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Molecular biodiversity of microbial communities in polluted soils and their role in soil phytoremediation

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Résumé

Les métaux lourds (ML) s'accumulent de plus en plus dans les sols à l'échelle mondiale, d'une part à cause des engrais minéraux et divers produits chimiques utilisés en agriculture intensive, et d'autre part à cause des activités industrielles. Toutes ces activités génèrent des déchets toxiques qui s'accumulent dans l'environnement. Les ML ne sont pas biodégradables et leur accumulation cause donc des problèmes de toxicité des sols et affecte la biodiversité des microorganismes qui y vivent.

La fertilisation en azote (N) est une pratique courante en agriculture à grande échelle qui permet d'augmenter la fertilité des sols et la productivité des cultures. Cependant, son utilisation à long terme cause plusieurs effets néfastes pour l'environnement. Par exemple, elle augmente la quantité des ML dans les sols, les nappes phréatiques et les plantes. En outre, ces effets néfastes réduisent et changent considérablement la biodiversité des écosystèmes terrestres. La structure des communautés des champignons mycorhiziens à arbuscules (CMA) a été étudiée dans des sols contaminés par des ML issus de la fertilisation à long terme en N. Le rôle des différentes espèces de CMA dans l'absorption et la séquestration des ML a été aussi investigué.

Dans une première expérience, la structure des communautés de CMA a été analysée à partir d'échantillons de sols de sites contaminés par des ML et de sites témoins non-contaminés. Nous avons constaté que la diversité des CMA indigènes a été plus faible dans les sols et les racines des plantes récoltées à partir de sites contaminés par rapport aux sites noncontaminés. Nous avons également constaté que la structure de la communauté d'AMF a été modifiée par la présence des ML dans les sols. Certains ribotypes des CMA ont été plus souvent associés aux sites contaminés, alors que d'autres ribotypes ont été associés aux sites non-contaminés. Cependant, certains ribotypes ont été observés aussi bien dans les sols pollués que non-pollués.

Dans une deuxième expérience, les effets de la fertilisation organique et minérale (N) sur les différentes structures des communautés des CMA ont été étudiés. La variation de la structure de la communauté de CMA colonisant les racines a été analysée en fonction

du type de fertilisation. Certains ribotypes de CMA étaient associés à la fertilisation organique et d'autres à la fertilisation minérale. En revanche, la fertilisation minérale a réduit le nombre de ribotypes de CMA alors que la fertilisation organique l'a augmenté. Dans cette expérience, j'ai démontré que le changement de structure des communautés de CMA colonisant des racines a eu un effet significatif sur la productivité des plantes.

Dans une troisième expérience, le rôle de deux espèces de CMA (*Glomus irregulare* et *G. mosseae*) dans l'absorption du cadmium (Cd) par des plants de tournesol cultivés dans des sols amendés avec trois niveaux différents de Cd a été évalué. J'ai démontré que les deux espèces de CMA affectent différemment l'absorption ou la séquestration de ce ML par les plants de tournesol. Cette expérience a permis de mieux comprendre le rôle potentiel des CMA dans l'absorption des ML selon la concentration de cadmium dans le sol et les espèces de CMA.

Mes recherches de doctorat démontrent donc que la fertilisation en N affecte la structure des communautés des CMA dans les racines et le sol. Le changement de structure de la communauté de CMA colonisant les racines affecte de manière significative la productivité des plantes. J'ai aussi démontré que, sous nos conditions expériemntales, l'espèce de CMA *G. irregulare* a été observée dans tous les sites (pollués et non-pollués), tandis que le *G. mosseae* n'a été observé en abondance que dans les sites contaminés. Par conséquent, j'ai étudié le rôle de ces deux espèces (*G. irregulare* et *G. mosseae*) dans l'absorption du Cd par le tournesol cultivé dans des sols amendés avec trois différents niveaux de Cd en serre. Les résultats indiquent que les espèces de CMA ont un potentiel différent pour atténuer la toxicité des ML dans les plantes hôtes, selon le niveau de concentration en Cd. En conclusion, mes travaux suggèrent que le *G. irregulare* est une espèce potentiellement importante pour la phytoextration du Cd, alors que le *G. mosseae* pourrait être une espèce appropriée pour phytostabilisation du Cd et du Zn.

Mots-clés : Champignons mycorhiziens à arbuscules (CMA), biodiversité, métaux lourds, fertilisation azotée, sols pollués, phytoremédiation, PCR, électrophorèse sur gel à gradient dénaturant (DGGE), clonage, séquençage.

Abstract

Trace metals (TM) are continually world-wide added to soils through the intensive use of mineral fertilizers and agriculture chemicals, together with industrial and other activities generating toxic wastes. Problems associated with metal-contaminated soil exists because TM are not biodegradable. TM that accumulate in soils affect the biodiversity of soil microorganisms.

Nitrogen (N) fertilization is a widespread practice to increase soil fertility and crop production. However, the long-term use of N fertilization causes many detrimental effects in the environment. The intensive use of N fertilization increase TM input in soils, and in extreme cases, N fertilization result in TM pollution of the surrounding soil and water and increase TM concentration in plant tissues. In addition, the long-term use of N fertilizers changes and declines the biodiversity of above and underground ecosystems.

The community structure of arbuscular mycorrhizal fungi (AMF) was investigated in TM contaminated and long-term N fertilized soils. In addition, the role of different AMF species in TM uptake or sequestration was investigated.

In the first experiment, AMF community structure was analyzed from non-contaminated and TM contaminated sites. We found the diversity of native AMF was lower in soils and plant roots harvested from TM polluted soils than from unpolluted soils. We also found that the community structure of AMF was modified by TM contamination. Some AMF ribotypes were more often associated with TM contaminated sites, other ribotypes with uncontaminated sites, while still other ribotypes were found both in polluted and unpolluted soils.

In the second experiment, the effect of different organic and mineral N fertilization on AMF community structure was investigated. Variation in root-colonizing AMF community structure was observed in both organic and mineral fertilization. Some AMF ribotypes were more affiliated to organic fertilization and other to mineral fertilization. In addition, mineral fertilization reduced AMF ribotypes number while organic fertilization

increased AMF ribotypes number. In this experiment, it was demonstrated that change in root-colonizing AMF community structure had a significant effect on plant productivity.

In the third experiment, the role of different AMF species (*G. irregulare* and *G. mosseae*) in TM uptake by sunflower plants grown in soil amended with three different Cd levels was evaluated. It was demonstrated that AMF species differentially affected TM uptake or sequestration by sunflower plants. This experiment supported a different effect of AMF in TM uptake based on Cd concentration in soil and the AMF species involved.

Our research demonstrated that TM and N fertilization affected and shifted AMF community structure within roots and soils. It was shown that change in root-colonizing AMF community structure significantly affected plant productivity. In this study, it was showed that the AMF species *G. irregulare* was recorded in all uncontaminated sites while *G. mosseae* was the most abundant AMF species in TM contaminated sites. Therefore, the role of *G. irregulare* and *G. mosseae* in Cd uptake by sunflower plants grown in soils amended with three different Cd levels was investigated. The results indicated that AMF species mediate different mechanisms to alleviate TM toxicity in host plants, depending on AMF species and soil Cd level involved. We hypothesize that *G. irregulare* is a potentially important species for Cd phytoextration processes, while *G. mosseae* might be a suitable candidate for Cd and Zn phytostabilization processes.

Keywords: Arbuscular Mycorrhizal Fungi (AMF), biodiversity, trace metals, N fertilization, polluted soils, phytoremediation, PCR plus Denaturing gradient gel electrophoresis (DGGE), cloning and sequencing.

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Chapter III

List of acronyms and abbreviations

ABC: ATP-binding cassette

Al: aluminum

AMF: arbuscular mycorrhizal fungi

ANOVA: analysis of variance

As: arsenic

Ba: barium

BCF: biological concentration factor

C: carbon

CCA: canonical correspondence analysis

Cd: cadmium

CDF: cation diffusion facilitor

CEC: cation exchange capacity

cm: centimetre

Co: cobalt

Cr: chromium

Cs: caesium

Cu: copper

DA: Discriminant analysis

DGGE: denaturing gradient gel electrophoresis

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

EC: electrical conductivity

FAME: fatty acid methyl esters

Fe: iron

Fig.: figure

g: gramme

h: hour

ha: hectare

Hg: mercury

HSD: honestly significant difference

HSP: heat shock proteins

IAA: indole acidic acid

IPC-MS: coupled plasma mass spectrometry

Kg: kilogramme

L: liter

LB: Luria-Bertani medium

mg: milligramme

mg kg^{-1}: milligramme per kilogramme

Mg: magnesium

MHB: mycorrhiza-helper bacteria

min: minute

ml: milliliter

mm: millimeter

mM: millimolar

Mn: manganese

Mo: molybdenum

MTs: metallothioneins

N: nitrogen (azote)

NCBI: national center for biotechnology information

Ni: nichel

nm: nanometer

Nramps: resistance-associated macrophage protein

NRC: National Research Council

NSERC: Natural Science and Engineering Research Council of Canada

NTA: nitrilotriacetic acid

Ra: radium

O.D. optical density

P: phosphor

pb: pair of bases

Pb: lead

PCR: polymerase chain reaction

PCs: phytochelatins

PGPR: plant growth promoting rhizobacteria

PLFA: phospholipids fatty acids

Po: phosphor organic

RNA: ribonucleic acid

rRNA: ribosomal RNA

Se: selenium

sec.: second

Sn: tin

sp.: species

Sr: strontium

SSU: small subunit

t: ton

TM: trace metal

U: uranium

µl: micro liter

µM: micro molar

μm^3: cube micro meter

UV: ultraviolet

v: volume

w: weight

yr: year

Z: zinc

ZIP: zinc transporter family

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CHAPTER I

General introduction

Trace metal (TM) contamination of soils originating from agricultural activities (e.g., fertilizers and sewage sludge) or industrial activities (e.g., metal mining and smelting) is one of the major environmental problems in many parts of the world (Gremion et al., 2004). These activities lead to the release of large quantities of hazardous chemicals into the biosphere; among these, trace metals constitute an important group of environmental pollutants (Kapoor et al., 2007). Problems associated with the contamination of soil and water such as animal welfare, health, fatalities, and disruptions of the natural ecosystems are well documented (He et al., 2005). TM such as Cd, Pb, Cu, and Zn persist in the soil and can either be adsorbed to soil particles or leached into ground water (Khan, 2006). Large amounts of trace metals are accumulated in soil. High concentrations of these metals disturb biological processes in both soil and living organisms. TM affects all groups of organisms and ecosystem processes including microbial activity (Giller et al., 1998). Moreover, lead, copper, zinc, and cadmium are also found naturally in the soil and they can cause significant damage to the environment and to human health as a result of their mobility and solubility; in addition, TM are non-degradable and need to be removed or immobilized from polluted areas (Khan, 2006).

Therefore, we need to remediate polluted soils that usually cover large land areas that are unavailable for human uses. Polluted soils can be remediated by chemical, physical, and biological techniques (McEldowney et al., 1993). Physico-chemical methods of remediation are very expensive because of the cost of excavating and transporting large quantities of contaminated materials for *ex situ* treatment, such as soil washing and chemical inactivation (Chaudhry et al., 2005; Pilon-Smits, 2005). Also, these methods change the soil physical, chemical, and biological characteristics (Khan, 2005). The physical and chemical methods of remediation of the contaminated soils are mainly used in relatively small areas and are unsuitable for large areas such as typical mining sites or industrially and agrochemically contaminated soils (Khan, 2005). Additionally, health hazards associated with soil contamination with trace elements having toxic effects together with high cost of removal and *ex situ* treatment of polluted soil have prompted the development of alternative technologies to recover the degraded lands (Khan, 2005).

Current research in this area now includes plants to remediate polluted soils and to facilitate improvement of soil structure leading to an innovative technique known as phytoremediation (Brooks, 1998).

I.1. Phytoremediation

Phytoremediation is defined as the use of plants and their associated microorganisms for environmental cleanup (Salt et al., 1995; 1998). This methodology makes use of the naturally occurring processes by which plants and their rhizospheric microorganisms degrade and sequester organic and inorganic pollutants (Pilon-Smits, 2005). Phytoremediation is an efficient cleanup technology for a variety of organic and inorganic pollutants (Pilon-Smits, 2005). Inorganic pollutants that can be phytoremediated include plant macronutrients such as nitrate and phosphate (Horne, 2000), trace elements such as Cu, Fe, and Zn (Lytle et al., 1998), nonessential elements such as Cd, Hg, Se, and Pb (Blaylock and Huang, 2000; Horne, 2000), and radioactive isotopes such as ^{238}U and ^{137}Cs (Dushenkov and Kapulnik, 2000; Dushenkov, 2003).

Moreover, phytoremediation is a much cheaper technology (approximately 10 fold) than engineering-based remediation methods, and its cost-effectiveness results from the fact that it is usually carried out *in situ* and use natural energy such as sun light (Glass, 1999). The use of *in situ* techniques to cleanup contaminated soils result in a reduction of pollution exposure to human, wildlife, and environment (Pilon-Smits, 2005). Phytoremediation also enjoys popularity with the general public as an "environmentally friendly" alternative to chemical and physical methods (Pilon-Smits, 2005). Thus, government agencies and companies like to include phytoremediation methods withen their green-cleanup strategies to stretch available funds (Pilon-Smits, 2005).

I.2. Phytoremediation strategies

Phytoremediation can be categorized under five major subgroups: (i) phytoextraction: removal and concentration of metals into the harvestable plant parts, (ii) phytosabilization: immobilization and reduction of the mobility and bioavailability of

contaminants, (iii) phytodegradation; degradation of organic contaminants, (iv) rhizofiltration; absorption of metals from contaminated water, and (v) phytovolatilization; volatilization of contaminants by plants from soil into the atmosphere (Chaudhry et al., 1998).

However, phytoremediation is a relatively slow process to reduce soil metal contents to safe and acceptable levels due to the small size and slow growth of most identified metal hyperaccumulator plants (Khan, 2005). Basically, to improve the effectiveness of phytoremediaton we have to select the favorable plant properties for phytoremediation, which in general are of fast growth, high biomass, extensive root system, competitive species, tolerant to pollution, high uptake level, translocation, and accumulation in harvestable tissues. Examples of plant species showing these properties are vetiver grass, hemp, sunflower, poplar, and willow (Khan, 2005; Pilon-Smits, 2005).

I.3. The role of AMF in phytoremediation

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microorganisms and constitute an important functional component of the rhizosphere (Smith and Read, 2008). These fungi form a symbiotic relationship with roots of approximately 80% of the plant species in natural, agricultural, and forest ecosystems (Smith and Read, 2008). AMF enhance the nutrition states of their hosts by acquiring phosphate, micronutrients, and water (Göhre and Paszkowski, 2006). The extraradical hyphae exploit a large volume of soil and bring to the host plant nutrients that are otherwise unavailable by roots alone (Smith and Read, 2008). Moreover, AMF occur in almost all habitats and climates (Chaudhry and Khan, 2002) and in the soil of most ecosystems, including trace metal-polluted soils (Göhre and Paszkowski, 2006). AMF isolated from polluted soil are more effective in transferring trace metal tolerance to plant than AMF isolates from non-polluted soils (Hildebrandt et al., 1999; Kaldorf et al., 1999). On the other hand, the diversity of AMF spores in trace metal soils is frequently lower than in non-polluted sites (Pawlowska et al., 1997). In line with this, only few AMF species and a comparatively low number of AMF spores were found in the rhizosphere of the yellow zinc violet plant in its natural habitat (Tonin et al., 2001).

However, a low number of spores do not necessarily reflect a limited AMF development (Hildebrandt et al., 1999; Kaldorf et al., 1999; Regvar et al., 2006). Additionally, AMF also play a role in the protection of roots against trace metal toxicity by mediating interactions between metal and plant roots (Leyval et al., 1997). AMF enhance uptake of trace metals by plants without showing any symptoms of trace metal toxicity (Weng et al., 2004). On the other hand, the reports revealed that AMF increase the stabilization of trace metals in polluted soil. Audet and Charest (2007) suggested that AMF increase the uptake at low metal soil concentration, while at high concentrations, AMF reduce metal bioavailability and protect plants against toxicity.

The AMF biodiversity in the most polluted ecosystems is still unknown. Studying the AMF biodiversity in TM contaminated ecosystems could, therefore, provide a more complete overview on the ecological role of these fungi in TM disturbed ecosystems. Because AMF play an important role in the mobilization and immobilization of metals in soil, they are also considered to be key factors in the bioavailability of metals to plants (Del Val, et al., 1999; Smith & Read 2008). AMF species differentially affect the translocation and accumulation of TM in the colonized plants. Consequently, a comparative analysis of AMF community structure in metal-polluted and unpolluted soils is essential for the identification of metal-tolerant AMF ecotypes and development of efficient phytoremediation techniques (Zarei, et al., 2008). Therefore, determining whether these fungi naturally occur in the most heavily polluted areas is potentially important to determine whether AMF can be used to sustain the practices of phytoremediation and solve the polluted ecosystem problems, and whether some AMF species are better than others for this specific purpose (Nicolas and Charest 2011).

AMF have been successfully used with different plant hyperaccumulator to increase metal tolerance and accumulation (Gaur and Adholeya, 2004). Among soil microorganisms, AMF provide a direct link between the soil and roots, and are known for their ability to improve plant mineral nutrients, including trace metals (Wang et al., 2007). AMF play an important role in the adaptation of plant to metal contaminated soils (Meharg and Cairney, 1999; Marques et al., 2007). AMF improve plant tolerance, attenuate trace

metal stress or enhance plant growth under metal contamination (Gaur and Adholeya, 2004). Citterio et al (2005) reported an increase in plant growth and metal transport from root to shoot of plants inoculated with AMF. Therefore, AMF are potential biotechnological tools for enhancing the phytoremediation of trace metal-contaminated soils (Gaur and Adholeya, 2004).

The specific role of AMF in plant exposure to metal stress depends on a variety of factors, including plant species, fungal species and ecotypes, the metal species, its availability, and its concentration (Del Val et al., 1999; Audet and Charest, 2007).

I.4. Objectives of the research

Phytoremediation in which plants and microorganisms are used to restore or decontaminate sites is respectful of the environment, efficient, and an innovative approach. The success of phytoremediation helps the polluted soils to find a certain level of ecological integrity, supporting an increased environmental quality. Although AMF are very beneficial for phytoremediation purposes, but their biodiversity in the most polluted ecosystems is still unknown. To date, few studies analyzed AMF diversity in TM contaminated sites only from soils using spores isolation and identification or only from roots using PCR-cloning methods. Investigation of the AMF community compositions that naturally found in the most heavily contaminated ecosystems from both roots and soils using several culture-independent techniques is beneficial to sustain phytoremediation. Under this global aim, the specific research objectives were:

1. To characterize and compare the *in situ* biodiversity of AMF in TM polluted and unpolluted fields, and to investigate the relationship between AMF community structure and TM pollution.
2. To characterize the impact of N fertilization regimes on AMF community structure.
3. To examine whether or not changes in AMF community structure result in variation of plant growth in response to different N-fertilization regimes.
4. To evaluate the variability of different AMF species in the phytoremediation of Cd polluted soils.

5. To investigate the effect of AMF on Cd bioremediation at different Cd concentrations in soil. In particular, we investigated the following questions: did AMF uptake/sequestration of TM depend on TM concentration in the soil; was there a variability in TM uptake/sequestration within AMF species, did AMF increase the transportation of TM from root to shoot system; and did AMF increase the biomass of plants which use in bioremediation?

I.5. Research hypotheses

1. AMF communities will be severely disturbed in the presence of TM, but some particular AMF species can thrive in these TM contaminated environments.
2. Some AMF species can only be more abundant to TM contaminated environments; in particular, some AMF species will be preferentially associated with particular TM pollutants.
3. Long-term use of N fertilization reduces root-colonizing AMF community structure as compared to unfertilized soils.
4. Manure fertilizer applications increase AMF community structure as compared to mineral N fertilizer.
5. AMF species differentially affect the translocation and accumulation of Cd in the colonized plants from Cd contaminated soils.
6. AMF colonization increases Cd accumulation in plant tissues at low soil TM concentration, but reduces Cd accumulation at high soil TM concentration.

CHAPTER II

Phytoremediation: biotechnological procedures involving plants and arbuscular mycorrhizal fungi

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Preface

Environmental contamination is a serious issue originating from variable sources and applications. Trace metal contamination is a significant environmental problem with a negative impact on human health and agriculture. Contaminated sites cover large areas of the world which become unsuitable for agriculture and other human uses. Thus, the polluted sites need to be decontaminated. Phytoremediation is the use of plants and their rhizosphere microorganisms to remove or immobilize contaminants from the environment. This chapter highlights the ecological role of plants and arbuscular mycorrhizal fungi (AMF) in phytoremediation process.

