O, 1.0 μM ZnSO₄·7H₂O, 0.05 mM H₃BO₃, NaCl 0.09 mM, 0.5 μM Na₂MoO₄·2H₂O, and 0.1 mM EDTA-Fe.

(b) 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 recorded. Fresh plants were then partitioned as flowers, shoots, or roots (excised from each respective compartment), and weighed separately. To determine the plant-Zn concentrations, 250 mg of dried flower and shoot samples (oven-dried at 70 °C for 72 h) were ground and placed in acid-washed Teflon[®] bombs (Nalgene[®], Rochester, NY, USA); root samples were not analyzed since they were used entirely for the AM colonization assay. Each flower or shoot sample was dissolved in 2 mL of 16 M HNO₃, heated for 10 h in a 80 °C water-bath, and diluted with 20 mL pure grade H₂O (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 HNO₃ and H₂O were also prepared to ensure the quality and accuracy of the metal analyses. 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 0.002313 µg g⁻¹.

(c) Soil chemical analyses

The pre-experimental (*i.e.* prior to Zn treatment) soil characteristics (Table 7.1) were determined from five 10 g soil samples dissolved in NaHCO₃ or NH₄ acetate solutions and analyzed using Atomic Absorption Spectrometry (Accutest Laboratories, Ottawa, ON, Canada). Further to these analyses, soil samples from all compartments from each treatment were collected at harvest, meanwhile the un-seeded pots where

sampled in the same manner at the beginning of the experimental period and at harvest. The soil-pH was analyzed directly from 5 g soil samples suspended in pure grade H₂O with a soil:solution ratio of 1:2.5 (*m/v*) according to Hendershot et al. (2008). To determine soil-Zn concentrations, 5 g soil samples were suspended in 0.1 M NaNO₃ with a soil:solution ratio of 1:2.5 (*m/v*) and filtered across an 11 µm grade #1 cellulose filter paper (Whatman Inc., Piscataway, NJ, USA) according to Wenger et al. (2002). Subsequently, 1 ml aliquots of the soil leachate were extracted, re-suspended in a 1 % HNO₃ solution, and analyzed using ICP OES. Five samples of Buffalo River sediment (Standard Reference Material #8704) from NIST along with five blanks consisting solely of 0.1 M NaNO₃ were prepared in the same manner and analyzed to ensure the quality and accuracy of the metal analysis. The instrumental limit of detection calculated from the procedural blanks and reference material was 0.004304 µg g⁻¹.

(d) AM root colonization analysis

At harvest, fresh root samples were carefully excised from all compartments for each treatment replicate, clean-rinsed, and stained with an aniline blue 0.02 % dye solution (6.78 mM aniline blue – 500 ml glycerol – 450 ml H₂O – 50 ml 1 % HCl) according to Dalpé (1993). Fifty ~1-2 cm long root segments *per* stratum sub-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 of 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 or

arbuscules (Dalpé 1993). The equations for the % frequency (1) and % length of root colonization (2) are defined as:

$$\frac{No\ Segments_{AM}}{No\ Segments_{Total}} \times 100\% \quad (1)$$

$$\frac{RootLength_{AM}}{RootLength_{Total}} \times 100\% \quad (2).$$

Together, these indicators provided insight into the relative distribution and intensity of the AM root colonization (Allen 2001).

(e) Statistical analyses

According to Zar (1999), one- and two-way ANOVA's with Bonferonni and Scheffé studentized range tests were performed for mean comparisons of plant growth parameters, AM root colonization, soil-pH, and soil-Zn data. 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).

7.3 Results

Overall, the Zn concentrations of both flowers and shoots (Fig. 7.2a and 7.2b) increased in relation to increasing soil-Zn levels. Although Zn concentrations did not vary significantly between the treatments at the 0, 50, and 200 soil-Zn levels, the AM plants had approximately 40% lower shoot Zn concentrations than non-AM plants at the 400 soil-Zn level. As for plant growth (Table 7.2), the flower, shoot, and root fresh masses generally decreased (up to 40%) with increasing soil-Zn levels. In this case, AM and non-AM fresh masses were not significantly different from one another at the 0, 50, and 200 soil-Zn levels; yet, both AM shoot and root fresh masses were greater than non-AM plants at the 400 soil-Zn level. As for to the distribution of root fresh mass across the stratum compartments (Table 7.3), both AM and non-AM plants for all of the soil-Zn treatments showed greater root mass in the top compartment with successively lower biomass in each compartment (0-7 cm > 8-14 cm > 15-21 cm). All roots from the AM treatments were shown to be well colonized as evidenced by the presence of hyphae, vesicles, and arbuscules (Table 7.4), meanwhile the non-AM treatments were confirmed to be non-mycorrhizal (data not shown). More specifically, the % frequency and distribution of hyphae and vesicles generally decreased with increasing soil-Zn levels, while arbuscules did not vary significantly. As found for the root fresh masses, the distribution of AM root colonization across the strata was also highest in the top compartment, then decreasing successively in each compartment (0-7 cm > 8-14 cm > 15-21 cm). Notably, no fungal structures were detected in the bottom compartment (15-21 cm) at the 200 and 400 soil-Zn levels.

The soil-Zn concentrations were determined in each compartment for all treatments (Fig. 7.3a, 7.3b, 7.3c, and 7.3d). The pre-experimental soil-Zn reference values (vertical lines) indicated a gradual increase from 8.8 to 395.4 mg Zn kg⁻¹ dry soil with increasing soil-Zn level. The post-experimental soil-Zn concentrations also showed this trend among all treatments, but the values were significantly lower than the pre-experimental ones (~25% to 40% lower) and decreasing in the order of un-seeded bare soil, AM plants, and non-AM plants. This distinct separation between the treatments was most prominent in the top compartment (0-7 cm) especially for the 200 and 400 soil-Zn levels, whereas the mid (8-14 cm) and bottom (15-21 cm) compartments showed no significant differences between the AM and non-AM treatments. The soil-pH values were also determined in each compartment for all soil-Zn treatments (Fig. 7.4a, 7.4b, 7.4c, and 7.5d). The pre-experimental soil-pH reference values (vertical lines) indicated a gradual decline from 6.23 to 5.31 with increasing soil-Zn levels. The post-experimental soil-pH also showed this trend among all treatments, but these values were again considerably lower than the pre-experimental ones and had a more prominent decrease in the top compartment (0-7 cm). In this compartment, the soil-pH values were generally lower in the decreasing order of non-AM plants, AM plants, and then un-seeded bare soil especially for the 50, 200, and 400 soil-Zn levels. As found for the soil-Zn concentrations, the soil-pH values were not significantly different between AM and non-AM treatments within the mid (8-14 cm) and bottom (15-21 cm) compartments.

Table 7.2 Plant Growth Parameters

Soil-Zn (mg kg ⁻¹ DS)	Inoculation	Fresh mass (g)			Shoot Height (cm)
		Flowers	Shoots	Roots †	
0	Non-AM	7.86 (1.51)a	24.1 (1.20)a	15.8 (0.19)a	25.0 (1.53)a
	AM	6.98 (0.64)a	22.2 (0.40)ab	17.9 (1.50)a	23.0 (1.15)a
50	Non-AM	6.15 (0.08)a	20.4 (0.54)bc	14.2 (0.88)ab	22.7 (0.33)a
	AM	5.49 (0.41)a	18.3 (0.27)c	14.7 (0.45)ab	21.7 (1.67)a
200	Non-AM	5.13 (0.40)a	15.7 (0.06)d	10.6 (0.76)b	19.0 (1.0)ab
	AM	6.66 (0.52)a	16.9 (0.35)d	12.8 (0.81)b	19.0 (2.0)ab
400	Non-AM	4.79 (1.58)a	12.7 (0.67)e	8.7 (0.32)c	17.3 (0.88)b
	AM	4.54 (1.12)a	14.9 (0.25)d	10.3 (1.19)bc	19.0 (2.08)ab
	Soil-Zn (Zn)	2.5ns	212.0***	24.7***	7.1**
	Inoculation (I)	0.2ns	11.8**	4.5*	3.2ns
	Zn×I	1.1ns	12.5***	1.5ns	3.2ns
	Block	3.6ns	9.0**	0.6ns	4.8*

Means (n=3) and SE (inside parentheses) are shown

Shared letters within each column designate treatments that are not significantly different according to Bonferonni and Scheffe mean comparison tests

† Combined roots from all 3 strata (see Table 2)

ns non-significant, * p<0.05, ** p<0.01, *** p<0.001

Table 7.3 Root Mass Distribution

Soil-Zn (mg kg ⁻¹ DS)	Stratum Compartment	Root fresh mass (g)	
		Inoculation	
		Non-AM	AM
0	0-7 cm	7.18 (0.15)a	8.49 (0.71)a
	8-14 cm	5.31 (0.26)b	6.70 (0.65)a
	15-21 cm	3.28 (0.24)c	2.66 (0.27)b
50	0-7 cm	5.91 (0.16)a	6.89 (0.20)a
	8-14 cm	5.52 (0.68)a	5.04 (0.75)a
	15-21 cm	3.29 (0.40)b	2.43 (0.11)b
200	0-7 cm	4.73 (0.23)a	5.87 (0.39)a
	8-14 cm	3.74 (0.37)a	4.39 (0.49)ab
	15-21 cm	2.10 (0.07)b	2.49 (0.67)b
400	0-7 cm	3.91 (0.32)a	5.35 (0.63)a
	8-14 cm	2.79 (0.13)ab	3.38 (0.81)ab
	15-21 cm	1.98 (0.22)b	1.58 (0.20)b
F-values and levels of significance			
	Soil-Zn (Zn)	38.8***	10.7***
	Stratum (S)	84.3***	60.4***
	Zn x S	3.64*	1.2ns
	Block	1.7ns	0.5ns

Means (n=3) and SE (inside parentheses) are shown

Shared letters within each soil Zn concentration grouping designate strata that are not significantly different according to Bonferonni and Scheffe mean comparison tests

ns non significant * p<0.05 ** p<0.01 *** p<0.001

Table 7.4 AM Root Colonization

Soil-Zn (mg kg ⁻¹ DS)	Stratum Compartment	Fungal Structures					
		Hyphae		Vesicles		Arbuscules	
		% Frequency	% Root Length	% Frequency	% Root Length	% Frequency	% Root Length
0	0-7 cm	67.3 (4.8)a	65.3 (4.3)a	53.3 (5.3)a	26.2 (4.7)a	38.3 (2.2)a	23.4 (1.5)a
	8-14 cm	47.3 (1.8)b	61.2 (1.7)a	37.3 (3.5)ab	27.6 (1.3)a	32.0 (2.0)a	23.3 (0.57)a
	15-21 cm	14.0 (7.0)c	11.0 (5.9)c	8.7 (4.4)c	5.2 (2.7)c	2.0 (2.0)b	0.7 (0.7)c
50	0-7 cm	52.7 (7.4)a	64.4 (1.6)a	38.0 (5.0)ab	23.6 (1.6)a	37.3 (5.7)a	23.4 (1.9)a
	8-14 cm	35.3 (5.2)b	54.8 (7.5)a	24.0 (1.2)c	17.8 (2.4)ab	21.3 (1.8)a	22.4 (1.8)a
	15-21 cm	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)	0.0 (0.0)c	0.0 (0.0)c
200	0-7 cm	49.3 (7.9)b	67.1 (3.9)a	32.0 (11.0)ab	19.5 (1.4)a	40.7 (3.5)a	23.2 (1.7)a
	8-14 cm	20.0 (6.1)c	29.1 (2.4)c	18.7 (6.7)b	14.9 (5.9)ab	9.3 (4.7)b	5.8 (1.8)b
	15-21 cm	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)c	0.0 (0.0)c	0.0 (0.0)c
400	0-7 cm	40.7 (1.8)b	67.5 (4.4)a	24.7 (0.7)b	11.8 (1.8)b	36.7 (0.7)a	23.5 (6.3)a
	8-14 cm	8.7 (4.4)c	13.8 (6.9)c	7.3 (3.7)c	7.9 (4.0)b	3.3 (1.8)b	2.1 (1.1)b
	15-21 cm	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)d	0.0 (0.0)c	0.0 (0.0)c	0.0 (0.0)c
F-values and levels of significance							
	Soil-Zn (Zn)	10.7*	0.31ns	14.8***	9.2**	1.26ns	11.64**
	Stratum (S)	92.3***	72.0***	70.5***	33.3***	79.6***	15.5***
	Zn x S	1.6ns	0.67ns	4.24*	2.64ns	0.01ns	11.37**
	Block	0.41ns	0.19ns	0.74ns	0.08ns	0.14ns	0.01ns

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 Scheffe mean comparison tests

ns non significant * p<0.05 ** p<0.01 *** p<0.001

Figure 7.2 Zn concentrations in flowers (a) and shoots (b). Means (n=4) and standard errors for the non-AM (grey bars) and AM (white bars) treatments are shown. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests ($p < 0.05$).

Figure 7.2

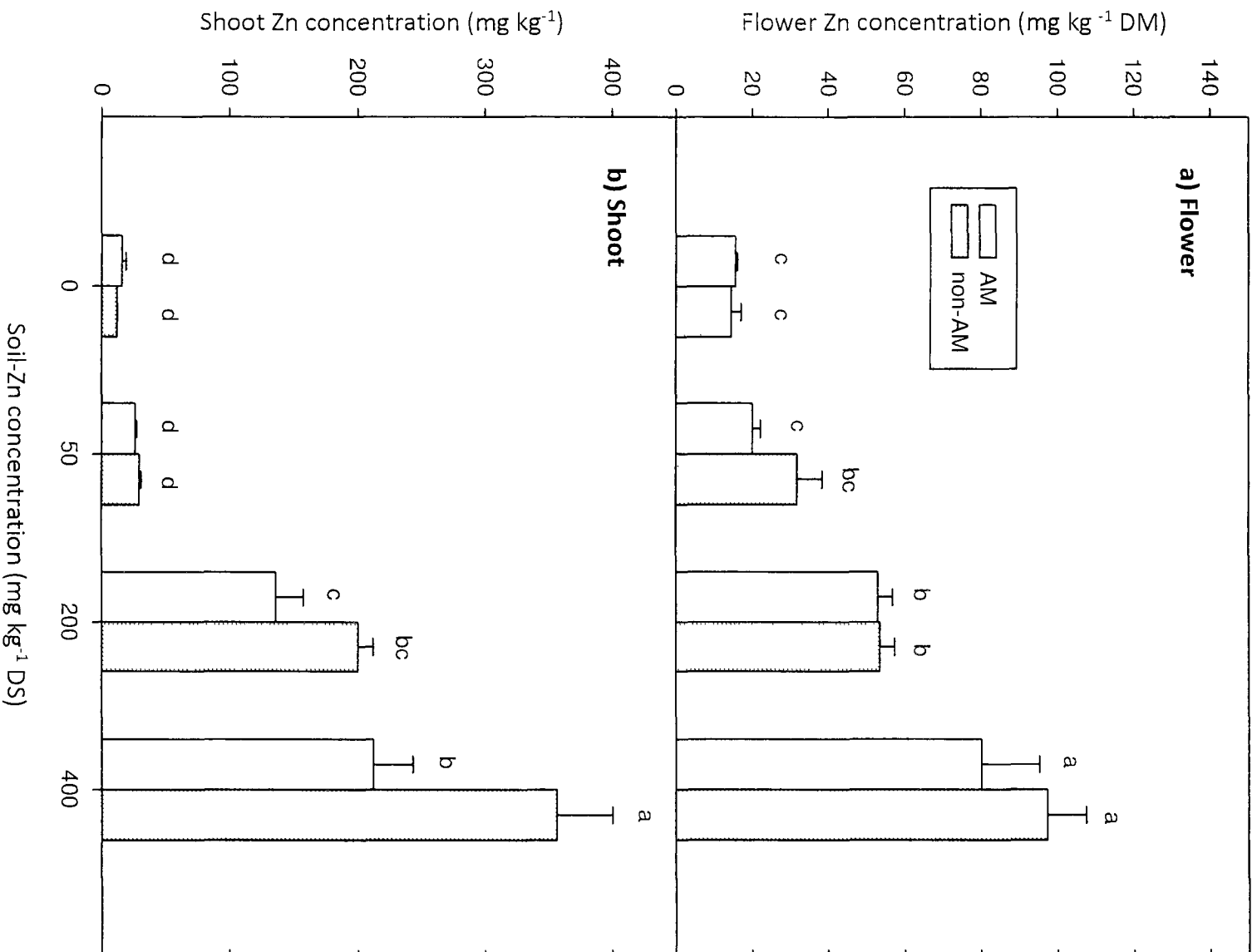


Figure 7.3 Soil-Zn concentrations in each strata compartment for the 0-control (a), 50 (b) 200 (c), and 400 soil-Zn treatments (d). Means (n=4) and standard errors for the non-AM (black circles), AM (white circles), and un-seeded soil (black triangles) treatments are shown. Vertical reference lines represent the mean (\pm S.E.) pre-experimental soil-Zn concentrations values. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests ($p < 0.05$).

Figure 7.3

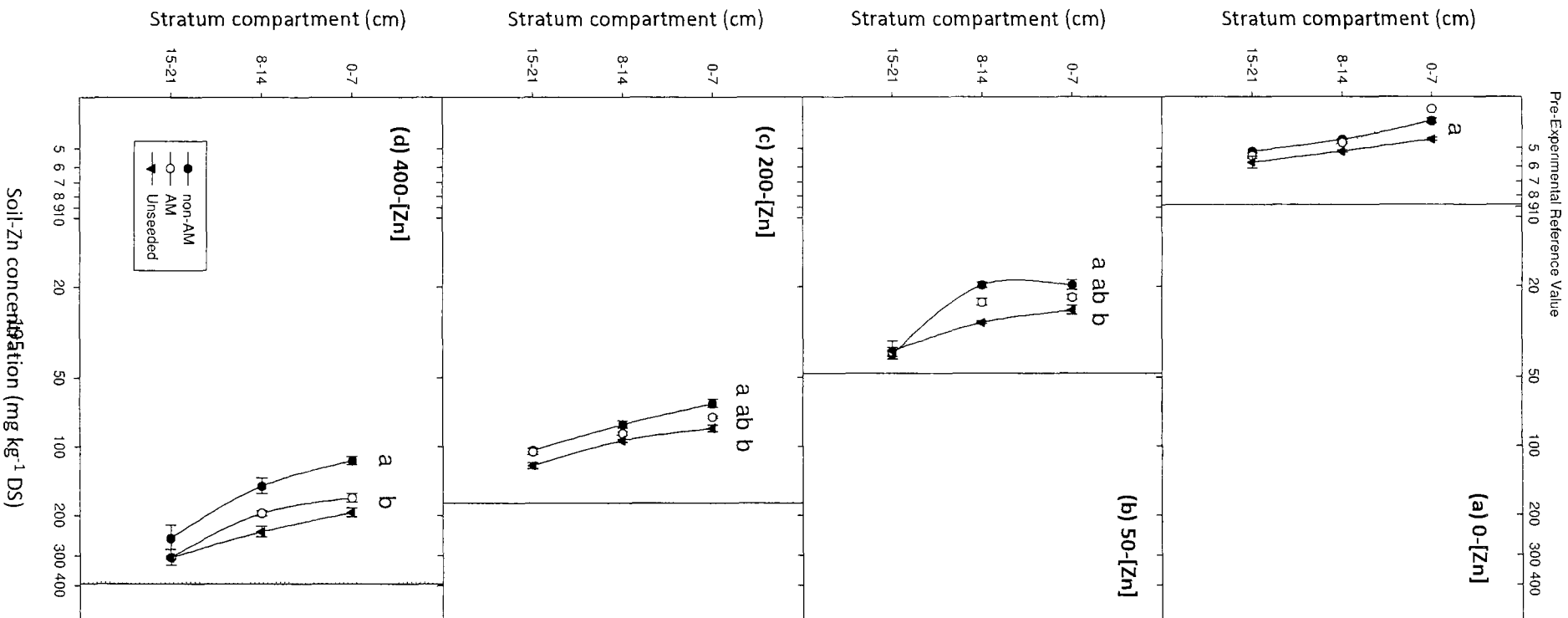
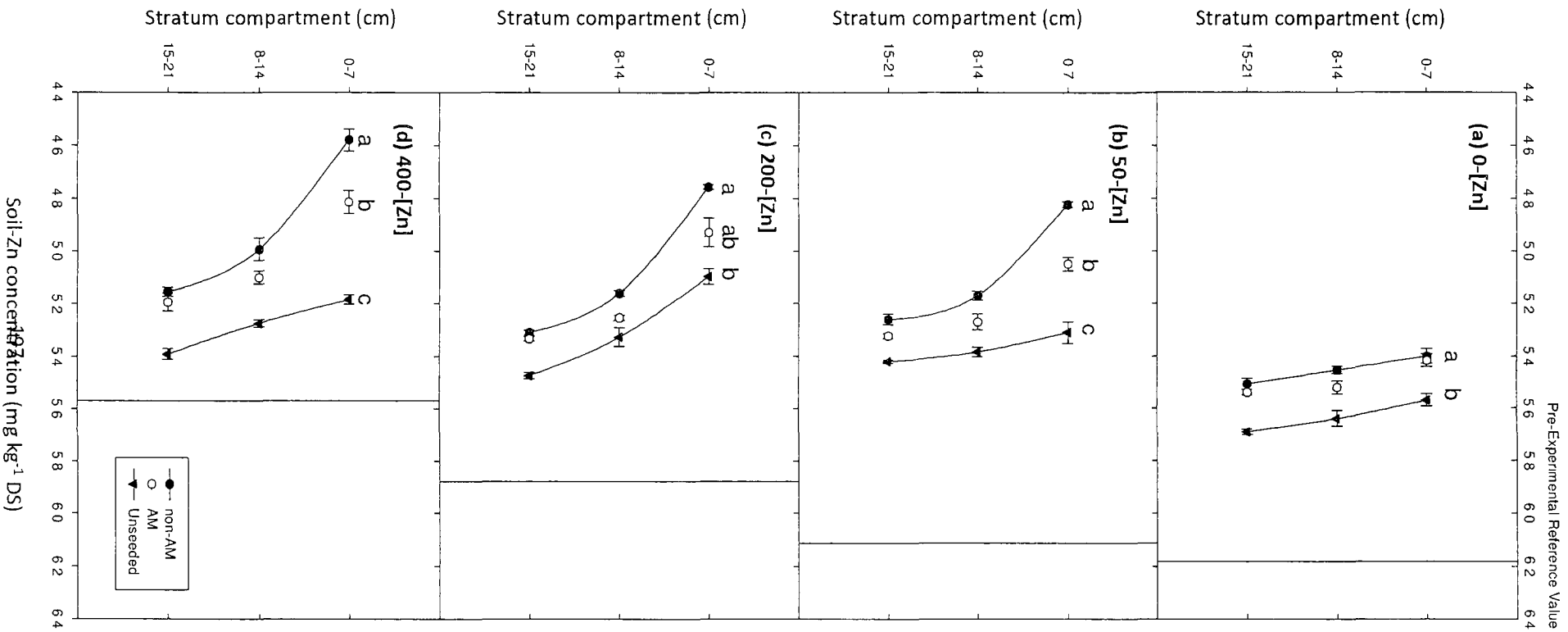