Abstract

Soil pollution is a serious concern in major parts of the Earth. Intensive use of mineral fertilizer and agriculture chemicals together with industrial activities, mining, oil and gas operations, traffic emission, and toxic wastes influences soil quality in both urban and rural areas. Despite the general decline in the use of agrochemicals, the problem of polluted soils still exists because agro-chemicals do not easily degrade and trace metals accumulate in soils. These contaminated soils have characteristics that may limit their re-use because of the potential presence of trace metals, and in the most extreme cases may prevent the establishment of spontaneous vegetation. The recent expertise in soil restoration developed in biotechnology and ecology allows the establishment of plants to rehabilitate certain portions of polluted soils. Phytoremediation, where plants and their associated microorganisms are used to restore or decontaminate such sites, is respectful of the environment, more efficient, and represents novel approach. The success of phytoremediation allows polluted soils to recover part of their ecological integrity. This review will discuss phytoremediation strategies, in particular the feasibility of implanting phytoextraction and phytostabilization procedures, and will focus on the potential role of plants and arbuscular mycorrhizal fungi (AMF) as bioremediative tools to cleanup a wide

range of contaminated soils, highlighting the complex relationship between AMF and trace metal contamination.

KEY WORDS

Phytoremediation, phytoextraction, phytostabilization, pollution, trace metals, arbuscular mycorrhizal fungi.

II.1. INTRODUCTION

Environmental contamination is a serious issue originating from various sources, such as the use of agrochemical fertilizers, sewage sludge, and pesticides, or industrial activities, in particular metal mining, smelting, oil and gas operations, and others human activities (Gremion et al., 2004; Khan, 2005). These applications release huge quantities of hazardous pollutants including organic and inorganic compounds into the air, water, and soil biospheres (Kapoor et al., 2007). Inorganic contaminants involving trace metals are natural components of the Earth's crust (Pilon-Smits, 2005). Trace metals freed from various sources represent approximately 22 000 t of Cd, 939 000 t of Cu, 783 000 t of Pb, and 1 350 000 t of Zn through the last decades (Singh et al., 2003). Trace metals are a group of 53 elements that have a specific density higher than 5 g/cm^3 (Göhre and Paszkowski, 2006). They can remain in the soil for long periods of time, adhering to soil granules or polluting the underground water (Khan, 2006). Among trace metals, Cu, Fe, Mn, Ni, and Zn are essential elements required for normal plant growth, and these compounds have fundamental functions in nucleic acid metabolism; moreover, they are important for electron transfer, enzymatic catalyzing, as well as redox reactions (Göhre and Paszkowski, 2006). Other trace metals like Cd, Pb, Hg, and As (As is a metalloid) are not required by living organisms.

Plant roots can uptake essential trace metals from soil through specific and non specific transporters (Göhre and Paszkowski, 2006), while non-essential elements are taken up by passive diffusion and wide specificity-metal transporters (Hall, 2002). Huge quantities of trace metals and metalloids (such as Cd, Pb, Zn, Cu, and As) hamper the biological activities of both higher organisms and soil microbes (Giller et al., 1998). High concentrations of trace metals shift enzymatic functions by changing protein structure and replacing necessary components, causing deficiencies (Göhre and Paszkowski, 2006). Also, plasma membrane and its permeability are highly sensitive to trace metal poisoning; hence, membrane functions have been influenced by the modification of the role of membrane's

protein transporters like H^+ ATPases (Hall, 2002). Furthermore, high levels of trace metals lead to oxidative damage of plant tissues as a result of the production of reactive oxygen species (Hall, 2002). Consequently, several toxicity symptoms in root and shoot systems may appear on plants in response to elevated trace metal concentration (Göhre and Paszkowski, 2006). Moreover, trace metals accumulated within soil may interfere in the food chain, leak into drinking water, and have a negative impact on human health, welfare, and the environment (Khan, 2005).

Basically, high concentrations of trace metals are probably carcinogens for human and animals causing nucleic acid deformations and mutations (Knasmuller et al., 1998). For instance, the World Health Organization (WHO) (1997) has showed that arsenic is a skin carcinogen and deleterious to the cardiovascular system, whereas cadmium and arsenic cause kidney damage as a result of its accumulation in kidney tissues; mercury has harmful effects on the neurological system including uncontrolled muscle movements, incomplete blindness, and malformation of newborn children. In this regard, Padmavathiamma and Li (2007) mentioned that lead exposure causes intensive damage to the nervous system, which lead to poor understanding, unconcentrated memory, and loss of leaning ability and social collaboration.

Increasingly, organic contaminants generating to environment from various sources such as usage of coal and fossil fuel as a source of energy, military activities, as well as agriculture and industrial application; in fact, most organic pollutants are toxic and carcinogens (Pilon-Smits, 2005). Organic lipophilic matters have potential risk effects on human health, as they can interfere with the food chain (Reilley et al., 1996). Therefore, such contaminated soils need to be remediated because they usually cover large areas of land.

II.2. PHYSICAL AND CHEMICAL APPROACHES OF REMEDIATION

Polluted sites can be cleaned up by physical, chemical, and biological techniques (McEldowney et al., 1993). The physico-chemical strategies include soil excavation and

storage, or transportation, washing, as well as chemical treatment (Göhre and Paszkowski, 2006). These *ex situ* treatments of disturbed soil remove pollutants but at the same time damage the soil microbial community. In addition, these approaches are very expensive (Chaudhry et al., 2005; Padmavathiamma and Li, 2007). Glass (1999) has summarized the high costly of physical and chemical methods of remediation, which have been estimated to approximately 75-425 US $/ton for vitrification, and 20-200 US $/ton for land filling and chemical treatments. These methods generate hazardous substances behind them, containing trace metals and additional pollutants, demanding further treatment. Furthermore, physico-chemical remediation approaches are unfit for very large areas of contaminated sites such as mining sites, industrially and agriculturally polluted soils, or areas wasted by oil and gas operation. These procedures are improper for plant growth, beside they are also damageable for almost all soil biological activities (Gaur and Adholeya, 2004; Khan, 2005). Additionally, *ex situ* remediation techniques modify and conversely harm physical, chemical, and biological traits of the treated soil (Khan, 2005). Recently, research has been oriented to an innovative field known as phytoremediation, in which plants and their associated soil microorganisms have been applied to remediate and improve disturbed soil (Brooks, 1998; Salt et al., 1998).

II.3. PHYTOREMEDIATION: APPLICATION AND DRAWBACKS

Phytoremediation is the use of plants and their rhizosphere-microbes to remove or immobilize contaminants from the environment (Salt et al., 1995; Salt et al., 1998). This technique is a natural, green-clean, and an eco-friendly process to treat a wide variety of polluted soils including organic and inorganic waste contaminants (Pilon-Smits, 2005). For instance, inorganic pollutants involving macronutrients such as nitrate and phosphate (Horne, 2000), essential trace elements like Cr, Ca, Mn, Mo, and Zn (Lytle et al., 1998), non-essential ones such as Cd, Co, F, Hg, Se, Pb, V, and W (Blaylock and Huang, 2000;

Horne, 2000), and radioactive isotopes such as ^{238}U, ^{137}Cs, and ^{90}Sr (Dushenkov and Kapulnik, 2000; Dushenkov, 2003) have been successfully remediated.

As phytoremediation is a natural process, it depends on solar energy and does not require the transfer of contaminated soil for outside cleaning. Phytoextraction cost has been approximately 5-40 US $/ton to get rid of phytomass products (Glass, 1999). In addition to the economical benefits of bioremediation, another benefit is minimizing exposure of humans, wildlife, and the environment to polluted products. Phytoremediation technology has received funding and is gaining popularity from many governments and environmental consultancy companies (Pilon-Smits, 2005). This green approach offer vegetation cover for a broad range of contaminated sites which produce extensive root systems and high biomass, thereby reducing erosion (Leyval et al., 1997; Glick et al., 1999; Gaur and Adholeya, 2004).

The application of phytoremediation, however, may be restricted by some drawbacks such as its slow process requiring many years to lower the pollutants concentration to safe levels, and as it is a biological process, phytoremediation relies on parameters such as soil features, toxicity level, bioavailability of pollutants, and climatic conditions (Pilon-Smits, 2005). Most of these factors should be well understood before phytoremediation be widely acceptable as a commercial technique. Basically, to improve the efficacy of *in situ* remediation and to reduce the long time periods required, it is important to ascertain the favourable plants traits to perform these purposes. The ideal plant is characterized by fast growth, efficiency in accumulating and concentrating contaminants and capacity to transfer them to aboveground parts, high tolerance to wide variety of pollutants, competitive, extensive root production, high shoot biomass, high levels of water and nutrients uptake, high transpiration rate, proper translocating or sequestrating of pollutants, as well as capacity to establish mutual relationship with rhizospheric soil microorganisms (Khan, 2005; Peuke et al., 2005; Pilon-Smits, 2005). Examples of plant species that possess these traits are vetiver grass, hemp, sunflower, poplar, and willow (Khan, 2005; Pilon-Smits, 2005).

Plants and their related rhizosphere-microorganisms have been used in several types of phytoremediation, which can be grouped into the following categories: phytoextraction, or disposal and accumulation of polluted compounds into aboveground plant tissues; phytostabilization, involving immobilization and sequestration of metals within soil and roots; rhizofiltration or absorption and adsorption of contaminants from water; phytodegredation and phytostimulation, the degredation of organic pollutants into soil or within plant parts; and finally phytovolatilization, or releasing and volatilizing polluted products via plant organs from wasted soils into the atmosphere as less hazardous compoungs (Chaudhry et al., 1998; Khan, 2005; Pilon-Smits, 2005).

II.4. VERSATILE FUNCTIONS OF PLANTS USED IN PHTOREMEDIATION

Hyperaccumulators are plants that can intake and accumulate large quantities of trace metals in their harvestable parts without the appearance of metal toxicity symptoms (Padmavathiamma and Li, 2007). About 400 plant species have been reported as hyperaccumulators; they belong to the following families: Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cununiaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Poaceae, and Violaceae. Environment Canada has developed the Phytorem database, including approximately 750 plants, lichens, algae, fungi and bryophytes that have demonstrated the ability to tolerate, accumulate or hyperaccumulate a range of 19 different metals, including wild and cultivated plants (Padmavathiamma and Li, 2007). Natural accumulator plants have shown concentrations of around 1% of Zn and Mn, 0.1% of Ni, Co, Cr, Cu, Pb, and Al, 0.01% of Cd and Se, and 0.001% of Hg of their dry weight shoot biomass (Padmavathiamma and Li, 2007). Labrecque et al. (1995) showed that fast growing willows (*Salix viminalis* and *discolor*) accumulated high contents of trace metals such as mercury, copper, lead, nickel, and zinc in their roots and stems from soils treated with wastewater sludge. The same authors suggested that the accumulation of trace metals within roots and shoots play a significant role in the immobilization of trace metals for

several years, in contrast to the accumulation of trace metals within the leaves because leaves fall down annually and their trace metals content returns to the soil. Trace metals sequestration in roots and stems provides a good route to decontaminate soils amended with trace metals-containing wastewater sludge. Thus, this would allow the reducing hazardous effects of pollution (Labrecque et al., 1995). At high levels of trace metals, both essential and non essential metals induce toxicity features and retardation of plant growth (Hall, 2002). These toxicity symptoms originate from interactions between metals and sulphydryl groups of proteins which causes tackling and deformation in activity and structure of potential proteins, or structural substitution of necessary elements causing poisonous damages (Hall, 2002). Moreover, elevated trace metal concentrations accelerate the constitution of free radicals and reactivate oxygen species causing oxidative stresses (Dietz et al., 1999). However, some plant species can survive on contaminated soils; in fact, they possess various tolerance mechanisms allowing them to thrive on polluted soils (Hall, 2002).

II.5. PLANT TRACE METALS TOLERANCE MECHANISMS

Plants have many strategies to resist trace metal toxicity (Clemens, 2001; Hall, 2002), reducing the poisonous impact of high concentrations of trace metals via mechanisms such as controlling trace metals intake, compartmentalization, translocation, and detoxification (Clemens, 2001). In this regard, plant trace metal tolerance involves circumventing toxic metals build-up or producing proteins that can withstand trace metals poisoning (Hall, 2002). Intercellular tolerance processes include plasma membrane in regulation of trace metals uptake or speeding up the efflux pumping of metals out of the cytosol, producing heat shock proteins or metallothioneins that are involved in repairing stress-disruption proteins, producing organic acids or amino acids that act as chelating agents, and compartmenting the metals within vacuoles (Hall, 2002).

Trace metals have a destructive influence on the structure and function of plasma membranes involving oxidative damage to membrane proteins, limiting the activity of H^+ ATPases transporters, and modifying the structure and viscosity of membrane lipids, thereby hampering plasma membrane permeability (Hall, 2002). However, plasma membranes have their own resistance to trace metal toxicity such as metal hemeostasis; the plasma membrane can control metals coming to or out of the cytoplasm, regulating the active efflux pumping (Hall, 2002). On the other hand, plasma membranes govern metal tolerance through the potential functions of membrane transporters, such as CPx-ATPases, Nramp (natural resistance-associated macrophage protein), CDF (cation diffusion facilitor) and ZIP (zinc transporter family) (Williams et al., 2000). Nramp occur in plants and microorganisms and perform an important role in transportation of trace metals (Williams et al., 2000). In addition, CDF, Nramp, and ATP-binding cassette play important functions in metal compartmentalization within vacuoles (Clemens et al., 2002; Singh et al., 2003).

Moreover, large groups of plants and other living organisms release heat shock proteins (HSPs) as a result of exposure to trace metals (Lewis et al., 1999). HSPs offer functional and repairing roles in trace metals damage (Hall, 2002). Another type of protein that has a crucial role in metal toxicity resistance is the phytochelatins (PCs), which are glutamylcysteins oligopeptides containing glycine or other amino acids attached to a carboxyl terminus (Peuke et al., 2005). PCs play an important role in detoxification of Cd and As, but do not have an important effect on Zn, Ni, and selenite (Hall, 2002). Metallothioneins (MTs) are sulphur rich proteins consisting, having the capacity to attach to trace metals (Cobbett and Goldsbrough, 2002). Interestingly, some genes contributing to metal tolerance have been recognized such as RAN1 (Hirayama et al., 1999), ATHMA3 (Gravot et al., 2004), phytochelatin genes (Clemens et al., 1999) and metallothionein genes (Goldsbrough, 2000). Intercellular resistance can also be contributed by dead xylem and phloem cells containing cellulose, hemicellulose, and lignin which have the capacity to trap metals, reducing their toxic impact to plants; also, trace metals can be captured inside trichomes (Choi et al., 2001).

On the other hand, plants achieve tolerance to the toxic effects of organic pollutants by uptake, transportation, volatilization, and sequestration inside vacuoles, or chemical modification involving oxidation, reduction, or hydrolysis and combination with glucose, glutathione, and amino acids (Salt et al., 1998; Meagher and Heaton, 2005; Dietz and Schnoor, 2001; Pilon-Smits, 2005). In addition, glutathione and glutathione-S-transferase play a functional action in the degradation of organic pollutants such as most pesticides (Dietz and Schnoor, 2001; Dixon et al., 2002). ATP-binding cassette (ABC) transporters have a crucial role in the export of organic molecules out of plant cells and their sequestration within vacuoles (Meagher and Heaton, 2005; Dixon et al., 2002). Chelating agents including metallothionein, glutathione, phytochelatin (Cobbett and Goldsbrough, 2002), phytosiderophores (Higuchi et al., 1999), nicotinamine (Stephan et al., 1996; von Wiren et al., 1999), and organic acids like citrate, malate, and histidine (Salt et al., 1995; von Wiren et al., 1999; Kupper et al., 2004) are produced by plant and are involved in trapping pollutants within vacuoles or exporting them to shoots.

II.6. PLANT ROOT EXUDATES AND TRACE METAL TOLERANCE

Plant root releases various organic acids like citrate, lactate, and malate, as well as flavonoid compounds (Ensley, 2000; Hutchinson et al., 2004), that as carbon sources for soil microbial populations (Kapulnik, 1996). In response, some soil microorganisms produce plant growth hormones, increase the efficiency of water and nutrient uptake, and inhibit the action of other intruding soil microbes, consequently improving the nutritional and health conditions of plants (Kapulnik, 1996). Indeed, root-released lipophilic substances enhance pollutant solubility and movement, and stimulate the activities of biosurfactant-producing bacteria (Siciliano and Germida, 1998). In this way, biosurfactants molecules promote the solubility of hydrophobic organic pollutants (Volkering et al., 1997). Additionally, plant roots together with soil microorganisms secrete degradable enzymes, using organic pollutants as substrates and speeding up the bioavailability of these

contaminants (Wolfe and Hoehamer, 2003). Root exudates influence soil pH; in fact, soil acidification has strong impact on the availability of nutrients and toxic trace metals (Meagher and Heaton, 2005). The substructural epidermal root layers possess enormous Golgi apparatus and plasma membrane vesicles that release siderophore products, capturing a wide range of metals like iron, zinc, and arsenic, and therefore minimizing their availability for root sorption (Meagher and Heaton, 2005). Beside, Robinson et al. (2006) suggested that root exudates ameliorate soil aeration through establishing avenues in soil for air and water exchange.

II.7. THE FUNDAMENTAL FUNCTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI IN PHYTOREMEDIATION

Arbuscular mycorrhizal fungi are ubiquitous soil microorganisms and a vital component of the rhizosphere. AMF form a putative interaction with the roots of approximately 80% of the terrestrial plants in nearly all ecosystems (Barea et al., 2005). Interestingly, AMF inhabit most of the habitatsharsh conditions and climates (Chaudhry and Khan, 2002), including soil contaminated with trace metals (Göhre and Paszkowski, 2006). AMF establish beneficial symbiotic relationships with plants and offer a physical bridge between the soil and plant roots (Barea et al., 2005). AMF constitute a large network of external hyphae within the soil, these hyphae extending into the soil, reach nutrients in soil zones unavailable for direct plant uptake and delivering these compounds to their host plants (Smith and Read, 2008). Therefore, AMF promote the nutrient supply to their hosts including phosphate, nitrogen, many micronutrients and other immobile molecule and water (Vivas et al., 2003; Smith and Read, 2008). Moreover, AMF improve soil texture and reduce erosion through aggregation of soil particles (Rillig and Steinberg, 2002; Steinberg and Rillig, 2003), and increase the immobilization of trace metals within soils by translocating of metals into hyphae or roots; in addition, AMF reduce metal moving from plants to soil and root-to-shoot translocation (Kaldorf et al., 1999).

Arbuscular mycorrhizal fungi can successfully colonize the root of some hyperaccumulator plant species and play a key role in metal tolerance mechanism and accumulation (Gaur and Adholeya, 2004). For example, AMF can establish symbiotic interaction with Ni-hyperaccumulator *Berheya coddi* (Turnau and Mesjasz-Przybylowicz, 2003), and As-accumulator *Pteris vittata* (Leung et al., 2006), *Cynodon dactylon* (hyperaccumulator for many trace metals) (Leung et al., 2006), and *Thaspi praecox* (Vogel-Mikus et al., 2006). This is an indication of the role of mycorrhizal hyperaccumulator symbiosis in phytoextraction processes; unfortunately, most of the hyperaccumulator plants produce small biomass and take up a high specific metal only (Wang et al., 2007). Wang et al. (2007) showed that AMF assist the ability of plants to uptake mineral nutrients, containing trace metals. Moreover, AMF have the ability to reclaim the trace metal contaminated soil to their host roots (Meharg and Cairney, 1999; Marques et al., 2007) and were shown to stimulate plant resistance, reduce trace metal toxicity impact, and promote plant growth under metal stress (Gaur and Adholeya, 2004).

Soil structure and aggregation of soil particles are important criteria of soil quality in stressed ecosystems (Miller and Jastrow, 2000). Soil microorganisms help small soil granules to aggregate and accumulate into larger particles within the rhizosphere. This function is carried out through particule adhesion with bacterial products (Barea et al., 2005), and branched AMF hyphal network (Miller and Jastrow, 2000). In this regard, glomalin released from the AMF extraradical hyphae has a huge involvement in the maintenance of soil aggregation (Wright and Upadhyaya, 1998). Thereby, the combination of AMF and rhizosphere bacteria contribute to soil aggregation and, as a consequence, improve water availability, the restoration, and the revegetation of contaminated soils (Requena et al., 2001).