Figure 7.4 Soil-pH in each strata compartment for the 0-control (a), 50 (b) 200 (c), and 400 soil-Zn treatments (d). Means (n=4) and standard errors for the non-AM (black circles), AM (white circles), and un-seeded soil (black triangles) treatments are shown. Vertical reference lines represent the mean (\pm S.E.) pre-experimental soil-pH values. Shared letters designate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests ($p < 0.05$).

Figure 7.4



7.4 Discussion

As predicted by the hypothetical model of AM plant metal uptake, the present findings demonstrate the phytoprotective effects of AM symbiosis which can enhance plant stress tolerance due to the metal biosorption properties of the mycorrhizosphere. In this regard, it is presumed that the extraradical hyphae can reduce metal bioavailability, especially when reaching potentially toxic exposure levels. As discussed in Chapters 5 and 6, these combined effects are considered to be indicative of an increased metal stress tolerance associated with AM-induced biosorption processes such as hyphal metal-binding (Gadd 1993; Galli et al. 1994; Gonzalez-Chavez et al. 2002) and metal sequestration (Chen et al. 2001; Gonzalez-Guerrero et al. 2007) that together reduce plant metal uptake to delay the onset of metal toxicity (Schutzendubel and Polle 2002). Indicative of this AM plant stress tolerance effect, up to 40 % lower Zn concentrations were found in the shoots of AM plants especially at the highest soil-Zn levels (200 and 400 soil-Zn) resulting in these plants having both increased root and shoot masses compared to non-AM plants. Accordingly, it is believed that the AM-mycorrhizosphere has a higher affinity for metal-binding than the rhizosphere due to the immobilization of excess metal ions to carboxyl, hydroxide, oxyhydroxide, and sulfhydryl constituents of the extraradical hyphae (Gadd 1993; Galli et al. 1994; Gonzalez-Chavez et al. 2002). Thereafter, metals may be sequestered by the mycelium through binding to the hyphal walls followed by diffusion into hyphal tissues or, alternatively, precipitated as metal-ligand complexes in the proximal soil environment (Jeffries et al. 2006; Joner et al. 2000; Leyval et al. 1997). Consequently, the fungal metal-binding process is believed to

decrease plant metal uptake by reducing the bioavailability of potentially toxic metals (Chapters 3-5; Cavagnaro et al. 2010). For these reasons, it was previously suggested that the mycorrhizal biosorption and hyphal sequestration processes are essential components of extrinsic plant stress tolerance which could complement intrinsic plant detoxification strategies, such as metallothionein and phytochelatin mechanisms (Cobbett and Goldsbrough 2002). In other words, the host plants could invest in an extrinsic stress tolerance strategy in order to maximize their growth and improve their health in relation to a number of environmental stressors. Notwithstanding, this symbiotic investment implies a significant metabolic cost (*i.e.* plant carbohydrates) on behalf of the host plant in order to maintain the mycorrhizospheric infrastructure: an investment which can represent up to 20 % of the plant's total carbon budget (Douds et al. 2000; Tinker et al. 1994). Corresponding with the considerable levels of AM root colonization reported here (*e.g.* as evidenced by a generally the high frequency and distribution of fungal hyphae, arbuscules, and vesicles), the allocation of plant carbohydrates to the AM symbiont could account for the slight decrease in AM-plant flower and shoot fresh masses compared to non-AM plants among the 0-control and 50 soil-Zn treatments (Chapter 8).

Of further interest, it is also reported here that AM plants have a distinctive AM root colonization pattern in relation to the increasing soil-Zn levels. As mentioned, the AM roots were found to be well colonized and showed an abundance of all fungal structures suggesting that the symbiosis was well active among all the treatments. However, the % frequency and distribution of hyphae and vesicles tended to decrease with increasing soil-Zn, which also corresponded with an overall

decrease in biomass for roots, shoots, and flowers. This finding points out the likelihood of a metal toxicity burden affecting both host plants and AM fungi (Chapter 2). In plants, this is expressed directly due to cellular oxidative stress (Baccouch et al. 1998; Cho and Seo 2005; Schützendubel and Polle 2002) or indirectly due to elemental nutrient imbalances arising in the rhizosphere (McBride 1994; Kabata-Pendias 2007). Meanwhile, the AM fungi are similarly affected through decreases in extraradical mycelium proliferation and sporulation (Chapter 5; Janouskova and Vosatka 2005; Pawlowska and Charvat 2004). For this reason, it is believed that such metal toxicity burdens would ultimately affect the symbiotic investment despite the many benefits of AM fungi to plant stress tolerance (Chapter 3). Besides the effects of metal exposure on the AM symbiosis, it was also observed the pattern of decreasing root colonization in each successive strata depths such that a greater frequency and abundance of fungal structures were measured in the top-most soil stratum. Hence, it is considered that the AM symbiosis was most active particularly in this mycorrhizospheric zone which then had significant implications toward the edaphic conditions, as discussed in more detail further on.

As for the soil conditions, the present findings demonstrate (if only in part) the roles of the AM symbiosis in buffering the soil environment to then potentially enhance its resiliency. Overall, the initial addition of Zn in the form of ZnSO_4 as well as the daily watering and fertilization regimes had significant consequences on the edaphic conditions. In this case, the proportional increase of SO_4^{2-} and $\text{H}^+/\text{H}_3\text{O}^+$ ions in the soil solution resulted in progressively lower soil-pH and differential soil-Zn bioavailability (Chapter 6; Chuan et al. 1996; Li and Christie 2001; Martinez and Motto

2000; Ross 1994; Tack et al. 1996). As in the previous compartmental greenhouse study, such inputs can detrimentally influence the soil's metal-binding capacity resulting in an increased rate of metal leaching (Apak 2002; Bradl 2004). What's more, the present results indicate that the presence of non-AM roots versus AM roots and extraradical hyphae further affected the edaphic conditions such that the non-AM treatments showed increasingly more acidic soil-pH as well as gradually lower soil-Zn bioavailability than AM treatments, particularly at the highest soil-Zn level. Although this finding may not be entirely conclusive on its own, the trend of increased soil acidity in non-AM versus AM treatments are similar to findings in the previous complementary greenhouse study (Chapter 6) in which the effects of the rhizosphere (roots), mycorrhizosphere (AM roots and extraradical hyphae), and hyphosphere environments (strictly extraradical hyphae) were isolated and compared for the same soil parameters (*e.g.* soil-pH and Zn bioavailability). While these tendencies were primarily attributable to the different rates of Zn uptake between the AM and non-AM treatments resulting in respectively different soil-Zn depletion zones, it is considered that the processes of hyphal alkalinisation and metal biosorption could also have influenced these soil parameters as in the case of phosphorus and nitrogen acquisition by mycorrhizae (Bago et al. 1996; Eckhard et al. 1995; Gahoonia and Nielsen 1992). In fact, the extraradical hyphae can induce the moderate alkalinisation of the proximal soil environment in relation to N and P uptake due to the exudation of specific organic chelators; this, unlike roots that tend to acidify it. This perspective is relevant since the processes of hyphal alkalinisation due to chelation could favour metal biosorption to reduce metal bioavailability, whereas root acidification may

facilitate metal leaching by increasing metal solubility. In this regard, it is recognized that further experimental investigation may be needed to verify this facet of the proposed model, for instance studies as to the impact of AM roots and extraradical hyphae on the mass balance of soil pollutants. Still, it is also recognized that the presence of non-AM roots versus AM roots and hyphae should likely play an important role in shaping the soil environment, thereby potentially influencing its resiliency when subjected to various environmental stressors. Accordingly, the stratified compartmental growth system present here enabled an assessment of the mycorrhizosphere's primary zones of influence across the successive soil strata. Here, the most considerable differences in soil-pH and soil-Zn bioavailability between the AM and non-AM treatments occurred in the top compartment, which coincided with the highest levels of root colonization. Such differences then became more marginal in each successive compartment, which also coincided with a sharp decrease in root colonization levels. These results could be associated with the greater distribution of roots and fungal inoculum in the top compartment which can increase the frequency of plant-fungus interaction to promote AM root colonization (Chapter 8). Thus, the mycorrhizosphere was likely primarily active in the topmost soil stratum where it had a greater impact on the edaphic growth conditions, particularly the soil-pH and Zn bioavailability.

7.5 References

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Section IV

Supplementary

Information

Note on publications from this Section

The contents in this Section were first published in the following proceedings:

- Audet P, Charest C (2010) Identification of constraining experimental design factors in mycorrhizal pot-growth studies. *Journal of Botany* doi:10.1155/2010/718013

Chapter 8

Identification of experimental design biases in mycorrhizal pot-culture

8.1 Objectives

Design parameters such as the size of the experimental microcosm (*e.g.* pot size) can have a significant influence on the interpretation of results regardless of the intended experimental conditioning (Bååth and Hayman 1984, Balali et al 2008, Ray and Sinclair 1998, Townend and Dickinson 1995). Specific to the study of mycorrhiza, it is believed that the pot size and fungal inoculum distribution may inadvertently affect plant growth and symbiotic interaction. Accordingly, it can be hypothesized that pot size influences the dynamics of resource allocation and mycorrhizal symbiosis by potentially restricting the proliferation of roots and extraradical hyphae (Koide 1991). Meanwhile, the distribution of fungal inoculum may also influence the frequency of interaction between roots and fungal propagules which then affects the level of root colonization level and symbiotic activity (Abbott and Robson 1991). To test these notions, I conducted a factorial greenhouse study of 'dwarf' sunflower and an arbuscular mycorrhizal (AM) fungus to discern how the pot size and fungal inoculum distribution affect plant growth and root colonization. Given that the AM symbiosis is widely recognized for benefiting plants under various environmentally stressful conditions, I then conducted a follow-up experiment incorporating a water deficit treatment to investigate the effects of pot size on the dynamics of symbiotic association and stress tolerance.