II.8. INTERACTION BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND RHIZOSPHERIC SOIL MICROORGANISMS

Soil microbes and plant roots can sense each other via the release of certain molecules within the soil, which stimulates the activity of particular microbial populations to colonize plant root surfaces which it inhibits other taxa. In fact, this plant-microorganisms communication is very complex and is governed by variabious biotic and abiotic factors. N-acyl-homoserine lactone is one of these signal molecules which is thought to be involved in quorum sensing and in regulating symbiotic relations between roots and soil bacteria (Barea et al., 2005). AMF contribute to plant productivity and health by favoring nutrition and providing tolerance against stressful conditions (Turnau and Haselwandter, 2002). AMF control the diversity and bioactivity of soil microorganisms within the rhizosphere (Khan, 2006). AMF colonization of root tissue can, in some cases, lead to change in morphological structure of root tissues, but more widely demonstrated is a changes in the root exudation, resulting in a modification in the rhizosphere microbial communities structure and interactions with roots, stimulation of plant growth, and increase plant resistance to harsh conditions (Barea et al., 2005; Lioussanne et al., 2008). AMF have several impacts on soil bacteria and fungi (Yergeau et al., 2006). It is also well established that AMF increase their host plants capacity to escape diseases caused by deleterious microorganisms (St-Arnaud and Vujanovic, 2007).

As AMF can acquire phosphorus from soil through their extraradical hyphal network, plant growth promoting rhizobacteria (PGPR) also increases the uptake of phosphorus (Rodríguez and Fraga, 1999). Accordingly, Vivas et al. (2003) demonstrated that AMF and PGPR isolated from polluted soils stimulate plant nutrition by improving N-fixation, producing plant growth hormones, and increasing P uptake. In line with this, *Pseudomonas* spp. as PGPR taxa, benefit plant growth through phytohormone production, enzyme secretion, N-fixation, induced resistance to plant pathogens, production of antibiotic and other pathogen inhibitor, and immobilizing trace metal through the function

of siderophores (Glick, 1995; Kapulnik, 1996; Chin-A-Woeng et al., 2003). Among antibiotics released by PGPR are acetylphluoroglucinol (Picard et al., 2004) and phenazine (Chin-A-Woeng et al., 2003). Furthermore, PGPR are antagonistic to a variety of plant pathogens and show increase nutrient uptake by plants and phytostimulation (Barea et al., 2005; Zahir et al., 2004). PGPR have an impact on biological diversity of other microbial taxa of the rhizosphere (Barea et al., 2005). Therefore, PGPR can be used as phytoremediative members that ameliorate plant traits including nutrition, health, and metal tolerance and detoxification.

Garbaye (1994) named the bacteria improving AMF hyphal growth and mycorrhizal establishment as mycorrhiza-helper bacteria (MHB). Vivas et al. (2003) demonstrated that inoculation of *Brevibacillus* spp. and *Glomus mosseae* isolated from Cd-contaminated soils increased the nitrogen and phosphorus contents and biomass of inoculated plants compared to control plants. Barea et al. (2005) concluded that microorganisms, including bacteria and AMF adapted to contaminated soils, enhanced the mechanism of plant tolerance to trace metals and thus have a beneficial role in the phytoremediation process. For these reasons, managing indigenous communities can contribute with plants to perform functions of vital importance in the revegetation and remediation of disturbed soil (Khan, 2002; Khan, 2004). Hence, the interaction of AMF, PGPR, and MHB can be exploited as factors into biofertilizer and biocontrol applications, as well as for phytoremediation implementation (Khan, 2006).

II.9. TRACE METAL TOLERANCE OF ARBUSCULAR MYCORRHIZAL FUNGI

AMF play a functional role in trace metal resistance and accumulation, but they vary in their contribution and tolerance to trace metal uptake and immobilization (Jamal et al., 2002; Hildebrandt et al., 2007). AMF have various mechanisms to protect themselves and their host against trace metal toxicity damages (Leyval et al., 1997). On one hand, immobilization and accumulation of trace metals into fungal tissue and the rhizosphere can

be exploited to withstand metal toxicity especially in highly contaminated soil (Hildebrandt et al., 2007). AMF can also act as a barrier in metal trafficking from root-to-shoot systems (Joner et al., 2000). Gaur and Adholeya (2004) suggested that this is consequent to intra-cellular precipitation of metallic cations with phosphate groups. In this regard, Turnau et al. (1993) showed higher accumulation of Cd, Ti, and Ba within fungal tissues than in host plant tissues. On the other hand, AMF can contribute several mecanisms contributing to adaptation to pollution stresses, including the crucial actions of the cell wall chitin (Zhou, 1999), extraradical hyphae, and AMF-released proteins such as siderophore, metallothioneins, and phytochelatins (Joner and Leyval, 1997). Trace metals such as Cu, Pb, and Cd can be trapped within the cell wall structure, including amino, hydroxyl, and carboxyl free radicals (Kapoor and Viraraghavan, 1995). In addition, glomalin is an insoluble glycopeptide and chelating factor secreted by AMF (Wright and Upadhyaya, 1998) that comes into the soil from AMF hyphae (Driver et al., 2005) and contribute to the immobilization of metals and to the decrease in bioavailability of metals (Gonzalez-Chavez et al., 2004), as well as help soil aggregation by adhering to soil particles (Khan, 2006). AMF-produced metal-binding glomalin have been extracted from contaminated soil under laboratory conditions, where 0.08 mg Cd, 1.12 mg Pb, and 4.3 mg Cu per gram of glomalin was extracted from AMF grown in polluted soils (Gonzalez-Chavez et al., 2004). Also, one gram of glomalin containing 28 mg of Cu was obtained from *Gigaspora rosseae* (Göhre and Paszkowski, 2006), so it is hypothetized that glomalin can significantly contribute to capture trace metals and sequester them within the soils. Beyond the role of fungal molecules such as chitin and glomalin in metal tolerance, the fungal hyphae offer a larger surface area than roots and a wide extent within the soils, where they can grow and spread nearly elsewhere between soil granules, where host root cannot grow (Khan et al., 2000). The extraradical AMF hyphae have higher metal attraction than host roots. For example, Chen et al. (2001) observed that the concentration of P, Cu, and Zn was higher in the fungal tissue than in roots and shoots of maize when maize plants were grown on modified glass bead compartment cultivation system with soil containing 0.80 of Cu mg/kg, 7.6 of Fe

mg/kg, 3.6 of Mn mg/kg, and 0.63 of Zn mg/kg and colonized by *Glomus mosseae* and *G. versiforme*. For instance, *G. mosseae* and *G. versiforme* accumulated respectively approximately 1200 mg/kg and 600 mg/kg of Zn in their tissues, while Zn concentrations in roots were lower than 100 mg/kg. Moreover, the variation in trace metal accumulation within AMF tissues depends on the difference of AMF species. For example, the concentration of nutrients and trace metals in *G. mosseae* were higher than those in *G. versiforme* because *G. mosseae* produces a higher external hyphae extension and lower spores count than those recorded in *G. versiforme* (Chen et al., 2001). This also confirms that trace metals accumulate at higher levels in AMF external hyphae than in AMF spores (Chen et al., 2001). As a consequence, AMF enhances root absorption efficiency, and thereby AMF assists in the uptake/immobilization of metals in the rhizosphere ecosystem and are significant contributors of trace metal fixation within soils (Joner et al., 2000; Gaur and Adholeya, 2004). In this regard, the external AMF mycelia act as a biological sink of metals (Turnau, 1998; Kaldorf et al., 1999) by adsorption into cell walls or glomalin (Wright and Upadhyaya, 1998; Zhou, 1999). Therefore, AMF diminish the exposure of plants to trace metal poisoning (Khan, 2006). A demonstration using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed that AMF mycelia can attach Cu (Gonzalez-Chavez et al., 2002).

AMF vesicles were shown to play a vital role in metal detoxification (Göhre and Paszkowski, 2006). Other indirect tolerance mechanisms have been shown, such as enhancing plant biomass, which dilutes trace metal concentration within tissues, precipitation of polyphosphate particles, and compartmentalization within vesicles and vacuoles (Turnau et al., 1993; Kaldorf et al., 1999).

AMF originating from deleterious soils enhances plant tolerance to trace metal further than those isolated from non-contaminated soil (Hildebrandt et al., 1999; Kaldorf et al., 1999). The abundance of AMF species in contaminated soils is significantly lower than that found in non-polluted soils (Pawlowska et al., 1997). In spite of the fact that frequency of AMF species and spores in metal-polluted soil is low, this smaller amount of AMF

spores in polluted soil does not appear to significantly restrict the establishment of mycorrhizal symbiosis (Hildebrandt et al., 1999; Kaldorf et al., 1999; Regvar et al., 2006). For instance, a low diversity of AMF species and spores was recorded in the rhizosheric soil of *Viola caluminaria* (yellow zinc violet plant) (Tonin et al., 2001) growing on highly Zn and Pb polluted soil. Spores of AMF isolated from Zn polluted soil have been shown to have a higher germination rate when compared with those isolated from non-contaminated sites when exposed to high Zn concentrations (Leyval et al., 1997). Investigation of the influence of trace metals on spore germination and symbiotic formation was performed on only two isolates and indicated that *Glomus irregulare* (DAOM 1811602) is more resistant to elevated trace metal concentration than *G. etunicatum* and that tolerance depend on the differences in fungal genotype (Pawlowska and Charvat, 2004). In addition, indigenous AMF populations can contribute to offset elevated trace metal stresses for plant growth (Del Val et al., 1999). The same authors have reported that although the number of AMF spores decreased with long-term application of sewage sludge containing high levels of Zn, Cd, Cu, Ni, and Pb, the AMF species did not disappear totally from polluted and stressful conditions.

Several trace metal-tolerant AMF species have been isolated from polluted soil. For example, Gildon and Tinker (1981) isolated *Glomus mosseae* from Zn contaminated soils that were expored to a concentration of approximately 100 mg/kg of Zn. Sambandan et al. (1992) also recorded 15 AMF species from metal polluted sites in India, where the percentage of colonized roots ranged from 22 to 71% and approximately 622 AMF spores were counted per 100 gram of soil, *Glomus geosporum* was found in all studied sites. Turnau and Haselwandter (2002) found that approximately 70% of *Fragaria vesca* roots were infected by *Glomus mosseae* in Zn contaminated soil. Del Val et al. (1999) identified *Glomus claroideum* from contaminated sludge soil.

High amounts of trace metals were shown to be accumulated in mycorrhizal structure (Gaur and Adholeya, 2004). Cavagnaro (2008) showed that AMF can enhance Zn uptake by plant at low soil Zn concentration. Deram et al. (2008) revealed that AMF-

increase the accumulation of Cd in shoots of *Arrhenatherum elatius*; their results suggested that the significant role of AMF in Cd assimilation varied with season and soil Cd concentration. There was a significant positive correlation between shoot Cd concentration and arbuscules occurance in roots of *Arrhenatherum elatius* (a perennial grass with high biomass and accumulate high concentration of Cd (Deram et al., 2007)) and a negative correlation between the frequency of AMF root colonization and soil Cd concentration. For example, arbuscules and vesicles of AMF disappeared when Cd concentration in soil reached their maximum value in May. This disappearance of AMF was accompanied with the decreasing of Cd concentration in shoots confirm that AMF symbiosis have a dynamic impact in the uptake and accumulation of trace metals by plants (Audet and Charest, 2007; Deram et al., 2008). The decrease in Cd concentration in shoot systems as a result of AMF disappearance indicate that seasonal variation of AMF lead to the protection of developing seeds from exposure to toxic injury of trace metal in contaminated soil (Deram et al., 2008). Seasonal AMF colonization may be concerning with phenology of AMF species (Deram et al., 2008). Moreover, an AMF inoculum composed of *Gigaspora margarita* ZJ37, *G. decipens* ZJ38, *Scutellospora gilmori* ZJ39, *Acaulospora spp*., and *Glomus spp*. have been shown to have a higher impact on phytoaccumulation of many trace metals (Cu, Zn, Pb, and Cd) by maize plants when compared to an AMF inoculum involving only *Glomus caledonium* 90036 (Wang et al., 2007). The consortia of AMF species contribute to a higher uptake and transportation of trace metals, as well as tolerance to trace metals toxicity than single AMF species and therefore a mixture of AMF may be more effective in phytoremediation (Joner et al., 2001). Mycorrhized *Populus* trees (*Populus alba* and *Populus nigra*) inoculated with *Glomus mosseae* have been recorded as suitable Zn-accumulator plants; however, there was variation in the ability of the two registered clones of poplars to extract trace metals from the soils (Lingua et al., 2008). Although AMF increases the accumulation of Zn in leaves of poplar, AMF causes biochemical modification to improve plant growth, and therefore enhances plant tolerance to high Zn concentration (Lingua et al., 2008). The changes in free putrescine (polyamine has a

significant role in the growth and development of higher plants) concentration in poplar inoculated with *Glomus mosseae* and grown on Zn polluted soil, where putrescine contributes to metal ion compartmentation (Sharma and Dietz, 2006).

On the other hand, Kozdrój et al. (2007) indicated that mycorrhizal fungi originating from highly spoiled soils improved Cd stabilization within sites which were planted with pines. Sudová R, Vosátka (2007) recorded that maize plants grown on gamma sterilized field substrate from a lead-polluted waste disposal site and inoculated with *Glomus intraradices* (isolate BEG 75 from non polluted soil) had a lower Pb concentration in their shoots than maize inoculated with *G. intraradicies* (isolates PH5-OS and PH5-IS from lead contaminated sites). The uninoculated maize plants had more than double the Pb concentration in their shoot biomass as compared with mycorrhizal plants, without any significant variation in the effect of *G. intraradicies* isolates. Increasing Pb accumulation in mycorrhizal colonized roots together with lower Pb levels in shoots of mycorrhized plants may confirm the significant role of intraradical fungal components in trace metal sequestration within the roots, either on cell walls or intracellullarly. In addition, extraradical mycelia of AMF contribute to trace metal immobilization (Joner et al., 2000). Similarly, Janousková et al. (2006) recorded higher accumulation of Cd in extraradical hyphae of AMF than in plant roots and lower Cd poisoning in mycorrhizal plant than non mycorrhizal plants. Hence, internal and external hyphal structures of AMF have a significant function in alleviating trace metal toxicity and increasing trace metal immobilization within soil (Sudová and Vosátka, 2007).

Since AMF decreases trace metal accumulation in plant shoots, AMF offers a protection role for their host against trace metal toxicity and result in high shoot yields (Gonzalez-Chavez et al., 2002; Chen et al., 2003). Wang et al. (2007) recorded that *Zea mays* colonized with *Acaulospora mellea* accumulated low amont of Cu which had no or less toxicity and did not cause damageable injury of plants, and thus this lead to higher plant shoot yields. On the other hand, AMF increases soil pH, changes the concentration of soil organic acids such as malic acid, citric acid, and oxalic acid, and influences the

exudation of carbohydrate compounds (Joner et al., 2000). These modifications may have a significant effect on metal availability (Wang et al., 2007). Vivas et al. (2006) observed that the co-inoculation of *Trifolium repens* with the AMF *G. mosseae* and the bacteria *Brevibacillus brevis* promoted plant growth, mineral nutrition uptake, and reduced nickel uptake. This suggests that these synergistic effects of AMF bacteria can be exploited in biotechnological approaches to increase the efficiency of phytostabilization.

In a phytagel experiment, *Glomus intraradicies* colonized carrot roots with high contents of Cd and Zn in M media, and caused carrot's roots were hyperaccumulator of Cd (90 µg/g Cd) and accumulator of Zn (550 µg/g Zn) (Giasson et al., 2005). AMF hyphae could transfer Zn to their host roots by the same transport pathway of phosphorus (Weissenhorn et al., 1995). Arbuscules of AMF increase the surface connection area between AMF and roots play a significant role in Zn uptake by roots (Smith and Read, 2008). The establishment of arbuscules and development of AMF within roots increases metal translocation in plant shoots (Turnau and Mesjasz-Przybylowicz, 2003). Although Cd is a nonessential nutrient, it can be translocated and accumulated within plants through the manganese and zinc transport systems (Giasson et al., 2005). Beside the role of metallothioniens and phytochelatins in the capture of trace metals in their cysteinyl radicles, trace metal can be accumulated in carrot's roots infected with *G. intraradicies* as a result of trace metal saturation in vegetation after long period of metal exposure (Giasson et al., 2005). The different strategies of detoxification are important for the successful thriving of mycorrhizal plants on trace metal polluted soils and in improving the restoration of contaminated sites (Wang et al., 2007).

II.10. PHYTOEXTRATION

Phytoextraction is a biotechnological approach in which contaminants are taken up from the soils by plants and stored within their harvestable tissues. Therefore, ideal plants for this biological approach should be more tolerant and adapted to trace metal stress, be

good metal accumulators, produce high biomass, possess an extensive root system, and be fast growing (Kramer, 2005; Pilon-Smits, 2005). When the harvestable plant tissues contain high quantities of trace metals, they can be extracted with a new fascinating technique called phytomining (Blaylock and Huang, 2000; Chaney et al., 2000). However, in order to eliminate the environmental risks of plant residues containing trace metals, the harvested plant parts must be used as a energy source by combustion and stored as very small amount of dry matter (Kramer, 2005; Peuke et al., 2005). Specific burning techniques are required to prevent metal losing with smokes (Keller et al., 2005). The use of phytoremediative plant cover in the forest industry and biofuel production is promising environmental routes to reduce the limitations of phytoextraction (Robinson et al., 2006).

The most important factor restricting the application of phytoaccumulation is metal bioavailability. The chemical and physical features of the soil along with other environmental conditions reduce the movement of contaminants within the soil (Pilon-Smits, 2005). Metal bioavailability is related to its solubility in the soil solution. It also depends on the metal status in the soil. Metal would not be bioavailabled when they form complexes with soil particles or when they are precipitated as carbonate, hydroxides or phosphates (Clemens et al., 2002). Yet, the induced accumulation of metals can be exploited by using synthetic chelating agents such as ethylene diamine tetraacetic acid (EDTA), nitrilotriacetic acid (NTA), thiosulphate, or thiocynate to increase the potential of metal extraction from contaminated soils (Moreno et al., 2005; Roy et al., 2005). Some environmental hazards associated with the use of these synthetic chelators involve metal leaching to underground water; moreover, some chelators like EDTA are not easily degraded and are still present within the soil after remediation, adding a pollutant to the environment (Robinson et al., 2006). Furthermore, many of chelators are used as sodium salts, and it is known that plant growth maybe reduced as a result of high Na concentration in the soil (Robinson et al., 2006). Finally, most synthetic chelating agents increase the solubility and availability of many metals other than those targeted by the remediation; as a consequence, new phytotoxicities arising from these non-target metals have been shown

(Robinson et al., 2006). Therefore, more research is needed to investigate the effects of the application of synthetic chelating agents.

Arbuscular mycorrhizal fungi may be applied to increase the efficiency of phytoaccumulation via their direct and indirect effects on trace metal uptake and accumulation, and on plant biomass production (Gaur and Adholeya, 2004; Giasson et al., 2005). In line with this, AMF enhance the plant contact area with soil through their extended hyphae and increase the root uptake area up to 47-fold (Smith and Read, 2008). Mycorrhizal colonization stimulates metal translocation to root in lettuce, while other results have revealed that AMF speed up the accumulation of trace metals within shoot systems of legume plants (Jamal et al., 2002). Similar results were recorded with other plants (Bi et al., 2003; Giasson et al., 2005). The combined inoculation of an AMF and a *Penicillium* isolate plus the application of chistosan (a chelating agent) promoted the shoot and root growth of *Elsholtizia splendens* and sped up the translocation of Zn, Pb, and Cd, but not of Cu to the shoots (Wang et al., 2007). Chitosan is a chelating agent for ions of trace metals because its free amine function combines with cations in polluted soils (Piron et al., 1997). Weng et al. (2005) recorded that chitosan increases the accumulation of Cu and Pb in roots and shoots of *Elsholtizia splendens*, and together with AMF increase the concentration of ZN and Pb in the roots. Chitosan has a low molecular weight, is water soluble and biodegradable, and does not hamper plant growth and AMF establishment (Wang et al., 2007). Furthermore, chitosan can be degraded after phytoremediation and can no longer chelate trace metals; thereby, it can be used as an eco-friendly chelator to increase the role of AMF in phytoextraction of trace metal polluted soils (Wang et al., 2007).

II.11. PHYTOSTABILIZATION

Stabilization of trace metals within the soil has been carried out by the precipitation of metals in the rhizosphere, adsorption onto root surfaces and soil particles, or absorption and accumulation within roots (Pilon-Smits, 2005; Göhre and Paszkowski, 2006). Trace metal immobilization for plants and their associated microorganisms (Berti and Cunningham, 2000). Phytosequestration of trace metals into soil results in the restriction of the spread of metal, its leaching to underground water, and finally reduces erosion (Pilon-Smits, 2005). Van Nevel et al. (2007) concluded that phytosequestration is suitable and an alternative route whenever phytoextraction is not feasible. As some trees produce high biomass, deep and branched root systems, have high transpiration rates, and provide metal-organic matters to the soil, they are suitable for the purposes of immobilization (Van Nevel et al., 2007). Mertens et al. (2007) have that trees that reduce soil pH and minimize the metal transition from root to shoot have an interesting function for phytostabilization. Yoon et al. (2006) reported 17 plant species that can survive on contaminated sites and show high metal concentration within roots and low metal translocation values from roots to shoots; they may suitable for phytostabilization.