Figure 8.1 Schematization of the pot size and inoculum distribution treatments.

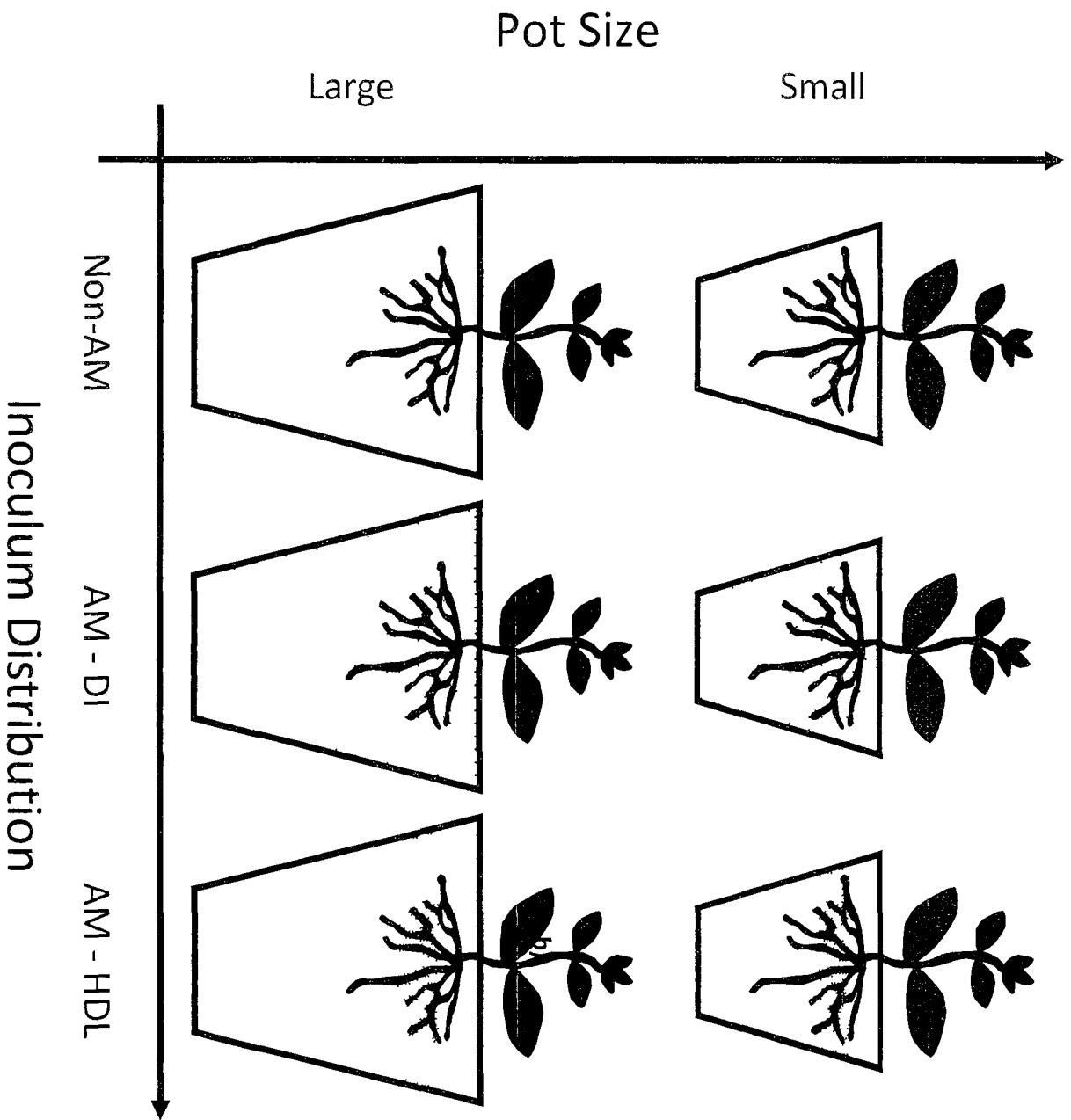


Figure 8.1

8.2 Materials and methods

(a) Experimental design

In the first of two pot-growth experiments, 'dwarf' sunflower (*Helianthus annuus* L. var. Pacino) plants were grown from seeds (McKenzie Seeds, Brandon, MB, Canada) for 10 weeks in a previously autoclaved, low mineral soil mixture (sand:potting soil, 1:1 v/v). Plants were grown in either small (2 L) or large-sized (7.5 L) pots containing the same base soil mixture but incorporating one of three fungal inoculum treatments (non-inoculated [Control]; dispersed inoculum [AM-DI]; or high density layered inoculum [AM-HDL]) which resulted in a total soil volume equivalent to the respective pot size capacity (Fig. 8.1). To test the effects of fungal inoculum distribution, the AM-soil treatments were achieved by incorporating fungal propagules (e.g. spores and hyphae) of *Glomus intraradices* Schenck & Smith (isolate DAOM-181602) from a commercial inoculum having a reported density of 15 propagules g⁻¹ dry substrate (Myke® Pro Endo, Premier Tech, Premier Tech, Rivière-du-Loup, QC, Canada). The AM-DI treatment consisted of the inoculum substrate being evenly mixed into the soil using an industrial mixer, whereas the AM-HDL treatment consisted of the same inoculum integrated as a 3cm layer of substrate. Due to the respective pot-size volumes, it was estimated that small pots contained ~750-1000 total propagules (65 g inoculum substrate dose) while large pots contained ~2750-3000 propagules (200 g inoculum substrate dose). The factorial design of the study (1 plant sp. x 3 soil treatments [Control; AM-DI; AM-HDL] x 2 pot sizes [Small; Large] x 5 reps) provided a total of 30 plants (1 plant *per* pot), each distributed in a factorial block design. The greenhouse conditions were maintained at 25°C / 23°C (day/night) with a 16 / 8 h

(light / dark) photoperiod, an average light intensity of $364.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, and a 65 % relative humidity. Over the course of the experimental period, all plants were watered daily as required avoiding any leaching and fertilized biweekly (100 ml *per* week) from weeks 4 to 10 using a low phosphorus Long-Ashton nutrient solution (K_2SO_4 2.0 mM, CaCl_2 anhydride 4.0 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5 mM, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 1.5 mM, NH_4NO_3 5.0 μM , $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.01 mM, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.0 μM , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 μM , H_3BO_3 0.05 mM, NaCl 0.09 mM, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.5 μM , and EDTA-Fe 0.1 mM).

In the second experiment, plants were grown in similar small and large-sized pots containing either the non-AM Control or AM-HDL inoculum substrates for 10 weeks under the same conditions as described above, but also incorporating a water deficit regime according to Ray and Sinclair (1998). This regime consisted of monitoring the water content of all soils via time-domain reflectometry (Dalton and Van Genuchten 1986), and specifically reducing the relative soil moisture from 90% to 20% for the drought-treated plants over the course of weeks 4 to 10. All plants received a biweekly fertilization (100mL *per* week) as described above. The factorial design of the study (1 plant sp. x 2 soil treatments [Control; AM-HDL] x 2 pot sizes [Small; Large] x 2 irrigation treatments [Well-Watered; Droughted] x 5 reps) provided a total of 40 plants (1 plant *per* pot).

(b) Harvest and determination of AM root colonization

Plants were harvested 10 weeks after seeding with roots being thoroughly cleaned with tap water. Plant organs were then partitioned as roots, shoots or flowers, oven-dried at 70 °C for 72 h, and weighed separately. At the time of harvest, fresh root samples (2 g) from each replicate were carefully excised from the apical zone near the taproot and stained with aniline blue 0.02% dye solution (6.78 mM aniline blue; 500 ml glycerol; 450 ml H₂O; 50 ml 1 % HCl) according to Dalpé (1993). One hundred ~1-2 cm long root segments *per* replicate were randomly selected, mounted on slides, and examined at 100X and 400X magnification using a compound microscope (CX41, Olympus Inc., Centre-Valley, PA, USA). Samples of non-AM roots were also prepared to ensure of their non-mycorrhizal status. Mycorrhizal colonization was estimated by determining the % frequency of colonization (Dalpé 1993), % length of root colonization, and relative density (# mm⁻¹ root length) of fungal structures (*e.g.* intraradical vesicles and arbuscules). The equations for the % frequency (1), % length of root colonization (2), and relative density (3) are defined as:

$$\frac{\# Segments_{AMstructures}}{\# Segments_{total}} \times 100\% \quad (1)$$

$$\frac{RootLength_{AMstructure}}{RootLength_{total}} \times 100\% \quad (2)$$

$$\frac{\# AMstructures}{RootLength_{colonized}} \quad (3).$$

(c) Statistical analyses

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

8.3 Results

In the first experiment, the root, shoot, and flower dry masses were over two-fold greater for large versus small-potted plants (Fig. 8.2*a*, 8.2*b*, and 8.2*c*). In this case, the flowers of large-potted plants were gradually smaller in the order of Control (7.1 g), AM-DI (4.5 g), and AM-HDL (2.8 g) treatments, while small-potted plants (ranging between 0.9 and 1.3 g) showed no such differences. Although small-potted plant roots showed some incidence of being pot-bound, none of these plants demonstrated any signs of nutrient deficiency. As for the root colonization (Table 8.1), all AM treatments were shown to be well colonized as evidenced by the presence of hyphae, vesicles, and arbuscules; however, the % frequency and length of root colonization were generally greater among large versus small-potted plants. In addition, the inoculum distribution also influenced root colonization as a greater length of colonization and density of vesicles were observed for the AM-HDL treatments; yet, a higher density of arbuscules for the AM-DI treatments.

In the second experiment, the root, shoot, and flower dry masses were again greater for large versus small-potted plants (Fig. 8.3*a*, 8.3*b*, and 8.3*c*). Furthermore, well-watered plants generally produced greater biomass than drought-treated ones (both large and small pots) which showed symptoms of wilting and chlorosis. Notably, the AM-droughted plants grown in large pots showed flower (3.4 g) and shoot (9.2 g) dry masses similar to those of well-watered plants (4.0 g flower, 9.9 g shoot), whereas non-AM droughted plants suffered a reduction in dry-mass. The AM-drought small-potted plants showed no such growth advantage. As in the first experiment, all AM plant treatments were well colonized and large-potted plants and

again generally tended to have a higher % frequency and length of root colonization than the small-potted plants (Table 8.2). Meanwhile, the overall AM root colonization of well-watered plants was greater than drought-treated ones.