Immobilization of contaminants within the soil reduces the exposure of humans and the environment to the hazardous effects of pollutants (Padmavathiamma and Li, 2007). Phytostabilization is most proper for soils with high organic and trace metal contents, and is suitable to cure a wide range of contaminated sites (Berti and Cunningham, 2000). Phytostabilization has another advantage that make this procedure is an eco-friendly method of restoration of polluted sites, as there is no need to get rid of shoot systems as a hazardous waste (Flathman and Lanza, 1998). The mechanisms of immobilization involve chelation of contaminants via root exudation, absorption, adsorption, and accumulation within roots, compartmentation inside vacuoles or combination with cell wall components, precipitation within the rhizosphere, and reduction of xylem transportation, which reduces the translocation of contaminants from root to shoot systems (Padmavathiamma and Li,

2007).

To increase the efficacy of phytostabilization, some amendments are used to increase the insolubility of metals and cause them unavailable to plant uptake; hence, trace metals cannot transfere to the food chain (Berti and Cunningham, 2000; Adriano et al., 2004). Phosphates as multi anions and organic compounds such as compost are some of these amendments that increase the immobilization and precipitation of trace metals (Bolan et al., 2003). Since phosphate raise metal adsorption by anion-induced negative charge and metal precipitation (Bolan et al., 2003; Padmavathiamma and Li, 2007), and organic compost enhances soil pH, these amendments may improve the physical and chemical soil properties, increase trace metal immobilization, and play an important role in the restoration of metal polluted soils (Bolan et al., 2003). Although phosphate addition increases the availability of arsenic from mine tailings, phosphate is required to facilitate the revegetation of mine tailing sites (Padmavathiamma and Li, 2007).

AMF can infect metallophyte plants and increase their ability to survive on highly contaminated soil by avoiding trace metal absorption (Weissenhorn et al., 1995; Leyval et al., 1997; Kaldorf et al., 1999; Ouziad et al., 2005; Vogel-Mikus et al., 2006; Hildebrandt et al., 2007). Trace metals immobilization has succeeded in acidic and wasteful wood sites (Robinson et al., 2006), as well as in sheep waste areas (Robinson et al., 2006). Increase in the accumulation of metals within the rhizosphere lead to the addition of further organic matter to the soil and establish vegetation cover on highly polluted soils (Robinson et al., 2006). The reduction of mycorrhizal colonization rates as a result of high metal availability may offer a mechanism for the restriction of trace metal uptake and the increase of fixation of trace metals within soil (Oudeh et al., 2002). In soils with high Cd and Zn contents, the hyphae of *Glomus mosseae* provide a barrier to trace metal translocation and reduce the trace metal uptake by *Phaseolus vulgaris*; and this is due to the capture of trace metals in hyphae-released slime (Guo et al., 1996). Furthermore, AMF have the ability to change the form of the contaminants; for example, AMF can reduce arsenate to arsenite and remove

arsenite from their hyphae (Sharples et al., 2000). Thereby, AMF can significantly take up macronutrients and exclude trace metals (Leung et al., 2007).

CONCLUSION

Phytoremediation is an attractive biotechnology to dispose or immobilize contaminants in polluted soils. One reason is because phytoremediation is an *in situ* approach that avoids the transportation of contaminated soil for *ex situ* treatment detoxification, making it an innovative, inexpensive, and popular approach. To enhance the efficacy of phytoremediative techniques and to minimize the long period of time required for cleanup, tolerant plants and their effective associated rhizospheric microorganisms are fundamental tools to reach this important environmental goal.

The use of arbuscular mycorrhizal fungi isolated from contaminated sites is a promising tool either in phytoaccumulation or phytosequestration techniques. The interaction between AMF and plant roots can be established in almost all habitats and because AMF exists in trace metal polluted soils, it can facilitate the growth of plants in polluted soils. Extraradical hyphae of AMF increase the ability of the roots to access unavailable nutrients and enhance water uptake. Hence, AMF plays an important role in enhancing the biomass of their host plants. Since AMF releases metal chelating agents such as glomalin, metallothionien, organic acid, and phytochelatin, it can increase the immobilization and sequestration of trace metals within soils. AMF can further reduce metal ions by the potential role of the specific plasma membrane metal reductases (Davies et al., 2001). Therefore, AMF filters the entrance of trace metals into plant shoots and increases the avoidance of trace metal toxicity. Moreover, indigenous AMF isolated from polluted sites show more resistance and adaptation to deleterious conditions than those isolated from non contaminated soils. Indigenous AMF isolated from polluted sites and the role of their vacuoles, vesicles, and arbuscules in the storage and translocation of trace metals, adds more advantages of AMF in the phytoremediation and restoration of polluted

sites. The protective role of AMF relies on diverse biotic and abiotic factors including the diversity of plant, fungal, and microbial species and varieties, the nutritional conditions of mycorrhized plants, the health of the plants, nutrients found within the soil, qualities of the soils, and metal bioavailability and concentration (Guo et al., 1996; Del Val et al., 1999; Joner et al., 2001; Audet and Charest, 2007).

CHAPTER III

Molecular biodiversity of arbuscular mycorrhizal fungi in trace metal polluted soils

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- Hassan SE, M Hijri, M St-Arnaud 2009 PCR-DGGE analysis of arbuscular mycorrhizal fungi diversity in heavy metal polluted soils. p. 69 in Program and Abstracts of the 59th Ann. Conf. Can. Soc. Microbiol., Jun 15-18, Univ. Concordia, Montréal, Canada.

Preface

The previous chapter introduced the ecological role of arbuscular mycorrhizal fungi (AMF) in phytoremediation. Although AMF are very beneficial for phytoremediation purposes, but their biodiversity in the most polluted ecosystems is still unknown. Determining the AMF community compositions that naturally found in the most heavily contaminated ecosystems is beneficial to investigate whether these fungi can be used to sustain phytoremediation and solve the pollution problem, and to determine whether some AMF species are better than other for phytoremediation purposes. In order to highlight these important aspects of AMF ecology, determining the AMF biodiversity across several metal contaminated areas was achieved in this chapter.

Abstract

We assessed the indigenous arbuscular mycorrhizal fungi (AMF) community structure from the roots and associated soil of *Plantago major* (plantain) growing on sites polluted with trace metals (TM), and on unpolluted sites. Uncontaminated and TM contaminated sites containing As, Cd, Cu, Pb, Sn, and Zn, were selected based on a survey of the TM concentration in soils of community gardens in the City of Montréal. Total genomic DNA was extracted directly from these samples. PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE), augmented by cloning and sequencing, as well as direct sequencing techniques, were all used to investigate AMF community structure. We found a decreased diversity of native AMF (assessed by the number of AMF ribotypes) in soils and roots harvested from TM polluted soils compared to unpolluted soils. We also found that community structure was modified by TM contamination. Various species of *Glomus*, *Scutellospora aurigloba*, and *S. calospora* were the most abundant ribotypes detected in unpolluted soils; ribotypes of *G. etunicatum*, *G. irregulare/ G. intraradices* and *G. viscosum* were found in both polluted and unpolluted soils, while ribotypes of *G. mosseae* and *Glomus* spp. (B9 and B13) were dominant in TM polluted soils. The predominance of *G. mosseae* in metal polluted sites suggests the tolerance of this species to TM stress, as well as its potential use for phytoremediation. These data are relevant for our

understanding of how AMF microbial communities respond to natural environments that contain a broad variety of toxic inorganic compounds and will substantially expand our knowledge of AMF ecology and biodiversity.

Introduction

Trace metals (TM) are continually added to soils world-wide through the intensive use of mineral fertilizers and agriculture chemicals, mining, oil and gas operations, traffic emission, incineration ash, and other industrial activities generating toxic wastes (Gremion et al., 2004). TM are extremely toxic to life. They alter the structure and function of essential enzymes by replacing the key elements or by changing protein structure. For example, TM modify the structure of plasma membrane proteins, thus harming the functionality of the plasma membrane (Hall 2002). Exposure to high concentrations of TM causes oxidative damage of vital tissues and DNA injury, through the production of reactive oxygen species such as the superoxide anion $O_2\bullet^-$, the hydroxyl radical HO•, and hydrogen peroxide (H_2O_2) (Schützendübel & Polle, 2002). Above a certain threshold, TM have direct toxic effects on the microbial community and modify soil properties. For instance, at high concentrations, TM lead to enhanced soil acidification, which in turn increases TM bioavailability (Del Val et al., 1999). Together, these effects impact negatively on plant viability and microbial biodiversity in the soil on a long-term basis (Del Val et al., 1999). Because TM are stable in the soil and do not readily decay, problems associated with TM pollution are on the rise (Jarup 2003). Such contaminated soils are limited in their reuse as a result of the presence of TM (Pilon-Smits 2005). In extreme cases, TM levels are so high that they can reduce or even prevent the establishment of natural vegetation (Pilon-Smits 2005).

Phytoremediation is the use of plants and their rhizosphere microbes to remove or immobilize contaminants from polluted sites (Göhre & Paszkowski 2006). Phytoremediation is an attractive technology for the disposal or stabilization (preventing spreading) of TM in derelict soils. Because phytoremediation is an *in situ* approach avoiding the transportation of contaminated soil for *ex situ* detoxification treatments, it is relatively inexpensive, and becoming increasingly popular (Pilon-Smits 2005). Tolerant plants and their associated rhizosphere microorganisms are both important for enhancing the efficacy of phytoremediation techniques and for minimizing the time required for cleanup, which is perhaps the main drawback of this technique (Göhre& Paszkowski 2006).

Plant species that possess these traits include vetiver grass, hemp, sunflower, poplar, and willow (Pilon-Smits 2005). In addition to plants, the rhizosphere microbial community is also an important component contributing to soil decontamination.

We initiated a study to address the role of the rhizosphere microbial community in TM decontamination by focusing our efforts on the arbuscular mycorrhizal fungi (AMF), an important division of this community for which biological information is still sparse. AMF are ubiquitous and colonize the roots of terrestrial plants in all ecosystems, forming an extensive network of hyphae that reaches nutrients in soil zones unavailable for direct uptake by plants (Smith & Read 2008). AMF improve nutrient and water supply to their hosts by delivering phosphorus, nitrogen, micronutrients and other immobile molecules directly to the root. They also reduce the damage caused by root pathogens (St-Arnaud & Vujanovic 2007; Smith & Read 2008). Finally, AMF were shown to improve soil texture and reduce erosion through soil particle aggregation (Rillig & Steinberg 2002). Interestingly, these organisms inhabit most climates and tolerate extremely harsh conditions, including soils contaminated by TM (Vallino et al., 2006; Zarei et al., 2008; Wu et al., 2010).

The mutualistic association between plants and AMF has been identified as an important mechanism for TM tolerance in plants (Hall 2002). Mycorrhized plants grow better on metal-contaminated soil than plants that lack AM colonization (Gonzalez-Chavez, et al., 2002). Furthermore, AMF isolates from metal-polluted soils show better resistance to TM toxicity as compared to isolates from unpolluted soils (Gonzalez-Chavez et al., 2002). The use of AMF strains isolated from contaminated sites is thus a promising tool for phytoaccumulation or phytosequestration techniques (Sudová et al., 2008; Wu et al., 2009).

Because AMF play an important role in the mobilization and immobilization of metals in soil, they are also considered to be key factors in the bioavailability of metals to plants (Del Val, et al., 1999; Smith & Read 2008). AMF species differentially affect the translocation and accumulation of TM in the colonized plants (Lingua et al., 2008). The level of uptake and immobilization of TM by plants depend on many variables including AMF species, plant species, nature of the TM and concentration in the soil (Audet &

Charest 2008). Although a negative correlation has been reported between TM concentration in soil and either AM colonization or the complexity of AMF population structures, AMF were never completely eliminated from soils, no matter how high the TM concentration measured in the field, suggesting that these fungi are extremely resilient (Vallino et al., 2006; Zarei et al., 2008; Wu et al., 2010). Consequently, a comparative analysis of AMF community structure in metal-polluted and unpolluted soils is essential for the identification of metal-tolerant AMF ecotypes and development of efficient phytoremediation techniques (Zarei, et al., 2008). In short, changes in AMF diversity caused by TM toxicity in soil can influence the bioremediation capacity of plants (Del Val, et al., 1999; Lingua et al., 2008).

The objective of this study was to compare AMF community structure between sites contaminated or uncontaminated with TM. To this end, the roots and rhizosphere soil of plantain were sampled to (i) determine AMF diversity in the rhizosphere (ii) evaluate the effect of TM contamination on AMF community structure, and (iii) identify which AMF strains frequently establish themselves at TM polluted sites.

Materials and Methods

Soil and root sampling

We studied three uncontaminated and six contaminated sites within the City of Montréal, Québec. The uncontaminated sites were Dupéré park (45°36’03.47’’N 73°32’01.55’’W) and Maisonneuve park (45°33’15.03’’N 73°32’30.57’’W) in the Mercier borough, and Étienne-Desmarteau park (45°33’27.93’’N 73°34’40.38’’W) in the Rosemont borough. The six TM polluted sites were Baldwin park (45°32’04.99’’N 73°33’47.91’’W) in the Plateau borough, La Mennais (45°32’01.34’’N 73°36’22.88’’W), Lafond (45°33’11.94’’N 73°34’07.55’’W), Laurier (45°33’03.77’’N 73°34’09.25’’W), L'Églantier (45°33’56.58’’N 73°34’06.37’’W), and Rosemont park (45°33’53.88’’N 73°34’07.46’’W) in the Rosemont borough. TM contamination resulted from demolition residues, ash from waste incineration, and various industrial wastes buried on these sites at different time periods, approximately from 1947 until 1961. Soil TM concentrations for the sampled sites were analyzed by a commercial laboratory (Maxxam Analytique Inc and Bodycote Groupe d’essais) using acid digestion and is reported for 0-60 cm depth (summarized in Table 1). In the six sites considered “contaminated by TM” for our study, the soil analyses showed that TM concentration for at least four out of the 12 metals tested exceeded the guideline values set by provincial regulations (Beaulien & Drouin 1999). For example, As, Ba, Cd, Pb, Zn and Cu all exceed guideline values at site 4. TM bioavailability in soils was also estimated in the present study using a Milli-Q water extraction method (Table 1) (Hendershot, et al., 2008). Trace metal concentration is defined as the total amount of TM that is present in soil measured by acid extraction while TM bioavailability is defined as the amount of TM soluble in water.

Plant species composition is an important ecological factor that affects the AMF community structure (Vandenkoornhuyse et al., 2002). In this study, we chose to sample plantain (*Plantago major* L.) because it was dominant in both contaminated and uncontaminated sites and it is a well-known mycotrophic plant. *P. major* can tolerate very high metal concentrations in the roots and is commonly used for monitoring changes in

AMF community structures (Orlowska, et al., 2002, Lázaro, et al., 2006). Dandelion and several grasses were also commonly found in these sites.

We also chose to analyze soil and roots separately because soils can have mixed AMF taxa but some of them can not colonize the roots due to possible competition. However, some AMF taxa colonizing the root are not sporulating or not forming a large extraradical biomass and therefore will not be able to be detected in the DGGE. Root systems and rhizosphere soil samples from *P. major* were collected at the nine different sites within the City of Montréal area in the summer of 2008. Three plantain plants were randomly chosen and sampled per site. The rhizosphere soil from the three plants at each site was individually passed through a 2 mm sieve, thoroughly mixed and stored at -20 °C. The root systems of the three plants were removed, separated from soil particles, and frozen at -20 °C until DNA extraction.

DNA extraction

DNA was extracted from root samples using the UltraClean microbial DNA isolation kit and from soil samples using the UltraClean soil DNA isolation kit (MoBio Laboratories) following manufacturer's instructions. All samples were crushed using a FastPrep^{TM} FP120 machine (MP Biomedicals) using Lysing Matrix A tubes at speed level 4, 3 times for 20 sec each.

Cloning and sequencing (initial step to establish references for PCR-DGGE)

PCR amplifications were individually performed on the DNA extracted from the root and soil samples using primer pair AML1 and AML2 (Lee et al., 2008) to amplify a 790 bp 18S rRNA gene fragment. The following cycling protocol was used: one initial cycle at 95 °C for 3 min, 30 cycles of denaturation (94 °C for 45 s), annealing (58 °C for 45 s) and extension (72 °C for 45 s), and a final extension at 72 °C for 10 min. One µL of diluted DNA (1:100) was used as template for PCR reactions in a 25 µL volume containing: 1x PCR buffer (Quiagen), 5 U of Taq DNA polymerase (Quiagen), 0.25 mM dNTP mixture, and 0.5 µM AML1 as well as 0.5 µM AML2. PCR products were run on a 1% agarose gel electrophoresis and stained with a 10% ethidium bromide solution, then

digitally captured using a gel imaging system (Gel Doc, Biorad). The PCR products were then pooled and cloned into pJET1.1 using a Clone JET^{TM} PCR cloning kit (Fermentas) following the manufacturer's instructions. Ligated plasmids were transformed into competent *E. coli* JM101 cells using a heat-shock approach. The transformed bacteria were plated onto LB (Luria-Bertani) medium containing ampicillin (100 μg ml^{-1}). PCR using AML1 and AML2 primers was performed directly on bacterial colonies to screen positive clones. Clones that showed fragments with the expected size were sent for sequencing at the Genome Québec Innovation Center facility (Montréal, Canada). Nucleotide BLAST searches were performed on the returned sequences using the NCBI website. Clones that matched known AMF sequences were selected and readied for DGGE. To do this, one clone of each AMF species was used as a DNA template for PCR amplification using primer pair AM1 (Helgason et al., 1998) and NS31-GC (Simon et al., 1992). The expected amplicon size was approximately 550 bp, which was suitable for DGGE analysis. PCR reactions for the DGGE were performed in 25 μl volumes under the same conditions described earlier in this section, and the amplicons were individually run through DGGE, as described below, to assess their electrophoretic mobility as a reference to the analysis of the field samples (Liang et al., 2008).

DGGE analysis

Nested-PCR was performed to amplify 18S rRNA gene fragments of AMF separately from each of the same original DNA samples extracted from root and soil samples. In DGGE, DNA fragments of the same length but different sequence (which are typically different alleles of the locus under study) are separated on a polyacrylamide gel contains a gradient of increasing concentration of the chemical denaturants formamide and urea (Helgason et al., 1998). This separation depends on the difference in mobility of the DNA fragments brought about by the fact that different nucleotide sequences have different melting points in the gradient. DGGE banding patterns can provide an analysis of microbial diversity and in addition, different DGGE bands can also be excised from the gels,

reamplified by PCR and sequenced. DGGE is therefore a reliable, reproducible, fast, and cost-effective method to examine the community structure of multiple samples.

The first PCR round was done using the primer pair NS1 and NS41 (White et al., 1990) to amplify an approximately 1.2 Kb fragment. The PCR mixture contained: 1×PCR buffer, 0.5 mM of MgCl2, 5 U Taq DNA polymerase (Qiagen), 0.25 mM dNTP, 0.5 µM NS1, 0.5 µM NS41, 0.5 µl Tween 1%, 1 µl DMSO, 0.125 µl bovine serum albumin (BSA), and 1 µl of extracted genomic DNA (diluted 1:100) in a PCR volume of 25 µl. The PCR cycling conditions were one cycle at 95 °C for 3 min, followed by 35× (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min) and a final extension at 72 °C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis. Products of the first PCR round were diluted to 1:100 and used as template in the subsequent nested PCR. The primer set for the second round was a mixture of equal amounts of the AM1 (Helgason et al., 1998), AM2, AM3 (Santos-Gonzalez et al., 2007) as the reverse primer combination and NS31-GC (Kowalchuk et al., 2002) as the forward primer. The second PCR round was conducted in 25 µl volumes of the following mixture: 1×PCR buffer, 5 U Taq DNA polymerase (Qiagen), 0.25 mM dNTP, 0.5 µM AM1, 0.5 µM AM2, 0.5 µM AM3, 0.5 µM NS31-GC and 1 µl of the first PCR product. PCR conditions were one cycle at 94°C for 3 min, followed with 30× (94°C, 45 s; 58°C, 45 s; 72°C, 45 s), and a final extension step at 72°C for 10 min. PCR products were analyzed in 1% agarose gel electrophoresis.

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad), with 10 µl of the second round (nested) PCR products just described. DGGE analyses were conducted in 1 x TAE buffer at a constant temperature of 60°C at 80 V for 20 min followed by 45 V for 17 h on a 6% (w/v) polyacrylamide gel (40% acrylamide/bis-acrylamide) with a 35-45% denaturant gradient (100% denaturant corresponding to 7 M urea and 40% (v/v) formamide). We used cloned DNA of *Glomus intraradices*, *G. viscosum*, and *Scutellospora calospora* as molecular markers for DGGE. Ten µl of each PCR product from cloned DNA were pooled and 10 µl of mixed PCR products were loaded on both side of each gel to facilitate gel-to-gel comparisons. Gels were stained in a

1:10,000 SYBR Gold solution for 15 min and visualized by UV illumination. Gel pictures were digitalized using a gel documentation system (GelDoc, Bio-Rad Laboratories).

Our initial investigations indicated that the similarity in the electrophoretic mobility of bands on a gel did not always reflect sequence identity of DNA fragments. Some dissimilar DNA fragments gave similar banding patterns on DGGE gel, thus resulting in an under-estimation of species diversity. We therefore opted for the reamplification and sequencing of all DGGE bands. Clear bands were excised from the UV illuminated acrylamide gels and DNA was extracted from bands by incubation in 30 µL ddH_2O at room temperature for 16 h. One µL of eluted DNA was used as a template for PCR amplification. PCR conditions were the same as described above for the second PCR round, except the number of cycles was reduced to 25. PCR products were run on DGGE gels using a 35%-40% denaturing range. When single bands appeared in each individual lane on the DGGE pattern, these individual bands were re-excised from the gel, and their DNA was extracted and amplified with primer set AM1, AM2, AM3/NS31 (without GC-clamp). The PCR products were sequenced at the Genome Québec Innovation Center facility (Montréal, Canada) with the NS31 primer.

Validation of the DGGE method

The reproducibility of DGGE was tested by loading three PCR products for each sample on DGGE gels. We did not observe any difference in the banding patterns of the three replicates for each sample. The DGGE identification method was also compared with the cloning and sequencing approach to assess its discrimination power against AMF ribotypes. One root and one soil sample from the Maisonneuve Park site were used in this comparison since they showed the highest AMF diversity using DGGE. One µL of each DNA sample was used as a template for PCR amplification. PCR conditions were the same as described above for the nested PCR protocol using primer pairs NS1/NS41 and AM1, AM2, AM3/NS31, respectively. The nested PCR products were individually cloned into pJET1.1 plasmid vector using a Clone JET^{TM} PCR cloning kit (Fermentas) following the manufacturer's instructions. One hundred and twenty-five clones from the root sample and

129 from the soil sample were positively screened by PCR and sequenced. DGGE bands of the two samples were excised and sequenced as described above in DGGE analysis. We then compared the recovered sequences of both methods using sequence similarity and rarefaction analysis as described below.

Sequence alignment and phylogenetic tree reconstruction

Sequences were aligned using Jalview (Waterhouse, et al., 2009). Highly variable, poorly aligned regions were removed from the final alignments using Gblocks (v0.91b) (Talavera & Castresana, 2007). The best-fit model of nucleotide substitution was chosen with jModeltest (v0.1.1) (Posada 2008). Phylogenetic analysis was performed in PhyML (Guindon & Gascuel, 2003) with 1000 bootstrap replicates. Recovered sequences were deposited in GenBank under accession numbers HQ258982-HQ258994, HQ323462-HQ323643, and HQ993213-HQ993466.

Statistical analysis

The presence of each ribotype was noted and a presence–absence matrix was built for statistical analyses. Most DGGE bands excised from the lower part of the gels showed high sequence identity to Ascomycota and Basidiomycota (data not shown). These bands were excluded from the further analyses. The Shannon-Weaver diversity index (H') (Rosenzweig 1995) was calculated to compare AMF ribotype diversity between sampling sites, using the following equetion:

$$H' = -\sum p_i \ln p_i$$

where the summation is over all unique ribotypes i, and p_i is the proportion of an individual ribotype relative to the total number of ribotypes in the population.

Analysis of variance was used to assess significant differences in ribotype richness (number of AMF ribotypes detected with DGGE) and Shannon diversity indices between uncontaminated and contaminated sites. Post-hoc comparisons between sites were done using Tukey's HSD test using SPSS v17 (SPSS Inc., Chicago, Illinois). Multiple linear

regression analyses were performed to evaluate the relationship between soil TM concentrations and AMF diversity index or AMF species richness using SPSS software v. 17 (SPSS Inc., Chicago, Illinois). Discriminant analysis was used to test for significant differences between AMF communities, with a Fisher test of the Mahalanobis distances between sites using XLSTAT v. 5.01 (Addinsoft Inc., Paris, France). Canonical correspondence analyses (CCA) was performed on the AMF ribotype presence/absence matrix based on DGGE banding patterns to test the significance of the relationship between TM concentrations and AMF community structure by permutation (n = 1000) using XLSTAT v. 5.01 (Addinsoft Inc., Paris, France).

Rarefaction analyses were performed on the clones from soil and root samples to estimate whether a representative portion of AMF soil and root diversity have been sampled, respectively. Ribotype frequencies were determined in DNAsp (Rozas et al., 2003) and the rarefaction analysis was performed under a Michaelis-Menten two parameter model with resampling. Abundance diversity estimates were calculated in EstimateS v8.2.0 (Colwell 2009).

Results

Clone library analysis

Sixty clones were analyzed from the pooled 18S rRNA gene fragments. Sixty-five percent of these clones matched to AMF ribotypes, while 10% did not, 9% yielded ambiguous sequencing, and 16% had no inserts. Of the 39 clones that matched to AMF taxa, there were 19 unique ribotypes (Table 2). These AMF ribotypes were used as references in the DGGE analyses of soil and root samples. DGGE of the clones produced 19 reference bands (Fig. 1S). Two pairs of clones (CL10 and CL11; CL17 and CL18) had the same electrophoretic mobility but different nucleotide sequences as shown by cutting and resequencing bands.

Identification of AMF taxa

Twenty-seven AMF ribotypes were identified from both DGGE and cloning/sequencing approaches (Fig. 1, Table 2). These ribotypes belong to the most important glomeromycotan families *Glomaceae*, *Gigasporaceae*, *Diversisporaceae* and *Archaeosporaceae* while no *Acaulosporaceae*, and *Paraglomaceae* ribotypes were detected. We constructed a maximum likelihood (ML) phylogenetic tree to correlate our ribotypes with previously described sequences (Fig. 2S). Seven *Glomus* ribotypes were related to known *Glomus* morphospecies including *G. irregulare* (formerly *G. intraradices* (Sokolski et al., 2010)), *G. mosseae*, *G. viscosum*, and *G. etunicatum* as supported with a bootstrap higher than 95%. Names of many AMF taxa have been recently changed and new names are shown in Table S1 (Schüßler and Walker, 2010). The rRNA gene tree topology resolves two *Glomus* species with significant bootstrap support, i.e. *Glomus viscosum* and *Glomus mosseae*. The species *G. etunicatum*, *G. lamellosum* and *G. claroideum* were not monophyletic. The sequences that clustered within *Gigasporaceae* and *Diversisporaceae* were supported by bootstrap values of 85% and 97%, respectively. Five clades with significant bootstrap support could not be assigned to known AMF species. The *Scutellasporacea* were not monophyletic.

Comparison of DGGE and cloning results

Samples from the Maisonneuve Park were used to compare the discrimination power between cloning-sequencing and DGGE approaches (Table 2S). For the root sample, 59 out of 125 clones (47%) were identified as AMF sequences. In the soil sample, 57 out of 129 positive clones (44%) were identified as AMF sequences. We used rarefaction analysis to compare AMF richness computed from recovered AMF sequences. This analysis allows the construction of rarefaction curves (Fig. 2). The number of unique ribotypes retrieved from root and soil samples showed a plateau around 7 and 8 ribotypes, respectively. Even though the saturation curves were dissimilar, the estimated diversity of ribotypes is not significantly different between root and soil samples. The Chao2 richness estimator for root samples was 24.55 (LB 95%: 12.95. UB 95%: 66.4) and for soil samples 25.3 (LB 95%: 13.31. UB 95%: 68.11). We used the classic calculation instead of the bias-corrected option in EstimateS because the CV for Incidence distribution was 0.647. Judging from the abundance estimators, at least 25 AMF clones are required to cover the ribotype diversity in our samples. The actual number of clones that would be required to capture AMF diversity is at least double the numbers shown in our rarefaction analysis because more than 50% of our clones did not yield AMF sequences.

In the root sample, four different ribotypes of *Glomus* species (B3, B5, B7, B8) were detected by DGGE, while seven *Glomus* ribotypes (Seq 2, 3, 4, 5, 8, 9, and 10) (Table 2S) were found by cloning. For example, Seq 2 was detected by DGGE as B3 and was also represented by 12 clones from roots, while Seq 9 was not detected by DGGE, but was found as five clones from roots. From the soil sample, four ribotypes of *Glomus* species (B2, B8, B10, and B11) were found in DGGE analysis and eight different ribotypes of *Glomus* species (Seq 1, 2, 3, 5, 6, 7, 8, and 11) were found by cloning. Overall, Seq 1-7 were detected by both approaches while Seq 8-11 were detected by cloning only. These results clearly show that while DGGE detects dominant taxa and is highly reproducible, the cloning and sequencing approach is much more sensitive.

Analysis of DGGE banding profiles from rhizosphere samples

DGGE profiles performed from DNA samples extracted from plantain roots and rhizospheric soil are shown in Figure 1. No difference in the DGGE patterns between the three replicates of each sample was observed (data not shown). A total of 18 different band positions corresponding to AMF ribotypes were detected (Table 2), of which 10 bands shared a common migration position with 10 among the 19 reference clones bands, allowing their preliminary identification as various species of *Glomus* and *Scutellospora calospora*. However, the remaining nine reference bands corresponding to clones identified as *Glomus* spp., *Scutellospora gilmorei, Ambispora fennica, Archaeospora* spp or *Diversispora* sp. did not match to any DGGE bands of rhizosphere samples. On the other hand, eight DGGE bands from root or soil samples did not match any clone band, and were identified by sequencing as 98-100% similar to *G. etunicatum, Glomus* sp, or *Scutellospora aurigloba.*

Of the AMF ribotypes detected by the DGGE analysis of rhizosphere samples, eight were found in roots of plants growing on uncontaminated sites, with an average of three ribotypes per sample, while seven other ribotypes were detected in roots of plants growing on TM contaminated sites, with an average 2.2 ribotypes per sample. In plant roots from uncontaminated sites, the most frequent ribotype was B3 (99% similarity to *G. irregulare/G. intraradices*), which was detected in all plantain roots from these sites, followed by B5 (99% similarity to *Glomus* sp.) detected in 66.6% of root samples from unpolluted sites. In addition, five bands of B1, B5, B8, B15 (various *Glomus* spp. with 98%-99% sequence identity) and B18 (99% similarity to *Scutellospora aurigloba*) were only recorded in roots from uncontaminated sites. In contrast, the most abundant AMF ribotype—within 77.8% of plantain roots—from TM contaminated sites was B6 (100% identity to *G. mosseae*). Four divergent *Glomus* spp. ribotypes (B4, B6 B9, and B12) were only recovered from roots in TM contaminated sites. Three ribotypes, B3 (*G. irregulare/G. intraradices*), B7 (*G. viscosum*) and B10 (*Glomus* sp.) were found in roots from plants growing on both TM contaminated and uncontaminated sites.

In soil, seven AMF ribotypes were detected in samples from uncontaminated sites, with up to three ribotypes per sample. Eight AMF ribotypes were detected in TM contaminated soil samples, with an average of 1.78 ribotypes per sample. B2 (*G. etunicatum*, 99% similarity) was the most dominant ribotype and was detected in 77% of uncontaminated soils. In addition, five ribotypes were identified as three *Glomus* ribotypes (B8, B10, and B11, 98-100% similarity) and two *Scutellospora* ribotypes (B17 and B18, 98% and 99% of similarity to *S. calospora* and *S. aurigloba*, respectively) were only observed in uncontaminated soils. In contrast, B13 (*Glomus* sp, 98% similarity), B6 (*G. mosseae*, 100% similarity), and B14 (*Glomus* sp., 98% similarity) were abundant in TM contaminated soils, with frequencies of detection varying from 33.3 to 38.9%. Furthermore, six ribotypes B3, B6, B13, B14, B15, and B16 were found only in TM contaminated soils. Only the two ribotypes B1 and B2 (*G. etunicatum*, 99% identity) were found in both uncontaminated and TM contaminated soils.

Discriminant analysis (DA) was used to test the significance of differences in AMF communities between TM contaminated and uncontaminated sites. The first two axes explained a total of 86.7% and 90.2% of the variation in AMF ribotype data sets within root and soil samples, respectively (Fig. 3). In root samples, DA showed a significant difference (P=0.03) between AMF communities in plantains growing on TM contaminated versus uncontaminated sites (Fig. 3A). In soils, DA showed a significant change in of AMF community structures (P=0.01) between uncontaminated and contaminated sites (Fig. 3B). Details are shown in supplementary information.

In root samples, discriminant analysis (DA) showed a significant difference (P=0.03) between AMF communities in plantains growing on TM contaminated versus uncontaminated sites (Fig. 3A). The significance of differences between AMF communities explained by the first two axes was further tested by analysis of Mahalanobis distances between the cluster centroids. According to this analysis, the AMF communities in plantain roots formed three clusters, which were significantly different across the first two axes. These clusters were AMF communities of uncontaminated sites 1 and 3, communities of TM contaminated sites 5, 6, 7, 8, 9, and communities of uncontaminated site 2 and metal-

contaminated site 4. From uncontaminated sites, samples from site 1 significantly differed (P=0.03) from site 2. In samples from metal-contaminated sites, AMF communities were significantly different (P=0.02) between site 4 and all other TM contaminated sites, which were similar to each other (P=0.1). Although a significant difference (P=0.03) in AMF communities in roots was found between all other uncontaminated and TM contaminated sites, the uncontaminated site 2 and the metal-contaminated site 4 had similar communities (P=0.4).

In soils, DA showed a significant change in of AMF community structures (P=0.01) between uncontaminated and contaminated sites (Fig. 3B). Three clusters were clearly separated across the first two discriminant axes: AMF communities in soils of all uncontaminated sites and TM polluted site 6 formed a first group, communities of metal-contaminated sites 4 and 5 formed a second group, and communities of metal-contaminated sites 7, 8 and 9 formed a third group. No significant difference (P= 0.07) was noticed between AMF communities of soils sampled from uncontaminated sites. Similarly, no significant difference was found between AMF community structures in soils of TM contaminated sites 4 and 5 (P> 0.1), and between communities of soils collected from polluted sites 7, 8 and 9 (P=0.2). Mahalanobis distance showed however a significant difference (P=0.04) between metal-polluted sites 4 and 5 and all other polluted sites. Although a significant difference was found between uncontaminated and metal-contaminated sites (P=0.01), the AMF community structure was similar between all uncontaminated sites and metal-polluted site 6 (P>0.08).

Multiple linear regression analysis confirmed a significant relationship (R^2 = 0.32, P= 0.04) between metal concentration and AMF species richness in roots. The beta coefficient values showed that species richness in roots decreased with increased Ba, Cd, Co, Sn, and Pb concentrations, whereas species richness increased with As, Cu, and Zn concentrations. Similarly, there was a significant relationship (R^2 = 0.42, P= 0.01) between metal concentrations in soils and AMF diversity index within roots, where the diversity index was negatively correlated with Cd, Co, Sn, and Pb concentrations. Multiple linear regression analysis revealed a marginally significant relationship (R^2 = 0.31, P = 0.053)

between metal content and diversity index in soils. AMF diversity index in soils decreased with increased Ba, Cd, Co, Sn, and Pb concentrations in soils. Thirty-five percent of the variation in species richness in soils was significantly (R^2 = 0.35, P= 0.03) explained by metal concentrations, where species richness decreased with increased Co, Sn, Pb, and Zn concentration in soils.

Canonical correspondence analysis (CAA) was used to assess the relationships between AMF ribotypes in plantain roots and the different TM concentrations (Fig. 4A). By this analysis, a total of 71.9% of the cumulative variance in the AMF ribotype dataset is explained by the first two canonical axes. CCA revealed a significant effect of TM contamination on AMF community structure within the roots (F= 1.7, P< 0.05). AMF ribotypes B1, B5, B15 and B18 found within roots of plantain from uncontaminated sites were represented in the upper left part of ordination, without clear association with TM. B6 ribotype was associated to Cd, Sn, and Zn contamination, while ribotype B9 was abundant in roots sampled from Cu and Cd contaminated sites, and ribotypes B10 and B12 were found in roots sampled from As polluted sites. No ribotype detected in plantain roots was found associated with Pb contamination in soil.

CCA also showed a significant modification of AMF communities in soils (F= 1.9, P< 0.05). The first two canonical axes explained 65.5% of the total variation in AMF community structure in soils with regard to TM contamination (Fig. 4B). The ordination diagram revealed that communities were separated across the first axis according to the pollution status of the sites. Ribotypes B8, B10, B11, B17 and B18 found in unpolluted sites are represented in the left part of the ordination, while ribotypes B3, B6, B13, B14, B15 and B16 found in polluted soils are represented on the right side of the ordination. Ribotypes B1, B2 which were found both in unpolluted and polluted soils are represented near the center of the ordination. Furthermore, the second canonical axis differentiated AMF communities in soils contaminated with Zn, Sn and Pb from those detected in soils contaminated with As, Cu, and Cd. Ribotypes B6 and B13 were represented in Zn, Sn, and Pb contaminated soils. Meanwhile, B3, B15 and B16 were found in As and Cd contaminated soils, where B10 and B11 were close to Cu axis.

AMF diversity index

The analysis of variance showed that AMF diversity index in root samples (Table 3) was not significantly different between TM contaminated and uncontaminated sites (P= 0.06). However, Tukey's HSD post-hoc test showed that AMF diversity index in sites 5 and 9 were significantly lower than other sites. Similarly, there was no significantly difference in species richness in plantain roots (P= 0.09). On the other hand, the AMF diversity index in uncontaminated soils was significantly higher than those in contaminated soils (P=0.02). Furthermore, species richness was significantly lower in contaminated soils than in uncontaminated soils (P= 0.015).

Table 1. Total concentration and bioavailability of trace metals in polluted and non-polluted sites.

	Sites[1, 2, 3]	As		Ba		Cd		Co		Cr		Cu	
		C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B
Non-polluted	1	<6	1.71±.02a	79	22.1±1.5ab	<0.5	.06±.01a	5	.42±.01a	17	1.61±.04a	23	15.5±.26a
	2	23	1.73±.01a	130	24.1±.43b	ND	.08±.01a	11	.39±.01a	38	1.89±.06a	25	12.6±.75a
	3	1.7	1.78±.04a	64	22.4±.38ab	0.8	.06±.01a	4	.43±.01a	10	2.49±.08b	12	12.8±.15a
Polluted	4	66	2.84±.03b	1700	27.1±.62c	2.2	.16±.01b	16	.89±.01b	52	2.52±.4b	550	37.7±.38e
	5	4.1	2.39±.03b	190	19±.67a	9.9	.30±.01c	6	.42±.02a	29	2.26±.14b	18	11.5±.05a
	6	<6	3.19±.02c	110	21.7±.25ab	<0.5	.20±.02b	9	1.09±.01c	19	2.88±.04b	130	28.5±.67d
	7	18	3.04±.01bc	140	21.9±.44ab	<0.5	.17±.01b	6	.80±.01b	21	2.82±.11b	1100	75.7±.44f
	8	<6	2.97±.01b	140	28.6±.88c	<0.5	.25±.01c	6	1.15±.04c	23	2.25±.17b	39	22.6±.03c
	9	<6	4.75±.10d	140	22.9±1.2ab	<0.5	.29±.01c	8	1.07±.03c	27	2.80±.21b	54	18.1±.19b

	Sites[1, 2, 3]	Sn		Mn		Mo		Ni		Pb		Zn	
		C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B	C_T	C_B
Non-polluted	1	<5	ND	310	11.7±.57a	<2	ND	14	2.85±.21a	27	1.52±.09a	59	6.68±.36a
	2	ND	ND	750	14.2±1.1ab	ND	ND	24	4.44±.35b	11	2.0±.09ca	91	7.95±.77a
	3	<5	ND	291	10.5±.36a	<2	ND	12	3.27±.11ab	32	1.67±.08a	40	7.45±.10a
Polluted	4	ND	ND	340	18.6±.98b	6	ND	31	4.96±.75b	1900	4.71±.33d	1800	39.6±.94e
	5	<5	ND	253	10.1±.75a	<2	ND	51	4.18±.22b	280	1.87±.14a	1050	31.1±.73d
	6	12	ND	430	24.2±1.7c	<2	ND	17	4.71±.02b	140	3.71±.04c	150	15.1±.42b
	7	76	ND	360	16.8±.51b	<2	ND	26	4.67±.2b	1700	4.06±.09cd	490	14.2±.5b
	8	46	ND	260	17.7±.99b	<2	ND	21	5.67±.13c	93	2.53±.21b	280	22.9±1.5c
	9	25	ND	410	27.2±1.8c	<2	ND	21	4.0±.10b	150	3.78±.07c	180	19.7±1.6c

C_T, total concentration of trace metal (mg kg^{-1}). C_B, bioavailabitity of trace metal (mg kg^{-1}).