Table 8.1 AM root colonization the pot size and inoculum distribution experiment.

Pot size	Inoculum	Frequency (%)			Root Length Colonized (%)			Density (mm ⁻¹)	
		Hyphae	Vesicles	Arbuscules	Hyphae	Vesicles	Arbuscules	Vesicles	Arbuscules
Small	AM-DI	21.0 (3.1)b	7.4 (1.0)c	21.0 (3.1)b	64.5 (3.9)a	8.7 (3.1)c	56.0 (4.1)a	1.5 (0.5)b	7.0 (0.6)a
	AM-HDL	22.8 (2.6)b	14.8 (2.3)bc	20.0 (2.5)b	76.8 (3.1)a	35.0 (5.2)a	59.1 (5.9)a	3.0 (0.2)ab	4.4 (0.3)b
Large	AM-DI	40.0 (4.1)a	20.0 (2.7)b	39.0 (3.5)a	75.3 (4.5)a	14.2 (2.2)b	58.9 (4.5)a	2.2 (0.1)b	7.0 (0.7)a
	AM-HDL	43.0 (4.4)a	37 (3.8)a	34.5 (3.5)a	82.1 (5.1)a	50.1 (6.1)a	53.4 (6.0)a	4.9 (0.6)a	3.8 (0.3)b
F-values and levels of significance									
	Pot size (P)	34.9***	49.6***	28.2***	3.3ns	5.8*	0.4ns	0.3ns	1.85ns
	Inoculum (In)	0.5ns	24.3***	0.8ns	4.7*	51.2***	0.1ns	33.3***	25.6***
	PxIn	0.1ns	4.8*	0.3ns	0.4ns	1.1ns	0.6ns	0.4ns	9.4**
	Block	3.2ns	3.6ns	1.8ns	0.0ns	2.7ns	0.1ns	0.3ns	0.3ns

Means (n=5) and SE (inside parentheses) are shown.

Shared letters within each column designate treatments that are not significantly different according to Bonferonni and Scheffe mean comparison tests.

'ns' non-significant

* p<0.05

** p<0.01

*** p<0.001

Table 8.2 AM root colonization for the pot size and water deficit experiment.

Pot size	Irrigation	Frequency (%)			Root Length Colonized (%)			Density (mm ⁻¹)	
		Hyphae	Vesicles	Arbuscules	Hyphae	Vesicles	Arbuscules	Vesicles	Arbuscules
Small	Drought	33.6 (6.7)a	24.4 (7.1)a	25.6 (4.8)a	54.0 (5.8)b	22.6 (5.2)b	28.0 (1.9)c	2.6 (0.2)b	3.2 (0.1)a
	Well-Watered	40.8 (3.0)a	37.6 (4.1)a	25.2 (2.6)a	76.4 (5.1)b	43.0 (3.6)a	35.7 (9.2)bc	3.2 (0.3)a	3.6 (0.4)a
Large	Drought	44.6 (9.7)a	34.6 (9.5)a	31.0 (8.3)a	71.6 (6.1)b	24.2 (5.7)b	39.5 (6.8)b	2.7 (0.2)b	3.5 (0.1)a
	Well-Watered	50.8 (8.4)a	38.8 (6.2)a	38.2 (7.1)a	85.2 (1.1)a	30.8 (1.3)b	56.1 (2.4)a	3.4 (0.4)a	3.7 (0.1)a
F-values and levels of significance									
	Pot size (P)	1.9ns	0.6ns	2.9ns	6.7*	1.4ns	12.6***	0.2ns	1.1ns
	Irrigation (Ir)	0.0ns	0.4ns	0.5ns	0.7ns	2.4ns	1.0ns	0.0ns	0.9ns
	Pxlr	0.8ns	1.4ns	0.4ns	12.3***	9.0**	7.4*	4.4*	1.6ns
	Block	0.8ns	0.6ns	2.2ns	0.7ns	0.7ns	4.0*	0.8ns	1.0ns

Means (n=5) 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' non-significant

* p<0.05

** p<0.01

*** p<0.001

Figure 8.2 Dry masses of flowers (a), shoots (b), and roots (c) from the pot size and inoculum distribution experiment. Means (n=5) and SE for the non-AM (empty bars), AM-DI (grey bars) and AM-HDL treatments (solid bars) are shown. Shared letters indicate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests.

Figure 8.2

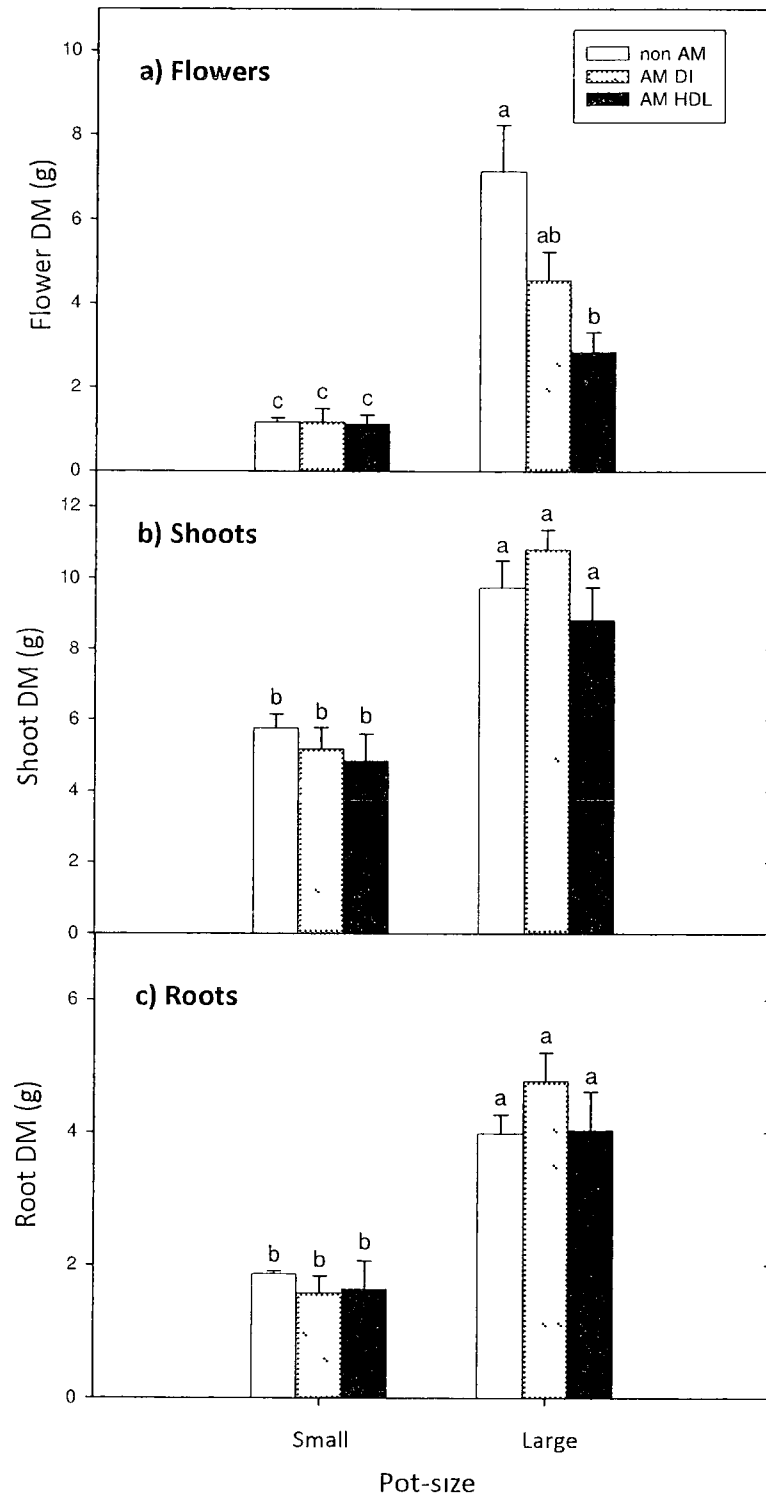
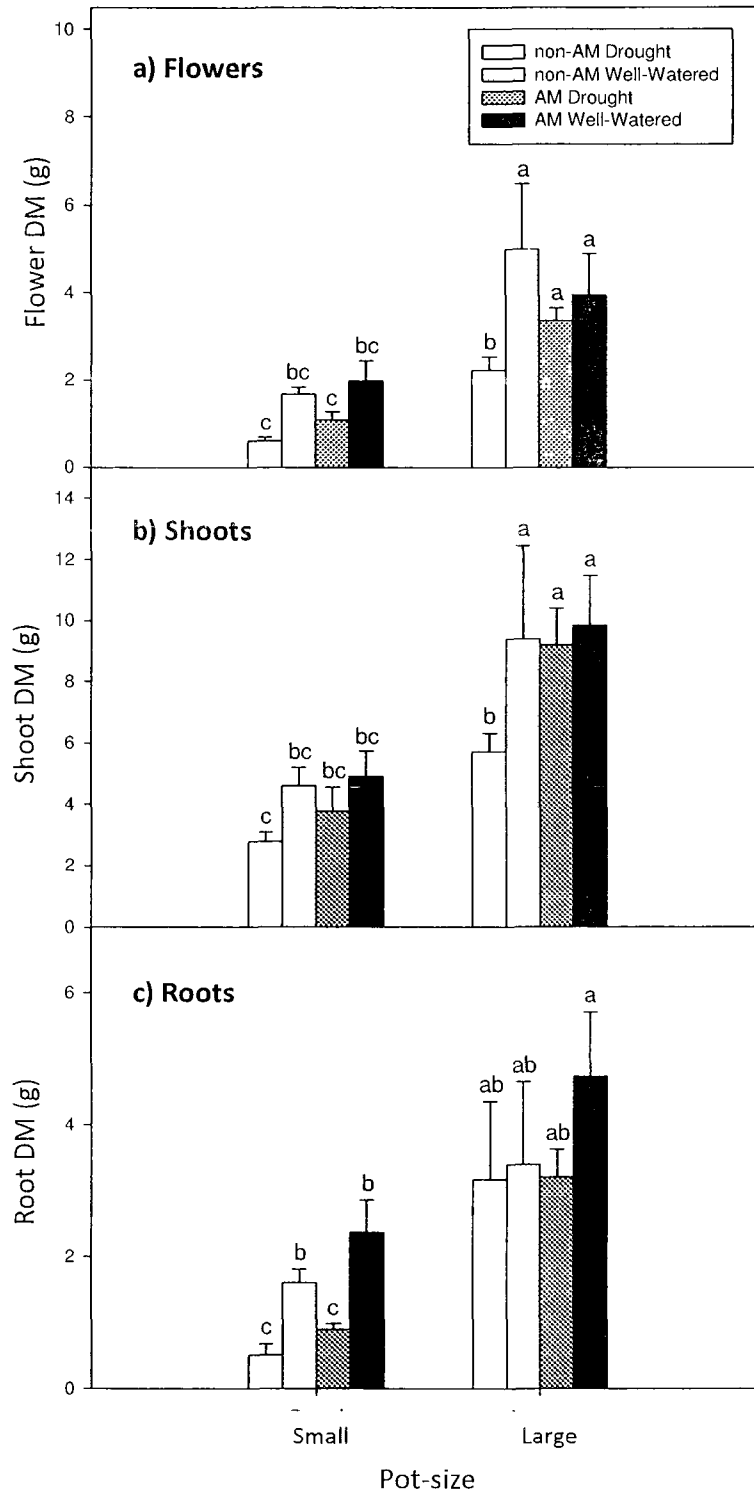


Figure 8.3 Dry masses of flowers (a), shoots (b), and roots (c) from the pot size and water deficit experiment. Means ($n=5$) and SE for the non-AM drought (empty bars), non-AM well-watered (light-grey bars), AM drought (dark-grey bars), and AM well-watered treatments (solid bars) are shown. Shared letters indicate treatments that are not significantly different according to Bonferonni and Scheffé mean comparison tests.

Figure 8.3



8.4 Discussion

It is reported here that pot size is a key experimental factor that, when limiting, can significantly reduce plant growth and AM root colonization. This finding is associated with the fact that the pot is an artificial microcosm having a smaller 'rootable' volume than natural environments (Townend and Dickinson 1995). This 'rootable' volume strictly determines the total nutrient supply in the rhizosphere and then limits the plants' relative growth potential (*i.e.* % of maximum growth) without necessarily expressing any noticeable symptoms of nutrient deficiency, as reported in studies of maize, potato, soybean, and young spruce (Balali et al. 2008; Ray and Sinclair 1998; Townend and Dickinson 1995). By restricting the proliferation of roots and extraradical hyphae, the 'rootable' volume also influences the investment in AM symbiosis since the primary benefit of mycorrhizal association (*i.e.* an enhanced resource acquisition capacity) is reduced, then resulting in a lower overall root colonization among small-potted plants (Bååth and Hayman 1984). Accordingly, when the pot size is less constraining, our results demonstrate that plant growth and AM colonization are both significantly increased.

Besides pot size, it was also determined that the dispersal of the fungal inoculum further affected AM root colonization such that plants grown in the high density layered (AM-HDL) substrate showed a higher frequency and density of vesicles than those grown in the dispersed inoculum (AM-DI) substrate. By contrast, those grown in the AM-DI substrate showed a higher density of arbuscules. The prevalence of vesicles (sites of lipid storage) over arbuscules (sites of resource exchange) indicates that the AM fungus had reached a more advanced stage of its life cycle

(Dalpé et al. 2005). As predicted, this tendency may be attributed to the fact that roots of AM-HDL plants likely had earlier colonization encounters with fungal propagules compared to roots of AM-DI plants which had a relatively more diffuse propagule distribution. For this reason, it is suspected that the higher density inoculum distribution could be more effective in promoting root colonization than a dispersed one despite having similar total fungal propagules *per pot*. Furthermore, it is also suspected that such differences in mycorrhizal investment could account for the gradually lower flower dry masses produced by non-AM control, AM-DI, and AM-HDL plants, particularly owing to the differential resource allocation associated with maintaining the symbiosis (Douds et al. 2000; Graham and Eissenstat 1998).

From the second experiment, it was demonstrated that the pot size effect may confound the dynamics of symbiotic association and plant stress tolerance. Besides having a greater overall biomass and root colonization for the large versus small-potted plants (as above), the AM-droughted plants produced similar shoot and flower biomass compared to the well-watered plants whereas non-AM plants suffered a growth reduction. Unlike small potted-plants which showed no such differences, large potted AM-plants especially benefited from the mycorrhizosphere's enhanced resource acquisition capability which provided them with a significant growth advantage over non-AM plants when subjected to water-deficit conditions (Al-Karaki 1998; Subramanian and Charest 1998, 1999). In addition, it is possible that the mycorrhizosphere also provided indirect benefits to host plants by enhancing soil stability (Augé et al. 2001; Bearden and Petersen 2000) and moisture retention (Miller and Jastrow 1990; Piotrowski et al. 2004) thereby buffering the soil environment. In

this regard, a more thorough investigation into the extent of the mycorrhizosphere would be beneficial to specifically verify these perspectives.

In conclusion, this study has shown that pot size and fungal inoculum distribution are key experimental factors that affect plant growth and AM root colonization by respectively influencing (a) the 'rootable' volume and (b) the likelihood of interaction between roots and fungal propagules. When limiting, these factors may influence the interpretation of results regardless of the intended experimental conditioning, such as drought resistance. By taking these factors into consideration, it is possible to reduce result biases and facilitate the comparison of findings between studies.

8.5 References

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Section V

Conclusion

Chapter 9

General discussion

9.1 Statement of originality

The purpose of this PhD thesis was to determine the impact of the AM symbiosis in plant stress tolerance across a wide range of metal stress, focusing especially on the antithetical roles of 'enhanced uptake' and 'metal biosorption' in plant metal uptake. To investigate these hypotheses, I first conducted a meta-analysis of the current body of phytoremediation literature to quantitatively assess trends in plant metal uptake in relation to soil metal conditions ranging from trace to toxic exposure levels. This review method was then used to investigate the inherent effects of the AM symbiosis in plant metal uptake and relative plant growth, and to determine the role of resource allocation plasticity and metal partitioning in plant stress tolerance. From these findings, I developed novel conceptual models of plant function integrating the key roles of AM 'enhanced uptake' and 'metal biosorption' by emphasizing how AM fungi can potentially shape the soil environment under various environmental conditions. Within this context, I then adapted relevant *in vitro* root-organ and *in vivo* whole-plant experimental systems especially designed to investigate the proposed dynamics of AM symbiosis and plant stress tolerance. These targeted experimental studies enabled me to test the conceptual models and further isolate their primary mechanisms of interaction, leading me to revise the initial hypotheses of AM-plant metal stress tolerance. Corresponding with these findings, I also expanded the scope

of the models to better depict the impact of the AM fungi on the soil environment. Besides these core research objectives, I conducted preliminary studies of pot-grown plants in the goal of improving our greenhouse experimental procedures. Here, I identified the size of the experimental microcosm (*e.g.* pot-size) and the distribution of the fungal inoculum as two experimental design factors that could potentially bias plant growth and development of AM root colonization. Overall, my primary statements of originality (Table 9.1) refer to the multi-disciplinary strategy of developing and adapting meta-analytical and experimental methodologies for assessing the role of the AM symbiosis in plant growth and metal-uptake. More specifically, in regards to the meta-analytical component of this thesis, I derived appropriate plant physiological metrics and established statistical methodologies for the analysis of meta-data extracted from over 30 published studies. Meanwhile, in regards to the experimental component, I designed and (or) adapted relevant experimental systems for subsequent investigations which included newly developed and optimized metal extraction procedures using ICP-OES metal analysis technologies. Altogether, these analytical tools enabled me to verify the proposed conceptual models and derive new perspectives as to the wide-ranging role of the AM symbiosis in plant development and ecosystem function. Notably, I contributed in addressing the questions of how mycorrhizospheric processes benefit plant stress tolerance, under what environmental conditions they occur, what mechanisms determine their function, and how they could impact the proximal soil environment.

Table 9.1 Summary of statement of originality

Section II – Meta-analysis

- | | | | |
|---|------------------|---|---|
| <ul style="list-style-type: none"> • First conducted meta-analysis of the current metal phytoremediation body of literature, | Chapter 2 | Trends and perspectives in metal phytoremediation | Audet P, Charest C (2007)
Environ Pollut 147 231-237 |
| <ul style="list-style-type: none"> • Development of appropriate physiological metrics and statistical methodologies for the analysis of meta-data, | Chapter 3 | AM symbiosis and plant metal uptake | Audet P, Charest C (2007)
Environ Pollut 147 609-614 |
| <ul style="list-style-type: none"> • Development of conceptual models of plant metal uptake and stress tolerance derived from the meta-analytical findings | Chapter 4 | Resource allocation plasticity and plant metal partitioning | Audet P, Charest C (2008)
Environ Pollut 156 290-296 |

Section III – Experimental analysis

- | | | | |
|---|------------------|--|---|
| <ul style="list-style-type: none"> • Adaptation of relevant <i>in vitro</i> root-organ and <i>in vivo</i> whole-plant experimental systems to address the proposed conceptual models of plant metal uptake and stress tolerance, | Chapter 5 | In vitro root-organ culture AM symbiosis and plant metal uptake | Audet P, Charest C (2009)
Botany 87 913-921 |
| <ul style="list-style-type: none"> • Development of plant and soil metal extraction procedures corresponding with ICP-OES analytical technologies, | Chapter 6 | Greenhouse culture Plant metal uptake and soil metal bioavailability in the mycorrhizosphere | Audet P, Charest C (2010)
J Bot doi 10 1155/268450 |
| <ul style="list-style-type: none"> • Development of appropriate metrics of physiological parameters for determining metal stress tolerance, | Chapter 7 | Greenhouse culture Assessing the mycorrhizosphere’s stratum of influence | Audet P, Charest C (2010)
Appl Environ Soil Sci,
doi 10 1155/812157 |
| <ul style="list-style-type: none"> • Re-evaluation of previous integrative conceptual models of corresponding with experimental findings | | | |

Section IV – Supplementary data

- | | | | |
|---|------------------|---|---|
| <ul style="list-style-type: none"> • Identification of pot-size and fungal inoculum distribution as experimental design factors having important consequences toward plant growth and AM root colonization | Chapter 8 | Identification of experimental design biases in mycorrhizal pot culture | Audet P, Charest C (2010)
J Bot doi 10 1155/718013 |
|---|------------------|---|---|

9.2 Implications for environmental plant physiology and soil ecology

As emphasized in throughout this thesis, the AM fungi are recognized for beneficially impacting a number of individual plant physiological and soil ecological processes; however, there are currently no unified perspectives depicting these combined effects across a broad-spectrum of environmental stress. By distinguishing between the direct and indirect benefits of AM symbiosis (Table 9.2), I have provided new perspectives in regards to plant stress tolerance and soil resiliency integrating the multi-lateral roles of the mycorrhizosphere in ecosystem function (Fig. 9.1). A cornerstone of this contribution is the depiction of the AM-induced processes of ‘enhanced uptake’ and ‘metal biosorption’ and their effects on plant resource acquisition and soil structure stabilization (Fig. 9.2 and 9.3). Here, I have shown how the role of AM fungi can shift dynamically in relation to these processes as soil metal exposure increases from trace to toxicity conditions. To my knowledge, this is the first representation of this kind which distinguishes between the direct and indirect benefits of the AM symbiosis, and then describes how they can contribute in shaping the plant growth environment. Accordingly, this nuance could account for the persistence of plant investment in mycorrhizal association in relation to a wide range of environmental stressors, as well as apparently non-stressful (*e.g.* optimal or non-limiting) growth conditions. In this regard, I have also suggested that plants could invest in the AM symbiosis as an extrinsic stress tolerance strategy to complement their intrinsic resistance mechanisms. Accordingly, by comparing the life history strategies of different plant guilds (*e.g.* metal excluders versus metallophytic species,

mycotrophs versus non-mycotrophs), I suggested that this investment is likely closely tied to the plants' respective stress tolerance strategy and resource allocation. Altogether, these overall findings provide new, testable hypotheses for future research regarding the role of symbiotic mutualism in plant stress tolerance and ecosystem function. Moreover, I consider that the approach of using meta-analysis to quantitatively assess the current state-of-the-art in a given field of research and addressing any subsequent questions using targeted experimental systems to be an effective strategy for a number of scientific questions (Fig. 9.4). With this framework, I have identified broad scale trends in environmental plant physiology and advanced integrative models of plant and ecosystem function which were not readily identified using conventional review or experimental methods. Accordingly, I consider that this research approach could be conducive for the eventual development of mathematical models of AM-plant interactions based on both conceptual and empirical findings. For these reasons, I consider the models depicting the impact of AM symbiosis on plant and soil systems to be relevant tools for improving sustainable agriculture and environmental remediation practices by conceptually determining the fate of metal contaminants in relation with the activities of plants and soil microorganisms.