[1]Sites are: 1, Dupéré; 2, Maisonneuve; 3, Étienne- Desmarteau; 4, Baldwin; 5, La Mennais; 6, Lafond; 7, Laurier; 8, L'Églantier; 9, Rosemont.

[2] Different letters in columns show significant difference by one-way ANOVA followed with Tukey's HSD post-hoc test at $p < 0.05$.

Mean ± SE, n = 3.

[3] ND=not determined, C_T =trace metal concentrations in soils, and C_B= trace metal bioavailability in soils.

Table 2: Arbuscular mycorrhizal fungal taxa detected by cloning sequencing and DGGE analysis of plantain roots and rhizospere soil sampled from trace metal polluted and non-polluted sites.

Bands[1]	Clones	Band frequencies [2] (%)				Holomogue sequences (sequence identity%)	Accession numbers
		Roots UN	Roots C	Soils UN	Soils C		
B1	CL1	33.3	0	44.4	16.7	*G. etunicatum* (99)	AJ852598
B2		0	0	77.8	16.7	*G. etunicatum* (99)	AJ852598
B3	CL2	100	33.3	0	22.2	*G. irregulare/G. intraradices* (99)	AJ852526, AY635831, FJ009617, FJ009616
B4	CL3	0	5.5	0	0	*G. lamellosum/G. claroideum* (99)	AJ276087, AJ276080
B5		66.6	0	0	0	*Glomus* sp. (99)	AM946871, AM946869
B6	CL4	0	77.8	0	0	*G. mosseae* (100)	NG_017178, AY635833, AY641819, AJ505618, AJ505616
B7	CL5	55.6	44.4	0	0	*G. viscosum* (98)	AJ505812
B8	CL6	11	0	33.3	0	*Glomus* sp. (98)	EF041097
B9	CL7	0	27.8	0		*Glomus* sp. (99)	EU332735, AJ506089, AJ505616
B10	CL8	11.1	11.1	66.6	0	*Glomus* sp. (99)	HM122275
B11		0	0	55.6	0	*Glomus* sp. (100)	EF177511, EF177503
B12		0	22.2	0	0	*Glomus* sp. (99)	AY916397
B13	CL9	0	0	0	38.9	*Glomus* sp. (98)	EF041077, EU340324
B14		0	0	0	33.9	*Glomus* sp. (100)	AF074371, GU353941
B15		11.1	0	0	11.1	*Glomus* sp. (99)	AJ563891
B16		0	0	0	5.5	*Glomus* sp. (98)	EU573765, AM946834, AM412534, AJ563908,
B17	CL18	0	0	11.1	0	*Scutellospora calospora* (98)	FJ009672
B18		11.1	0	11.1	0	*Scutellospora aurigloba* (99)	AJ276092
	CL10					*Glomus* sp. (99)	EU340324
	CL11					*Glomus* sp. (97)	DQ085254, DQ085251
	CL12					*Glomus* sp. (97)	EU340312, DQ085247
	CL13					*Glomus* sp. (99)	GU059542, GU059535
	CL14					*Glomus* sp. (96)	EU152192
	CL15					*Ambispora fennica* (94)	AM268192
	CL16					*Archaeospora* sp. (94)	DQ396691
	CL17					*Diversispora* sp. (95)	AM713425
	CL19					*Scutellospora gilmorei* (99)	AJ276094

[1] Band positions are labeled in Figure 1

[2] UN: uncontaminated sites; C: contaminated sites

Table 3: Diversity of AM fungal communities associated with sampling sites.

	Shannon-Weaver diversity index [1, 2, 3]	
	Roots	Soils
1. Dupéré	1.16 ± 0.23 c	0.92 ± 0.23 d
2. Maisonneuve	1.29 ± 0.10 ac	1.39 ± 00 d
3. Étienne- Desmarteau	0.69 ± 00 bc	0.68 ± 0.39 c
4. Baldwin	0.69 ± 00 bc	0.72 ± 0.36 c
5. La Mennais	0.59 ± 0.31 b	0.82 ± 0.42 d
6. Lafond	1.19 ± 0.10 c	0.46 ± 0.23 b
7. Laurier	0.82 ± 0.13 c	0.73 ± 0.36 c
8. L'Églantier	0.72 ± 0.46 bc	00 a
9. Rosemont	00 a	00 a

[1] Shannon-Weaver diversity index, $H' = -\Sigma pi \ln pi$

[2] Different letters in columns show significant difference by one-way ANOVA, Tukey's HSD post-hoc test at $p < 0.05$.

[3] Sites 1, 2, and 3 are uncontaminated, and sites from 4 to 9 are TM contaminated.

Table 1S. List of new species and families according to Schüßler A, Walker C (2010).

New species classification	**Synonyms (former classification)**	**Family**
Ambispora fennica	*Ambispora fennica*	*Ambisporaceae*
Claroideoglomus claroideum	*Glomus claroideum*	*Claroideoglomeraceae*
Claroideoglomus etunicatum	*Glomus etunicatum*	-
Claroideoglomus lamellosum	*Glomus lamellosum*	-
Rhizophagus intraradices	*Glomus intraradices*	*Glomeraceae*
Rhizophagus irregularis	*Glomus irregulare*	-
Funneliformis mosseae	*Glomus mosseae*	-
Scutellospora aurigloba	*Scutellospora aurigloba*	*Gigasporaceae*
Scutellospora calospora	*Endogone calospora*	-
Scutellospora gilmorei	*Gigaspora gilmorei*	-
-	*Cetraspora gilmorei*	-

Table 2S. Comparison of DGGE and cloning approaches using root and soil samples from Maisonneuve park.

Clones		Root		Soil	
	Accession numbers	DGGE [1] (L6)	Cloning [2]	DGGE [1] (L32)	Cloning [2]
Seq1	AJ852598	no	no	B2	6 clones (4.6%)
Seq2	AJ852526, AY635831, FJ009617, FJ009616	B3	12 clones (9.6%)	no	5 clones (3.8%)
Seq3	AM946871, AM946869	B5	13 clones (10.4%)	no	5 clones (3.8%)
Seq4	AJ505812	B7	11 clones (8.8%)	no	no
Seq5	EF041097	B8	10 clones (8%)	B8	13 clones (10%)
Seq6	HM122275	no	no	B10	12 clones (9.3%)
Seq7	EF177511, EF177503	no	no	B11	13 clones (10%)
Seq8	AM946883	no	4 clones (3.2%)	no	2 clones (1.5%)
Seq9	GU353899	no	5 clones (4%)	no	no
Seq10	GU059539	no	4 clones (3.2%)	no	no
Seq11	AM946874	no	no	no	2 clones (1.5%)

[1] L6 and L32 refer to Lane 6 and lane 32; respectively, as shown in Figure 1.
[2] Numbers in brackets refer to the percent of each detected clone in the conducted clone library

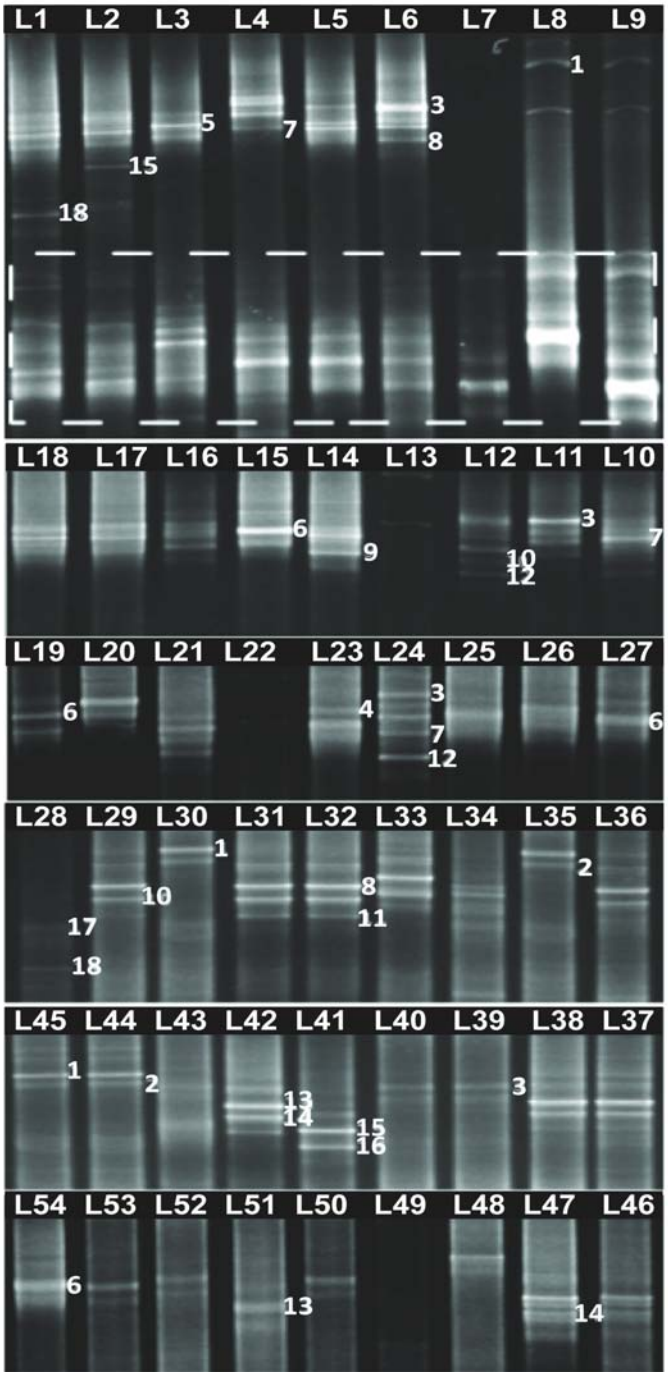

Figure 1. DGGE patterns of partial 18S rRNA gene amplified from root and soil samples from the rhizospere of plantain plants. Triplicate samples were analysed from each location. L1 to L27 are samples from roots and L28 to L54 are soil samples. Lanes: L1 to L3 and L28 to L30 are samples of site 1; L4 to L6 and L31 to L33 are samples of site 2; L7

to L9 and L34 to L36 are samples of site 3; L10 to L12 and L37 to L 39 are samples of site 4; L13 to L15 and L40 to L 42 are samples of site 5; L16 to L18 and L43 to L45 are samples of site 6; L19 to L21 and L46 to L48 are samples of site 7; L22 to L24 and L49 to L51 are sample of site 8; L25 to L27 and L52 to L54 are of site 9. Bands numbering refers to AMF ribotypes identification given in Table 2. The white box surrounds bands corresponding to non AMF ribotypes.

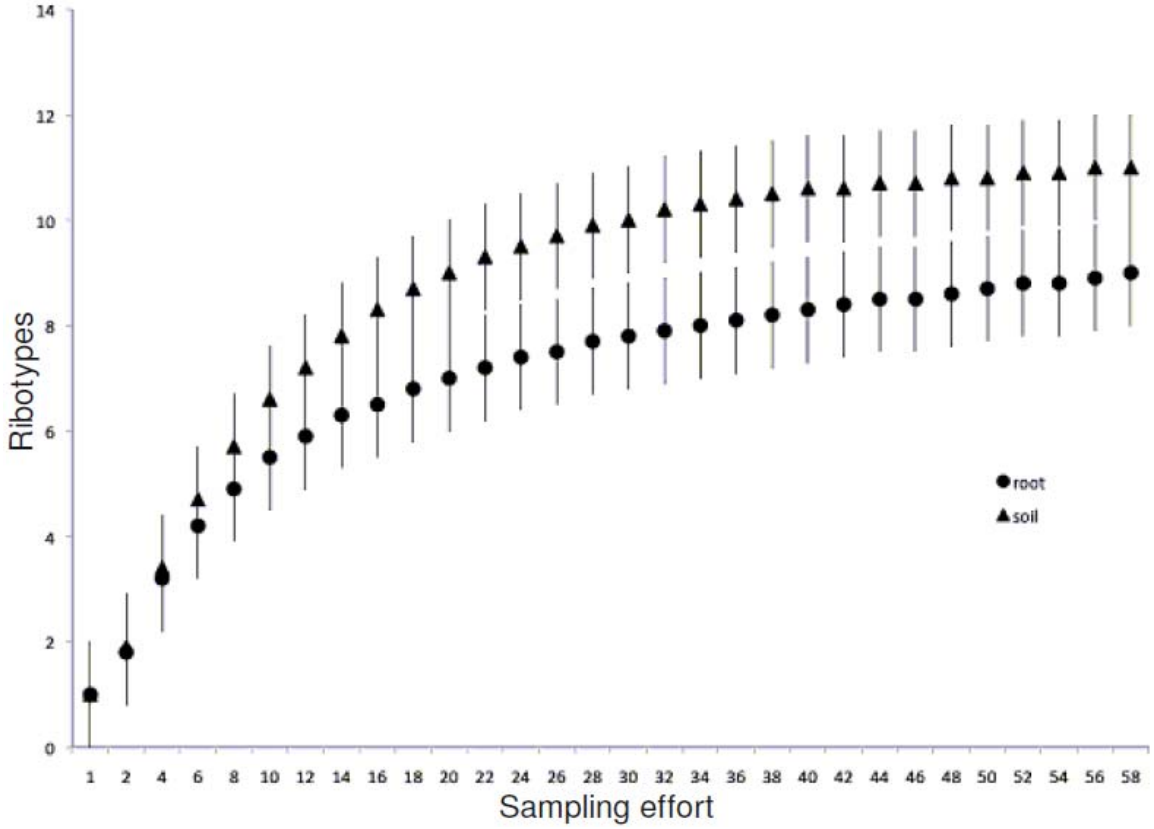

Figure 2. Rarefaction analysis of root (circles) and soil (triangles) samples. The analysis was performed with 1000 bootstrap replicates. Higher and lower 95% confidence intervals are indicated as bars above and below the data points, respectively.

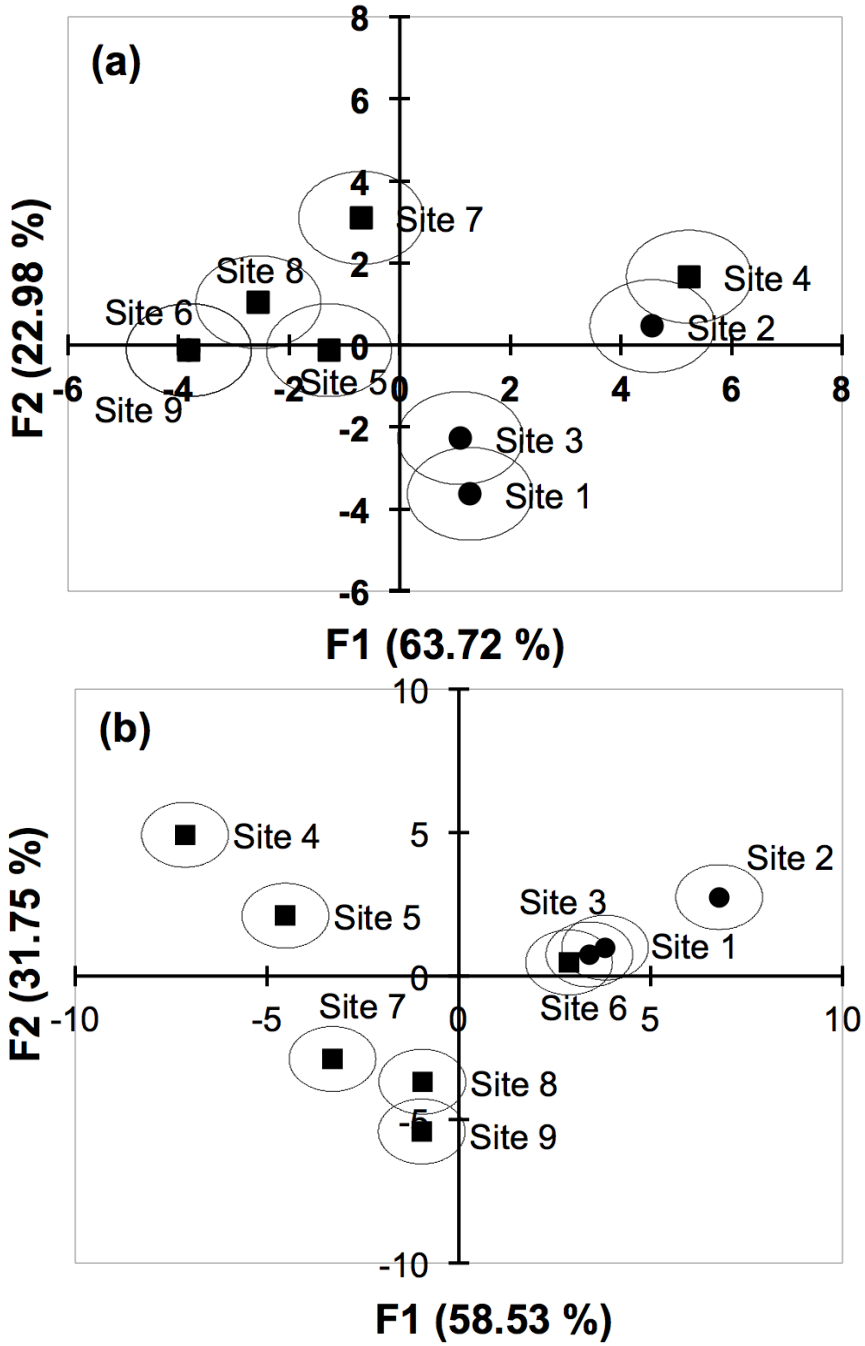

Figure 3. Discriminant analysis (DA) showing the relationship between AMF sequence types and sampling sites. A. DA of AMF community structure within roots samples. B. DA of AMF community structure within soil samples. Circles are uncontaminated sites and rectangles are metal contaminated sites.

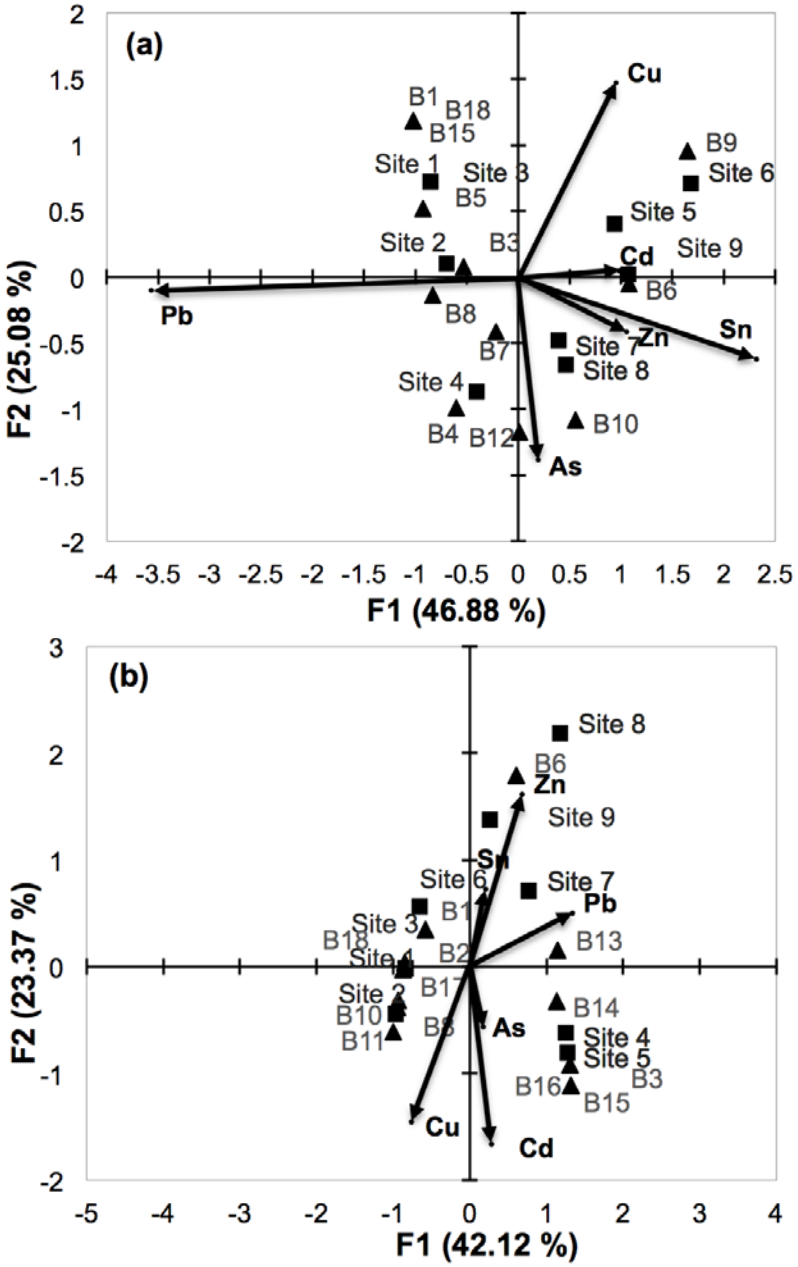

Figure 4. Canonical correspondence analysis (CCA) biplot of species-trace metal variables showing the relationship between the AMF ribotype assemblage of each site and trace metal concentrations. A. CCA of AMF community structure within root samples. B. CCA of AMF community structure within soil samples. Sites from 1 to 3 are uncontaminated. Sites from 4 to 9 are metal contaminated. Triangles are AMF ribotypes.