Table 9.2 Summary of the impact of AM symbiosis on plant physiology and soil ecology

Mechanism	Target	Description	References	
Direct benefits	Enhanced resource acquisition capability	Essential soil resources (non-metals, metals, and water)	Preferential uptake of nitrogen (NO ₃ /NH ₄) and phosphorus (P _i)	Bolan (1991)*, Chapman et al (2006)*, Mosse (1973)*
			Mobilization and uptake of trace essential elements having low bioavailability (i.e.), particularly under nutrient deficiency conditions	Jeffries et al 2003*, Koide (1990)*, Marschner & Dell (1994)*
			Enhanced water use efficiency and drought recovery	Auge et al (2001), Auge (2002)*, Subramanian and Charest 2009*
Indirect benefits	Soil structure stabilization	Metal bioavailability	Metal-binding due to negatively charged surface constituents of extraradical hyphae (i.e. hydroxides, oxy-hydroxides, sulfhydryls), Reduction of plant metal uptake to delay phytotoxicity, particularly at high soil exposure levels (i.e. Zn, Pb, Cd, Ni)	Leyval et al (1997)*, Galli et al (1994)*, Gadd (1993)*
		Soil retention capacity	Enhanced soil aggregation properties, increased water and nutrient retention capacity, decrease of nutrient leaching	Augé et al (2001), Auge (2002)*, Bearden (2001, 2002), Miller & Jastrow (1990)
	Mycorrhizal exudation	Soil microbes	Promotion of beneficial bacteria (i.e. nitrogen fixation), competitive exclusion of soil pathogens	Chapman et al (2006)*, Barea et al (1995)*, Johanson et al (2004)*
		Metal bioavailability	Precipitation of metal-ligands, modulation of soil-pH	Leyval et al (1997)*, Galli et al (1994)*, Gadd (1993)*

*Denotes review publications

Figure 9.1 Schematization of the mycorrhizosphere illustrating the wide-ranging impact of AM-roots and extraradical hyphae on the proximal soil environment.

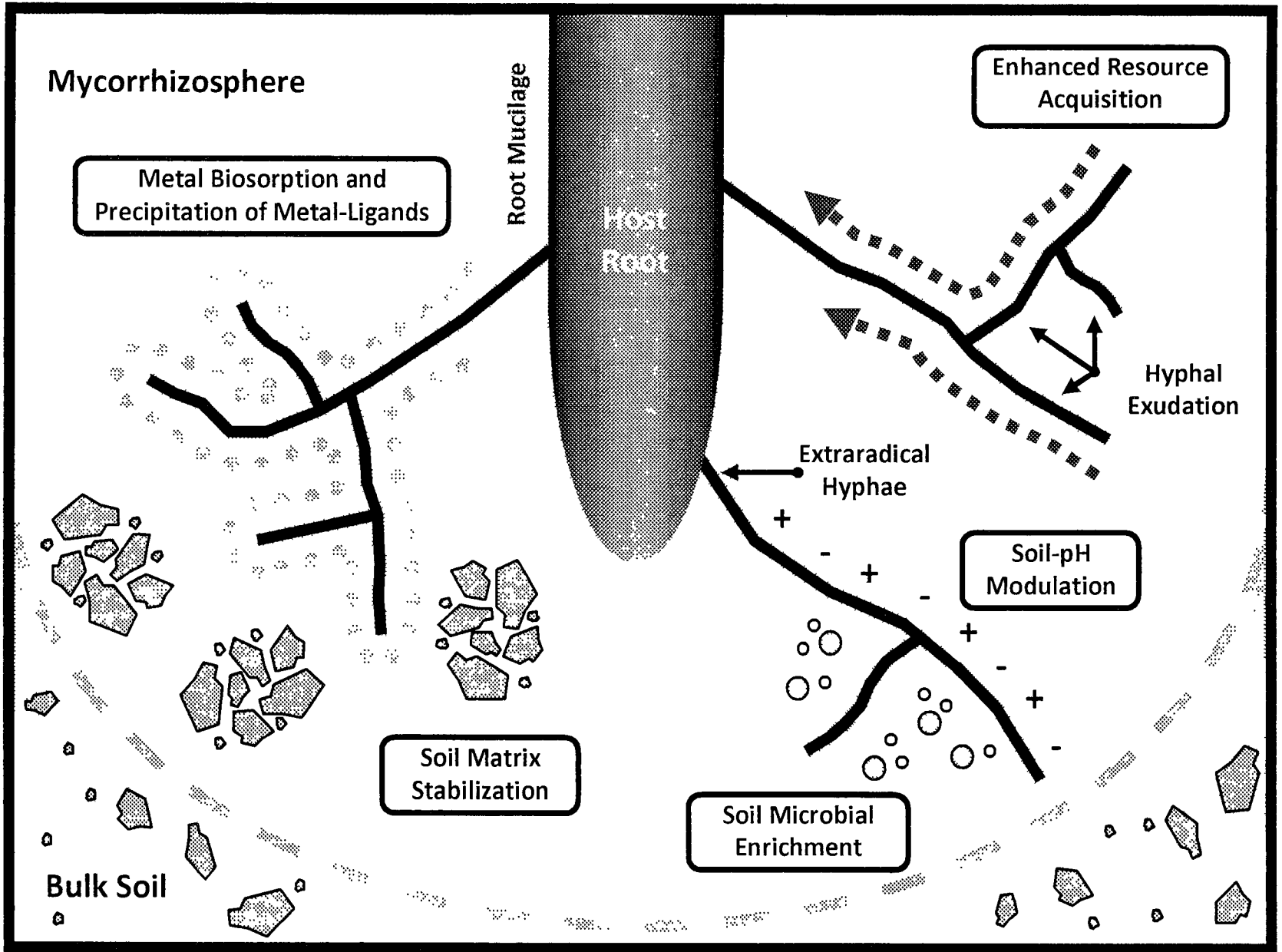


Figure 9.1

Figure 9.2 Revised conceptual model of AM-plant metal uptake showing the effects of AM-enhanced uptake and biosorption on metal bioavailability.

Figure 9.2

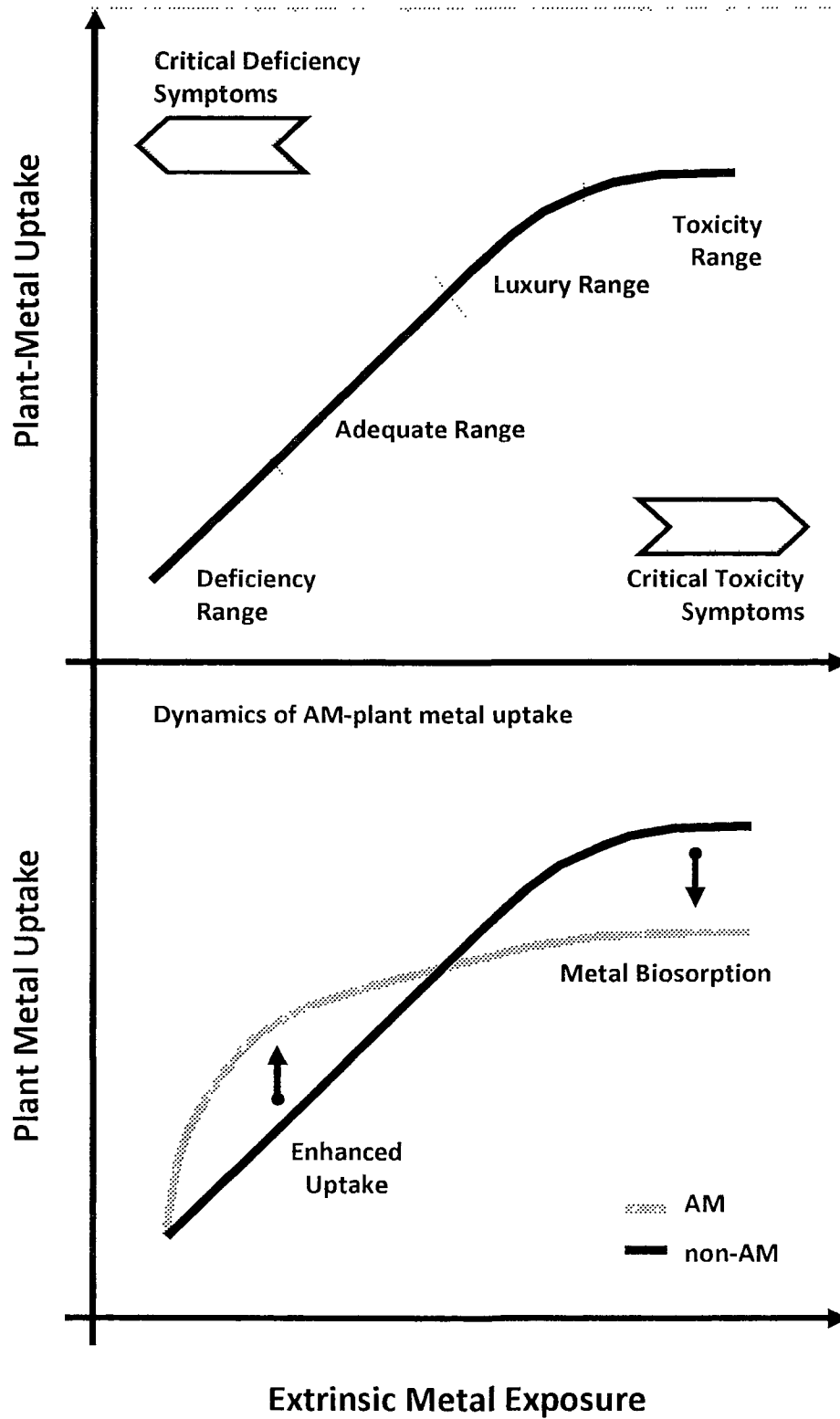


Figure 9.3 Revised conceptual model of AM-plant relative growth.

Figure 9.3

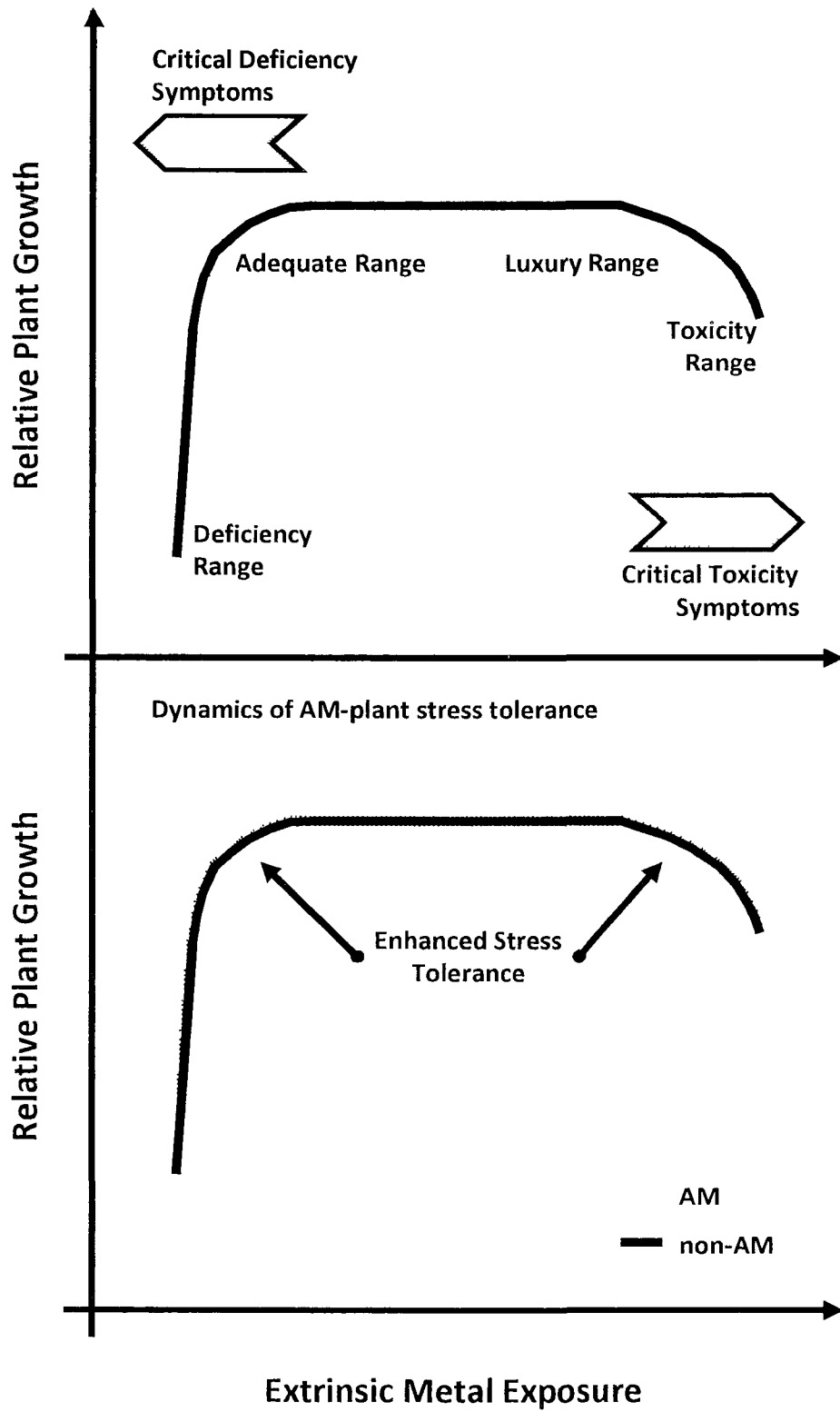
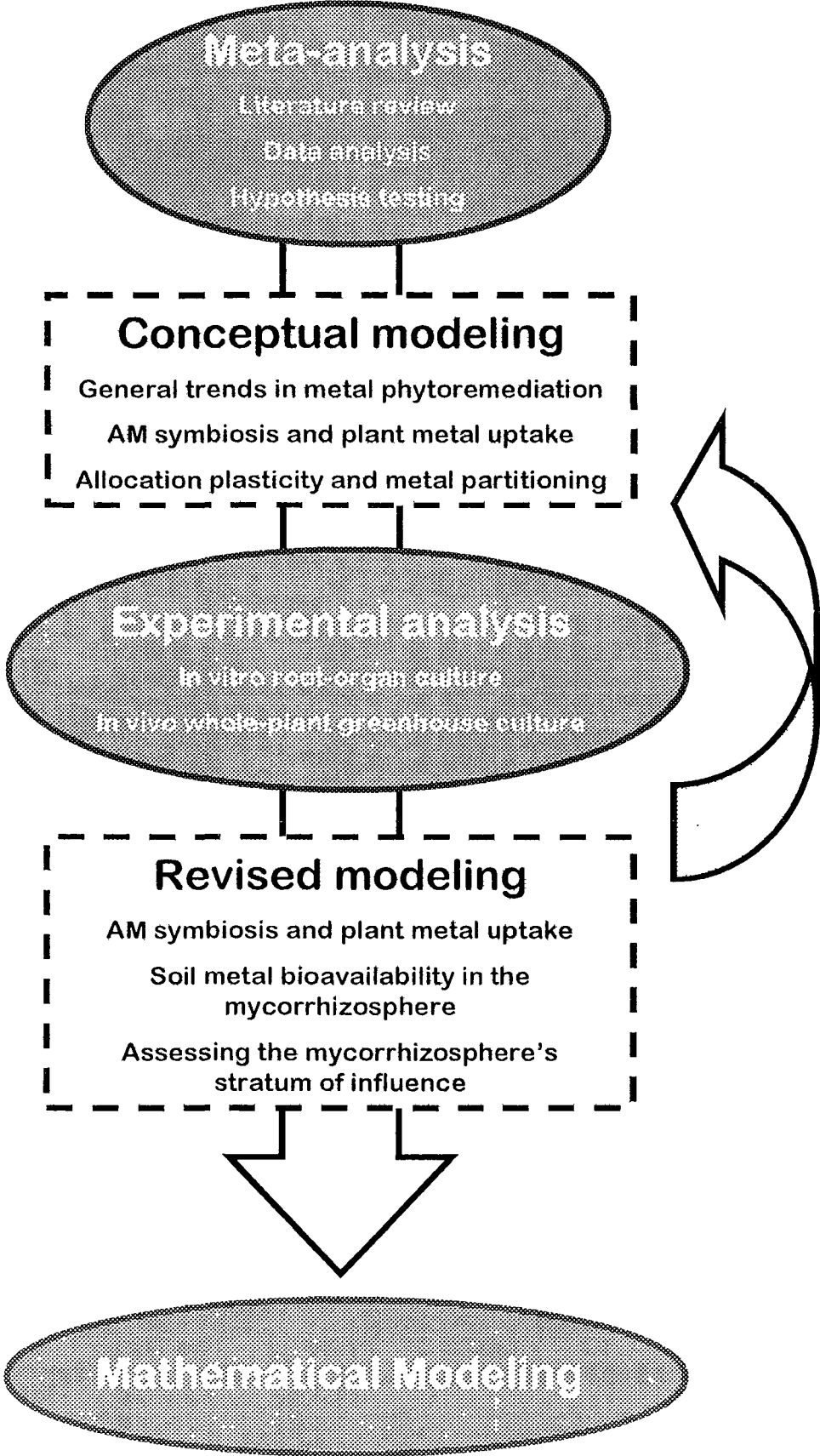


Figure 9.4 Summary of the research approach used in the present PhD thesis.

Figure 9.4



9.3 Considerations for future research

Building from the proposed models of AM-plant interactions, I consider certain research perspectives to be intriguing aspects for future investigations. The first of these perspectives pertains to the quantification of the mycorrhizosphere and the determination of its environmental parameters, particularly the estimation of the mycorrhizospheric volume and the inherent cost of maintaining the symbiotic infrastructure. More specifically, this would involve assessing the relative expansiveness of the rhizo-, mycorrhizo-, and hyphospheric networks and comparing their relative impacts on the growth environment in relation to their respective developmental costs. By distinguishing between the direct and indirect benefits of the interaction, I anticipate that this approach would be relevant in weighing the costs versus benefits of investing in AM symbiosis, and then assessing the plant's relative 'mycorrhizal dependency' when subjected to a number of environmental stressors. I also anticipate that this approach could contribute in more accurately determining the mycorrhizosphere's zone of influence, predicting its multi-lateral impact on various plant and soil ecological factors, and identifying its developmental limitations when subjected to critical stress conditions; an aspect briefly touched upon in Chapter 7. In this regard, an assessment of the impact of the mycorrhizosphere toward the speciation of metals (*e.g.* defining the liquid- and solid-partitioning of metals in soils) as well as a more in depth analysis of the metal biosorption processes (*e.g.* preferential binding of metals to extraradical mycelium and isolation of internal sequestration sites) would greatly expand the scope of the models and increase their

pertinence as an environmental risk assessment tool. Altogether, the comprehensive representation of these physiological and environmental parameters would be essential for either mathematical modeling strategies or field-level applications in sustainable agriculture and environmental remediation.

Another research perspective deserving of further investigation involves the connection between plant investment in intrinsic versus extrinsic stress tolerance mechanisms in relation to the plant's life history strategy. By assessing plant physiological tendencies among different plant families, I suggested that the exclusive investment in intrinsic stress tolerance could account, in part, for the non-mycotrophic status of some metallophytic species, unlike the metal excluders that may rely on mycorrhizal symbiosis (among other extrinsic association) for enhanced metal stress avoidance services (*e.g.* metal biosorption and modulation of metal bioavailability). A similar physiological dynamic may exist when comparing the life history strategies of typically mycorrhizal dependent indigenous plant species versus less mycorrhizal dependent invasive species. As in the case of AM-plant metal stress tolerance, I pose the question whether endemic plants invest in extrinsically mycorrhizospheric processes to increase their competitiveness and survivorship compared to invasive species that may rely more on intrinsic strategies such as rapid growth and accelerated reproduction mechanisms to colonize foreign soils. For this reason, it is intriguing to investigate the role of plant life history strategy in determining mycorrhizal dependency in relation to various other environmental stress

factors. In this regard, I consider the conceptual models of AM-plant interactions to represent useful starting points for these future investigations, among others.