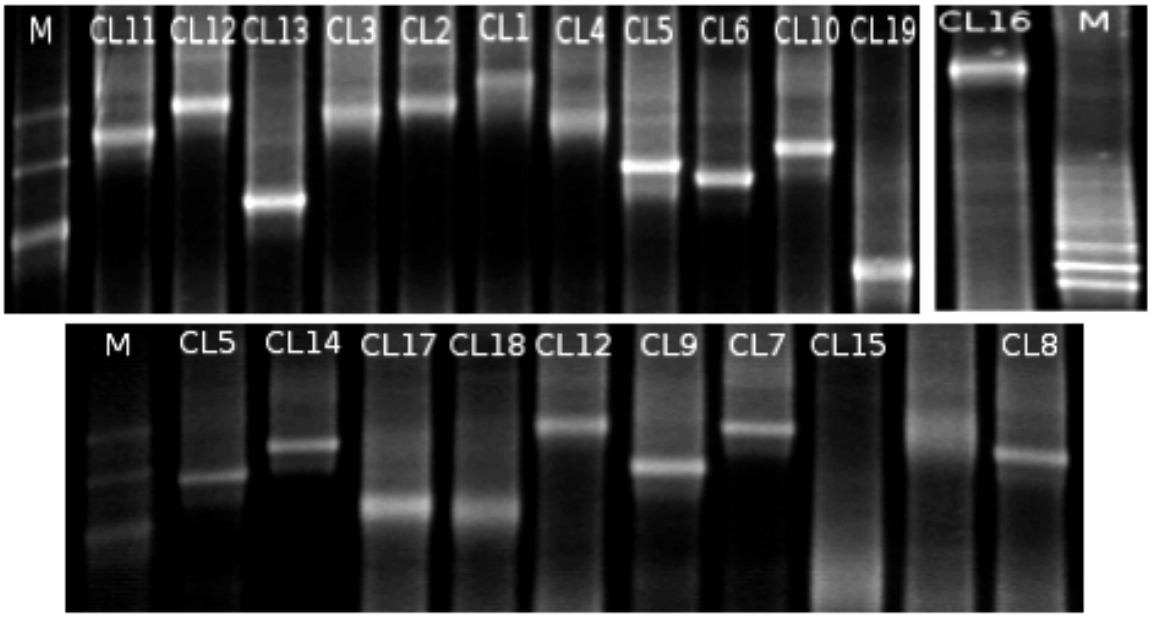

Figure 1S. DGGE banding patterns of partial 18S rRNA gene from clones of AMF taxa, Lanes: M, marker; numbers denote the AMF ribotypes identified in Table 2. PCR product of all clones were run on DGGE gels using a 35%-45% denaturing range, except of CL16 for which a 35%-38% denaturing range was used.

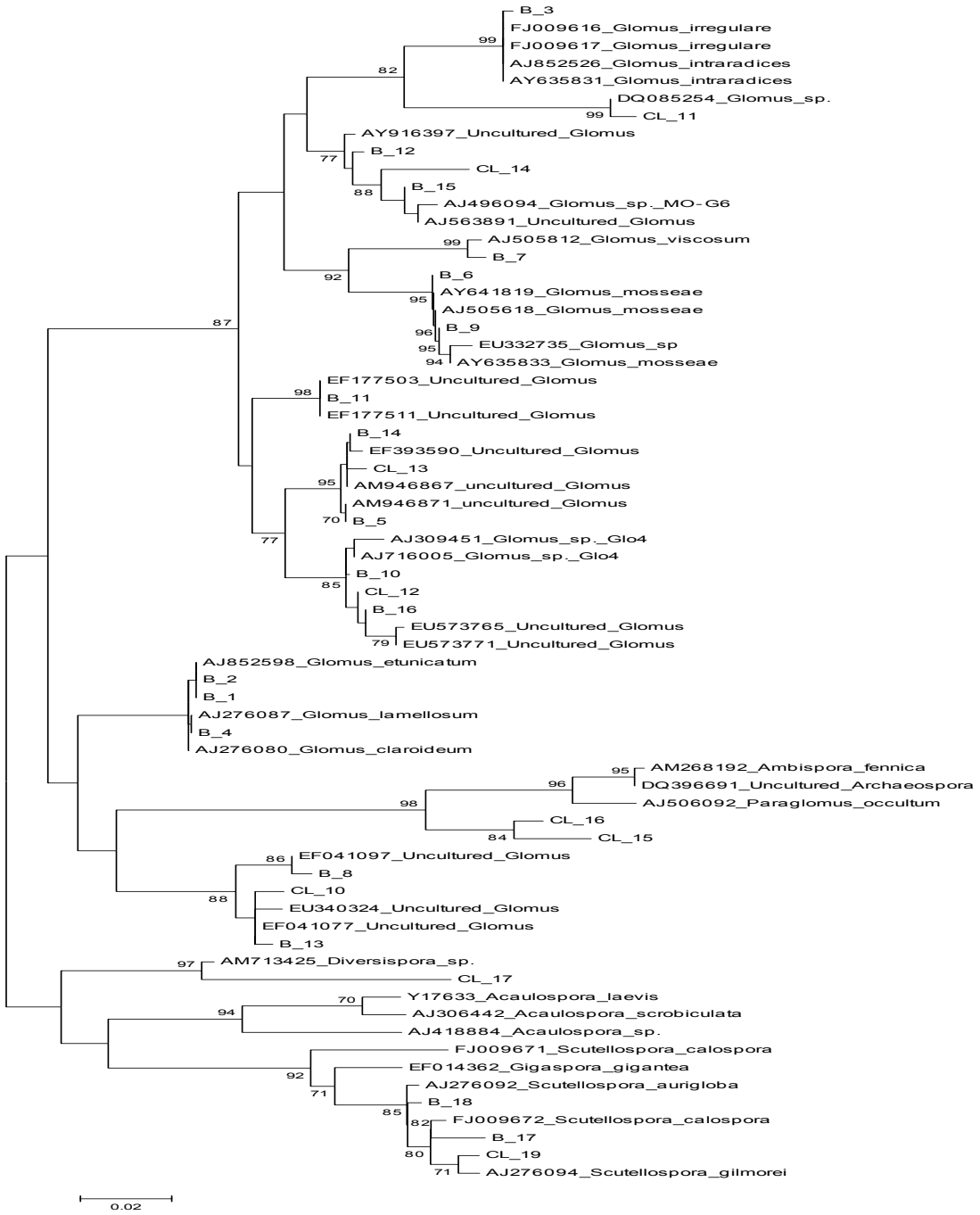

Figure 2S. Phylogenetic analysis by Maximum Likelihood. This unrooted bootstrap consensus tree was inferred from 1000 replicates and based on the GTR+G+I model. Only bootstrap values higher than 70 are depicted. Branch lengths are measured in the number of substitutions per site.

Discussion

Our results clearly show that trace metals reduce AMF diversity and modify community structure in roots and rhizospheric soil of plantain plants compared to those detected in uncontaminated soil. Interestingly, we found that some AMF ribotypes were preferentially associated with TM contaminated or uncontaminated sites, while other ribotypes were detected in both TM contaminated and uncontaminated sites.

Identification of ribotypes

Our data showed a predominance of *Glomus* ribotypes in plantain rhizospheres. Sixteen *Glomus* ribotypes out of 18 different glomeromycotan ribotypes were recovered using DGGE, while 14 *Glomus* ribotypes out of 19 different glomeromycotan ribotypes were detected in plantain rhizospheres by cloning. The predominance of *Glomus* species has been reported in other studies performed on various habitats, such as geothermal soils (Appoloni et al., 2008), tropical forests (Wubet et al., 2004), agricultural soils (Daniell et al., 2001), and phosphate contaminated soils (Renker et al., 2005). Interestingly, dominance of *Glomus* species has also been found in metal contaminated sites; for instance, Yang et al. (2010) found that *Glomus* species were the only AMF taxa recorded in roots of *Elsholtzia splendens* growing on Cu contaminated soil, and Vallino et al. (2006) detected 12 *Glomus* ribotypes out of 14 AMF ribotypes within roots of plant growing on metal polluted soils. No AMF ribotypes corresponding to *Acaulosporaceae, Paraglomaceae* families were detected within plantain rhizospheres. They may be absent from this type of disturbed environment, or present in such low numbers that they could not be detected using PCR-DGGE or cloning/sequencing using 60 clones as used here. These results are in accordance with studies using restriction fragment length polymorphism and sequencing (Vallino et al., 2006) that found no AMF sequences belonging to *Acaulosporaceae* and *Archaeosporaceae*, and only one *Paraglomus* sp. out of 14 different glomeromycotan sequence groups recovered in plant roots growing on metal contaminated sites.

It has been proposed that AMF may tolerate metal contaminated environments more easily when hyphae grow from colonized roots rather than germinating from spores

(Pawlowska & Charvat 2004). Since *Glomus* species have the ability to propagate by mycelial fragments and mycorrhizal root fragments, they can be more fit than other AMF that require spore germination (such as *Gigaspora* sp). Alternatively, a predominance of *Glomus* species may be due to a higher sporulation rate (Daniell et al., 2001), favoring their survival in disturbed environments. Our results reinforce the notion that *Glomus* species are frequently found in TM polluted sites, indicating that they are tolerant to polluted environments.

The presence of diverse AMF communities within TM contaminated areas suggests these species can tolerate harsh metal stress. It was hypothesized that metal stress not only induces the disappearance of less tolerant AMF species, but also promotes species that are more tolerant (Del Val et al., 1999). This was strongly supported by our data, where seven different *Glomus* ribotypes were specifically detected in the rhizospere of plantains growing on TM contaminated sites but not found in uncontaminated sites, suggesting the ability of these species to tolerate the toxic effects of TM while being less competitive in uncontaminated conditions.

In this study, ribotypes resembling *G. mosseae* (B6 and B9) were the most dominant in the rhizosphere of plantain growing on Cd, Cu, Sn and Zn contaminated sites. *Glomus mosseae* is commonly found in soil contaminated with Zn and Pb (Turnau et al., 2001; Vallino et al., 2006; Zarei et al., 2008). Consequently, the dominance of *G. mosseae* in TM contaminated soils suggests a better tolerance of that species under TM pollution stress. In addition, ribotype B13 (identified as *Glomus* sp.) was also frequent in Pb contaminated sites, while ribotypes of *Glomus* spp. (B4, B10, and B12) were abundant in As contaminated sites. These results showed that TM pollution modified AMF diversity in roots and rhizospheric soil.

The *G. irregulare /G. intraradices* ribotype was not only the most frequent AMF ribotype detected in plantain roots growing on uncontaminated sites but was also observed within the rhizosphere of plantain plants growing on metal contaminated sites, suggesting its tolerance to a wide range of TM concentrations. The tolerance of *G. intraradices* to Zn, Pb, and Cd was previously examined (Pawlowska & Charvat 2004). Several biological

growth parameters such as spore germination, internal and external hyphal extension, and sporulation of *G. intraradices* were less affected by TM in this species than in other AMF species. *G. intraradices* is commonly found in diverse habitats including non-contaminated (Turnau et al., 2001) and moderately contaminated soil, but never in sites with high concentrations of TM (Zarei et al., 2008), suggesting a limited tolerance to TM toxicity. Wong et al., (2007) reported that *G. intraradices* was sensitive to Pb stress but tolerant to Zn. Colonization of the same ribotype in both contaminated and uncontaminated sites was also observed in the present study for other ribotypes such as different *Glomus* spp., *G. etunicatum*, and *G. viscosum*, suggesting a partial tolerance of these taxa to TM toxicity. However, five AMF ribotypes, identified as *S. calospora*, *S. gilmorei*, and several different *Glomus* ribotypes were found only in uncontaminated sites. This suggests a higher sensitivity of these taxa to metal stress but a lower competitivity in less contaminated soil.

As can be inferred from the phylogenetic tree (Fig. S2), the use of ribosomal markers to identify AMF taxa is not without problems. Ribosomal repeats in AMF lab cultures, so called 'isolates', show high levels of intra-isolate variation for the large subunit (LSU), the small subunit (SSU) and the internal transcribed spacer (ITS) regions both on a genomic level (Hijri et al., 1999; Kuhn et al., 2001) and, for LSU, in the transcriptome (Boon et al., 2010). Moreover, copy number variation has been demonstrated for the LSU and SSU between isolates of *G. irregulare* (Corradi et al., 2007). These properties of ribosomal variation in AMF have not been investigated exhaustively, but the data available so far shows that alleles are highly variable, with some alleles being more divergent within compared to between isolates from such distant locations such as Switzerland and Canada (Boon et al., 2010). In all, this means that a single allele cannot be representative of a taxon, and our assignment of ribotypes to particular species should be interpreted as an approximation that facilitates comparison to other ecological studies. There is a dire need for better molecular markers for AMF ecological studies. However, the lack of other nuclear or mitochondrial markers means the rRNA genes remain the best option to compare field samples inhabited with unknown AMF communities.

Usefulness of DGGE for molecular identification of AMF

Both cloning and DGGE were successful in detecting different AMF species, and the structural differences in AMF communities within roots and rhizospheric soils between TM contaminated and uncontaminated sites. DGGE proved to under-estimate species diversity compared with the cloning and sequencing approach. However, if a high number of samples need to be analyzed, such as in most environmental study, DGGE still remains a far more affordable method than cloning and sequencing and allows the identification of dominant ribotypes which are probably those having the largest effect on the associated plant. As noted here and previously (Öpik et al., 2003; Liang et al., 2008), both clones with high sequence similarity and clones displaying significant sequence divergence sometimes migrated to identical locations on the gel. To address this problem, the identification of AMF communities in our study was confirmed by the excision, reamplification, and sequencing of the original DGGE bands from different positions on the gel, rather than comparison of migration position with known reference sequences only. New affordable methods to rapidly and accurately assess AMF species complexity in high sample numbers are still needed, and cloning/sequencing as well as direct sequencing techniques appear to be viable options to complement DGGE before the cost of sequencing thousands of samples will continue to drop to very low levels.

Trace metal contamination and AMF diversity

Trace metal contamination reduced AMF diversity in polluted sites. Using multiple regression analysis, we showed that Ba, Co, Cd, Pb, Sn, and Zn concentrations negatively affected AMF ribotype richness and diversity indices in plantain rhizospheres. Our results are in agreement with Zarei et al., (2008) who found that a decrease in AMF spore numbers was associated with high concentrations of Pb and Zn within soil. Del Val et al. (1999) also found a significant decrease in AMF populations caused by an application of sludge containing high concentrations of TM, in particular Pb and Zn. Similarly, our results showed that AMF ribotype numbers in plantain roots growing on contaminated sites were lower than those of uncontaminated sites. Mean ribotype numbers were 1.78 in TM

contaminated soil, while in uncontaminated soil we found three ribotypes on average. Based on spore morphology, six unique AMF species were found in unpolluted soil in contrast to only two different species in Cd, Pb, and Zn contaminated sites (Pawlowska et al., 1997). The toxic effect of TM in soil was proposed to prevent various AMF species from colonizing root systems or propagating in the rhizosphere, causing a decrease in species richness in metal contaminated soil compared to uncontaminated soil (Del Val et al., 1999).

The presence of diverse AMF in the roots and associated soil of plantain plants on TM contaminated land might be due to their tolerance to polluted environments. AMF might not only tolerate TM toxicity but also help their host plant to tolerate and establish themselves in TM contaminated soil (Hall 2002). Furthermore, species isolated from TM contaminated sites have shown a higher capacity to take up or sequester TM than those isolated from uncontaminated sites (Kaldorf et al., 1999; Orlowska et al., 2005; Sudová et al., 2008). Consequently, accurate identification of AMF taxa or strains found in the rhizosphere of plants growing on TM contaminated sites is an important step toward improving bioremediation techniques.

Conclusion

Trace-metal contamination is one of the environmental factors that influence and modify AMF community structure in the plant rhizosphere. Although TM contamination reduced AMF diversity in the rhizosphere communities examined here, it did not completely inhibit growth or establishment of mycorrhizae. Furthermore, the presence of various AMF in the roots and associated soil of plants growing on TM contaminated sites suggests that AMF diversity contributes a critical functional component in disrupted environments. The predominance of *G. mosseae* in TM polluted sites suggests the tolerance of this taxon to TM stress. Therefore, understanding the capacity of *G. mosseae* regarding TM uptake or immobilization would be an important aspect of phytoremediation. Indeed, *G. mosseae* could prove to be a powerful tool to improve phytostabilization technology (i.e, to prevent the spread and leakage of TM into the soil environment or underground water).

G. irregulare/G. intraradices was broadly found in diverse habitats including TM polluted soil suggesting the wide tolerance of this species to TM toxicity and thereby the useful application of this species in phytoremediation.

Acknowledgments

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CHAPTER IV

Impact of long-term manure and inorganic nitrogen fertilization on the community structure of arbuscular mycorrhizal fungi

This chapter will be submitted to an international Journal.

Also, it was presented in the following confereces:

- Hassan SE, M Hijri, A Liu, T Forge, M St-Arnaud 2009 Impact of long-term manure and inorganic nitrogen fertilization on the community composition of arbuscular mycorrhizal fungi using PCR-DGGE analysis. pp. 30 In Abstracts of the 6th International Conference on Mycorrhiza, August 9-14 2009, Belo Horizonte, Brazil.
- Hassan SE, M St-Arnaud, M Hijri 2010 The long-term effect of manure and inorganic nitrogen fertilization on the biodiversity of arbuscular mycorrhizal fungi. In 10th Agricultural Biotechnology International Conference (ABIC) 2010 Conference, Sept. 12–15, Saskatoon, Canada.

Preface

In the previous chapter the identification of AMF community composition was determined from metal contaminated and non contaminated areas. Identification of AMF community compositions from metal contaminated sites is an essential step to develop mycorrhizal inoculation to sustain practices of phytoremediation. Since the long-term use of nitrogen (N) fertilizers has caused metal contamination of agricultural soil and water, trace metals entrance in food chain, and AMF biodiversity change. To date, little is known about the biodiversity of these fungi under the overuse of N-fertilizer. Thus, in this chapter, the effect of N-fertilization on AMF community composition was achieved to investigate the ecological importance of mycorrhizal inoculations and N-fertilizers interaction. In addition, identification of AMF community compositions under N-fertilization is an essential purpose to develop mycorrhizal inoculation to improve the use efficiency of N-fertilizer, and to minimize soil metal pollution that resulted from the overuse of these fertilizers.

Abstract

The long-term effect of manure or mineral NH_4 fertilizer application on the arbuscular mycorrhizal (AM) fungal community structure was analysed in a pot experiment. Soil and grass roots were harvested from a forage field experiment treated for 12 yrs with equivalent doses of (i) inorganic nitrogen (NH_4) or (ii) dairy manure slurry (manure) or (iii) no N fertilization (FertCtrl). Sunflower plants were sown in this soil and submitted to three mycorrhizal inoculum treatments: (i) high level of native AMF inoculum (MycHigh), consisting of unfumigated field soil + mycorrhizal grass roots, (ii) low AMF inoculum level (MycLow), consisting of fumigated field soil + mycorrhizal grass roots, or (iii) no AMF control (MycCtrl), consisting of fumigated field soil + sterilized mycorrhizal grass roots. Four months after sowing, roots and rhizosphere soil were separately harvested and the total genomic DNA was directly extracted from samples and subjected to PCR-DGGE and sequencing approaches targeting an 18S rRNA gene fragment. Twelve AM fungal ribotypes were detected within roots or soil and were identified as different *Glomus*

spp. and *Acaulospora* spp. Under high inoculum level, the percentage of root length bearing mycorrhizal colonization was significantly higher in plants grown in soil fertilized with NH_4 or manure than in soil from plots with no N-fertilization. However, under low inoculum level, root colonization was significantly higher in manure than other treatments. Plant biomass was significantly higher in plants grown in manure-fertilized soil compared to NH_4 and no N fertilization treatments. In general, plant biomass was also significantly higher under low AM inoculum and no AM inoculation than under high AM inoculation level, while, there was no significant difference between low AM inoculum and no inoculation treatments. In manure-fertilized soil, plant biomass was significantly higher under low-inoculum than high inoculum treatment; however, there was no significant difference in plant biomass under low-inoculum or no inoculation treatments. However, the low inoculum or no inoculation treatments caused a significant increase in biomass of plants grown in soil with no fertilization or fertilized with NH_4 compared with plants grown in the high inoculum treatment. In plant roots inoculated with the higher inoculum dose, we found that *Glomus* spp. ribotypes B9 and B10 were the most frequent taxa in plants grown in unfertilized soil, B2, B7, and B8 in manure-fertilized soil, and B7 and B8 in NH_4-fertilized soil. Additionally, under the lower inoculum dose, *Glomus* spp. ribotypes B1, B9, and B10 were abundant in unfertilized and NH_4-fertilized plant roots, while *G. intraradices*/ *G. irregulare* ribotype B2 was the most frequent taxa in roots of manure-fertilized plants. Our results showed that the manure-fertilized soil produced the highest increase in plant biomass under low AM inoculum level, and that *G. intraradices*/\ *G. irregulare* was the most frequently detected AM fungal taxon under these conditions.

Introduction

Nitrogen (N) fertilizers are applied to increase soil fertility and crop production. However, the intensive use of N-fertilization causes many detrimental effects to the environment. For instance, long-term use of N-fertilizers result in increasing the soil content of phosphorus (P) and N into the environment, and in extreme cases causes N and P pollution (Gyaneshwar et al., 2002; Sharpley et al., 2003). Other environmental problems coincide with the overuse of N-fertilization, including changes in soil pH and increased salt concentration, production of greenhouse gases, global warming and acid rain, and reduction of both plant and soil biodiversity (Adesemoye and Kloepper, 2009). Moreover, different mineral N-fertilizers may contain trace metals and affect the soil metal concentration (Rui et al., 2008); for example, the long-term use of mineral N-fertilizers was shown to result in an increase in Cd concentration in soil and wheat grains (Wångstrand et al., 2007). Additionally, since organic manure may also contain different metals, its use can cause trace metal pollution of soil and water (Long et al., 2004; Qureshi et al., 2008).

The long-term use of N-fertilizers also affects the biodiversity of aboveground and underground ecosystems. N-fertilization was shown to reduce plant biodiversity (Bobbink, 1991; Fenn et al., 1998), and to cause a shift and reduction of the soil bacterial and arbuscular mycorrhizal fungi (AMF) community structures (Toljander et al., 2008). Mycorrhizal root colonization, AMF sporulation, and AMF community structure were all recorded to be influenced by N-fertilization (Egerton-Warburton and Allen, 2000; Santos et al., 2006).

Arbuscular mycorrhizal fungi are ubiquitous soil microorganisms and colonize the roots of most terrestrial plants in nearly all ecosystems (Smith and Read, 2008). AMF exert an important ecological role in the nutrient supply to their hosts, in particular phosphorus, nitrogen, many micronutrients, other immobile molecules, and water, and reduce the root pathogen infections, as well as affect plant growth, productivity, and diversity (van der Heijden et al., 1998; Vivas et al., 2006; St-Arnaud and Vujanovic, 2007; Smith and Read, 2008). AMF are inhabitants of most climates, resisting harsh conditions (Chaudhry and Khan, 2002), including trace metal contaminated soils and long-term N- or P-fertilized soils

(Beauregard et al., 2010; Bhadalung et al., 2005; Vallino et al., 2006; Zarei et al., 2008; Wu et al., 2010).

One important goal of modern agriculture is to decrease the harmful effects of N-fertilization while maintaining crop productivity (Adesemoye and Kloepper, 2009). Management of AMF communities is one way to achieve this goal since they affect nutrient uptake and plant growth, and was show to influence N plant nutrition (Mäder et al., 2000; Cruz et al., 2004; Adesemoye and Kloepper, 2009). AMF may increase fertilization efficiency, reduce effective fertilizer doses, and therefore reduce the harmful effects of long-term N-fertilization. However, some AMF species are more sensitive to N-fertilization than others; in addition, AMF species isolated from N-fertilized or unfertilized soil differentially affected plant growth in response to N-fertilization (Johnson, 1993; Bhadalung et al., 2005). N-fertilization selects the AMF species most tolerant to these conditions (Johnson et al., 2003). Thus, monitoring the native AMF communities under long-term N-fertilization regimes and identifying species or isolates with high potential to increase plant productivity, appear to be important factors to improve N-fertilization efficiency.

The first objective of this study was therefore to compare the effect of the long term use of organic (manure) and mineral (NH_4) N-fertilization on AMF community structure and mycorrhizal colonization of sunflower plant roots and rhizosphere soil. Secondly, we aimed to evaluate the impact of different levels of mycorrhizal inoculum on plant growth under these soil fertility conditions, to examine whether changes in AMF community impact plant growth in response to different N-fertilization regimes. Finally, we aimed to identify AMF taxa associated with increased plant productivity under the application of different forms of N-fertilizers.

Materials and Methods

Experimental setup

A greenhouse experiment was conducted using a 3×3 factorial scheme in a completely randomized design, with the following factors: three long-term N-fertilized soil levels: NH_4-fertilized soil (FertInor), dairy manure slurry-fertilized soil (FertOrga), or unfertilized control soil (FertCtrl), and three AMF inocula levels: high native AMF inoculum level (MycHigh), low AMF inoculum level (MycLow), and no AMF inoculation (MycCtrl), with six replecates. Thus, there are 54 pots in the experiment which were seeded with sunflower seeds. After germination, four plants were kept in each pot (one plant in the center of the pot, and three others at four cm from the pot edge, forming a triangle around the central plant). Sunflower seeds were germinated for 3 days before planting. Pots were placed in a greenhouse under full sun supplemented with mercury-vapor lamps, with a day/night regime of 16h/8h at a temperature of 20-22 ºC.

Long-term N-fertilized soil and AMF inocula preparation

Soil was collected from a forage field experiment treated for 12 yrs with N-equivalent doses of (i) NH_4-fertilizer (FertInor), applied at a rate of 100 kg N/ha, four times/year, plus P and other nutrients once per year or (ii) dairy manure slurry (FertOrga), applied at a rate equivalent to 100 kg N/ha, four times/year, P and other nutrients once per year or (iii) with no fertilization (FertCtrl). Soil analysis is given in Table 1. Each soil was split into three parts: two parts were fumigated with Basamid® at a rate of 1 g/kg soil in plastic bags and then left to vent to remove the remaining gas, while the third soil part use not fumigated.

Roots of grasses growing in the same fields were sampled, chopped in small fragments, and autoclaved or not; 100 g were mixed with the soil withing each pot in order to increase the AMF inoculum level. There were three mycorrhizal inoculum levels: (i) high level of native AMF inoculum (MycHigh), consisting of unfumigated soil complemented with unsterilized grass roots, (ii) low AMF inoculum level (MycLow),

consisting of fumigated soil plus unsterilized grass roots, or (iii) no AMF inoculation (MycCtrl), consisting of fumigated soil plus sterilized grass roots.

Plant tissues and soil analyses

Shoots and roots were separately harvested four months after sowing. Roots were carefully washed under tap water to remove soil particles, cut into 1-cm segments and mixed in water. A first subsample from each pot was stored in 50% ethanol for mycorrhizal root colonization assessment. A second root subsample from each pot was froozen at -20°C until DNA extraction. Fresh weights of each plant tissue were estimated before the tissues were oven dried for 48h at 60 ºC to determine dry weights. The rhizosphere soil was harvested from each pot and stored at -20 °C until DNA extraction.

To determine mycorrhizal root colonization, roots were cut into 1-cm segments, cleared in 10% KOH (10 min at 100 ºC), and stained in Schaeffer black ink in a 5% vinegar solution (3 min at 100 ºC) (Vierheilig et al., 1998). Mycorrhizal root colonization percentages were assessed at 20-50× magnification using the gridline intercept method (Giovannetti and Mosse, 1980).

DNA extraction and PCR amplification

DNA was extracted from the root samples using the UltraClean microbial DNA isolation kit, and from soil samples using the UltraClean soil DNA isolation kit (MoBio Laboratories), following manufacturer's instructions except that all samples were crushed using a FastPrep^{TM} FP120 machine (MP Biomedicals), using Lysing Matrix A tubes at speed level 4, 3 times for 20 sec each.

Nested-PCR was performed to amplify 18S rRNA gene fragments of AMF from root and soil samples as described in Yergeau et al. (2006). The first PCR round was done using the primer pair NS1 and NS41 (White et al., 1990) to amplify an approximately 1.2 Kb fragment. The PCR mixture contained: 1×PCR buffer, 0.5 mM of $MgCl_2$, 5 U Taq DNA polymerase (Qiagen), 0.25 mM dNTP, 0.5 µM NS1, 0.5 µM NS41, 0.5 µl Tween 1%, 1 µl DMSO, 0.125 µl bovine serum albumin (BSA), and 1 µl of extracted genomic DNA

(diluted 1:100) in a PCR volume of 25 µl. The PCR cycling conditions were one cycle at 95°C for 3 min, followed by 35× (94°C, 1 min; 50°C, 1 min; 72°C, 1 min) and a final extension at 72°C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis to confirm the amplification of a DNA fragment of the targeted length. Products of the first PCR round were diluted to 1:100 and used as template in subsequent nested PCR. The primer set for the second round was AM1 (Helgason et al., 1998) and NS31-GC (Kowalchuk et al., 2002). The second PCR round was conducted in a 25 µl volume in the following mixture: 1×PCR buffer, 5 U Taq DNA polymerase (Qiagen), 0.25 mM dNTP, 0.5 µM AM1, 0.5 µM NS31-GC and 1 µl of the diluted PCR products. PCR conditions were one cycle at 94°C for 3 min, followed with 30× (94°C, 45 s; 58°C, 45 s; 72°C, 45 s), and a final extension step at 72°C for 10 min. PCR products of the second round were analyzed in 1% agarose gel electrophoresis and then subjected to DGGE analysis as described below.

DGGE analysis

Using a DCode Universal Mutation Detection System (Bio-Rad), 10 µl of the second round PCR products of each of the root and rhizosphere soil samples were analyzed. DGGE analyses were conducted in 1× TAE buffer at a constant temperature of 60°C at 80 V for 20 min followed by 45 V for 17 h on a 6% (w/v) polyacrylamide gel (40% acrylamide/bis-acrylamide) with a 38-50% denaturant gradient (100% denaturant corresponding to 7 M urea and 40% (v/v) formamide). Gels were stained in a 1:10,000 SYBR gold solution for 15 min and visualized under UV illumination. Gel pictures were digitalized using an imaging system (GelDoc, Bio-Rad Laboratories).

Sequencing of DGGE bands

Three to five clear DGGE bands from each different migration positions were excised from UV illuminated acrylamide gels and DNA was eluted from bands in 30 µL ddH_2O at room temperature for 16 h. One microlitre of eluted DNA was used as a template for PCR amplification. PCR conditions and mixture were the same as described above for

the second PCR round, except that the number of cycles was reduced to 25. PCR products were run on DGGE gels using a 35%-45% denaturing range. When single bands appeared in each lane on the DGGE pattern, these individual bands were excised from the gel and their DNA extracted and amplified with primer set AM1/NS31 (without GC-clamp). The PCR products were sequenced at the Genome Quebec Innovation Center facility (Montreal, Canada) with the AM1 primer.

Sequence analysis and AMF ribotype identification

Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) through the NCBI GenBank database, and using the MEGA4 software (Tamura et al., 2007). A distance analysis was performed using the neighbor-joining method (Saitou and Nei, 1987) of Kimura-2-parameter (Killham and Firestone, 1983), with 1000 Bootstrap replicates.

Statistical analysis

The effect of mycorrhizal and N-fertilization treatments on plant fresh and dry weights and the mycorrhizal root colonization percentage were analyzed by two-way analysis of variance (ANOVA). The data were then subjected to one-way ANOVA within each mycorrhizal or N-fertilization levels. Post-hoc comparisons of means were determined using the Tukey's HSD test ($P \leq 0.05$). All statistical analyses were performed using the SPSS software v. 17 (SPSS Inc., Chicago, Illinois).

Bands that migrated to different positions were considered different ribotypes. The presence of each ribotype was determined and coded in a presence–absence matrix for statistical analyses. The Shannon-Weaver diversity index (H') (Rosenzweig, 1995) was calculated to compare AM fungal ribotypes diversity between treatments. Diversity indices were calculated based on the number of observed DGGE band positions, where each unique DGGE band position represented a particular ribotype. The Shannon-Weaver index was used as a diversity index and was calculated as follows:

$$H' = -\Sigma pi \ln pi$$

where the summation is over all unique bands *i*, and *pi* is the proportion of an individual band relative to the sum of all band positions (i.e., total number of bands).

Analysis of variance was used to examine the significant differences in species richness (number of AM fungal ribotypes detected on DGGE) and Shannon diversity indices between different treatments, and post-hoc comparisons between the treatments were done using the Tukey's HSD test. Discriminant analysis (DA) was used to test for significant differences in AMF communities between different mycorrhizal and N-fertilization levels using a Fisher test of the Mahalanobis distances in XLSTAT v. 5.01 (Addinsoft Inc., Paris, France). Canonical correspondence analyses (CCA) were performed on the AMF ribotypes presence/absence matrix of DGGE banding patterns to test the significance of relationships between the different treatments and AMF community compositions with permutation test (n = 1000) using XLSTAT v. 5.01.

Results

Plant growth and mycorrhizal root colonization

There was a significant effect of N-fertilization and mycorrhizal inoculation treatments on plant biomass ($P<0.001$ and $P<0.01$, respectively), while no significant interaction between treatments was found for plant tissue biomass (Tables 2 and 3).

Regardless mycorrhizal inoculation treatment, the FertOrga fertilization significantly increased plant biomass compared to FertInor and FertCtrl treatments. No significant variation in plant biomass was observed between FertCtrl and FertInor treatments. Plants inoculated with MycHigh had significantly lower dry tissue biomass than plants grown with MycCtrl and MycLow mycorrhizal treatments, while plants inoculated with MycCtrl or MycLow mycorrhizal treatments had similar dry plant biomass.

Both N-fertilization and mycorrhizal inoculation treatments had a significant ($P<0.001$) effect on root colonization (Table 2 and 3). A significant interaction ($P<0.05$) was also found between N-fertilization and mycorrhizal inoculation treatments on root colonization. All plants grown in the non-inoculated treatment showed no mycorrhizal colonization or only colonization traces (<1%). Under the MycLow treatment, plants grown in unfertilized soil or in soil fertilized with NH_4 had similar mycorrhizal root colonization extent, while root colonization of plants grown in the manure-fertilized soil was significantly increased (by a 3-fold magnitude). However, in plants grown in MycHigh treatment, no significant differences in root colonization levels was found between the manure and NH_4 fertilization treatments, but plants grown in unfertilized soil had a significantly lower root colonization percentage. In unfertilized soil, plants inoculated with the highest dose had significantly higher root colonization (2×) than plants inoculated with the lower dose. Similarly, in NH_4-fertilized soil, plants inoculated with the higher dose had significantly (2.8×) higher root colonization extent than plants grown with lower dose. However, in manure-fertilized soil, no significant effect on root colonization was found between plants inoculated with the two inoculum treatments.

AMF diversity indices and species richness

A significant effect (P=0.047) of N-fertilization on AMF diversity indices in roots was noted, but no effect of mycorrhizal inoculation was found (Table 2). The AMF diversity indices in roots was marginally increased (P= 0.052) in the FertInor compared to FertOrga treatment, but no difference between the FertCtrl and FertInor or FertOrga treatments was found. the AM species richness in roots was not modified by any treatment. However, there were marginally significant interactions of mycorrhizal and N-fertilization treatments on AMF diversity indices (P=0.062) and species richness (P=0.07). No difference in AMF diversity indices and species richness in soil was observed between the different fertilization and inoculation treatments.

AMF community structure

The nested PCR and DGGE analysis of 18S rRNA gene fragments allowed us to detect AMF community structure defferences in roots and rhizosphere soils of sunflower plants grown in different N-fertilization and mycorrhizal inoculation treatments. DGGE banding profiles are shown in Figure 1. As expected, no AMF ribotypes was detected from roots and soil samples from fumigated soil inoculated with MycCtrl inoculum (FertCtrl/MycCtrl, FertOrga/MycCtrl, and FertInor/MycCtrl). Twelve bands were identified as different AM fungal ribotypes (Table 4). These ribotypes belong to the families *Glomaceae* and *Acaulosporaceae* (Fig. 4). Sequence homology showed that ten AM fungal ribotypes were affiliated with to *Glomaceae*, as supported by bootstrap values higher than 85%, while twoAMF ribotypes clustered within *Acaulosporaceae*, with a bootstrap value of 99%. The excised DGGE bands which migrated to the lower part of the gel (Fig. 1) all belong to non-AMF sequences and showed high homology to Ascomycetes and Basidiomycetes taxa (data not shown); these sequences were excluded from the multivariate analyses.

In plant roots inoculated with the highest dose, six, eight, and seven AMF ribotypes were found in FertCtrl, FertOrga and FertInor treatments, respectively. In plants inoculated with the lower dose, six, five, and four bands were recovered from FertCtrl, FertOrga and

FertInor treatments. The most abundant AMF ribotypes in roots of the higher inoculum dose with no fertilization (FertCtrl/MycHigh) were B9 and B10 (96% - 97% similarity to *Glomus* spp.) which were observed in 66.7% of roots (Table 4). AMF ribotypes B2 (99% similarity to *G. intraradices*/ *G. irregulare*), B7, and B8 (99% similarity to *Glomus* spp.) were detected of 50% in roots harvested from higher inoculum dose fertilized with manure (FertOrga/MycHigh), while B7 and B8 were found in 66% and 83% of plant roots grown in the higher inoculum/NH_4-fertilization treatment (FertInor/MycHigh). In addition, B9 was the most frequent AMF ribotype found in 83% of roots collected from the low-inoculum/no fertilization treatment (FertCtrl/MycLow), and B2 was the most observed ribotype in roots (83.3%) of low-inoculum/manure treatment (MycLow/FertOrga), while ribotypes B1 (87% similarity to *G. etunicatum*), B9, and B10 were recovered from all root samples harvested from the low-inoculum/NH4-fertilization treatment (FertInor/MycLow). On the other hand, in soil inoculated with the highest dose, ribotypes B7 and B8 were found in all samples from manure and NH_4-fertilization treatments, and 50% of unfertilized soil samples, while B9 and B10 were frequent in 50% of samples from the unfertilized soil.

In root samples, DA analysis showed a significant difference ($P<0.001$) in AMF community structure between plants grown in FertOrga/MycLow and plants from all other treatments, as shown by the separation of this treatment in the lower left quadrant of the ordination (Fig. 2A). Mahalanobis distances analysis also showed that AMF community structure in roots of FertInor/MycLow and FertCtrl/MycLow were similar ($P=0.7$), but that these communities were significantly different ($P<0.05$) from the remaining treatments. Further, AM fungal community of the FertCtrl/MycHigh treatment in roots was significantly different ($P<0.01$) from all other treatments, and clustered in the lower right part of DA ordination. However, no difference ($P=0.07$) occurred in AMF community structures of MycHigh/FertInor and FertOrga/MycHigh treatments, while Mahalanobis distances showed a significant difference ($P<0.05$) between these treatments and the other treatments. In contrast, DA analysis did not reveal any significant difference ($P=0.357$) in AMF community structure in non-fumigated soils fertilized with manure, NH_4 or unfertilized (Fig 2B).

The relationship between AMF ribotypes in roots and the different treatments of N-fertilization and mycorrhizal inoculation was also investigated using CCA. By that analysis, a significant effect ($F=1.4$, $P<0.01$) of mycorrhizal and N-fertilization treatments on the root-colonizing AMF community structure was found (Fig. 3A). The mycorrhizal inoculation treatments had a greater influence on AMF community structure than N-fertilization treatments, as shown by the length of the vectors. The first two axes described 88.7% of the cumulative difference in AMF ribotypes dataset, and showed 60.8% and 28% of the variation in AMF community structure, respectively. CCA ordination showed that AMF ribotypes B2, B5, B7, and B8 were more associated with manure-fertilized plants at the highest inoculum level, while B1, B4 B9, and B10 were more linked to unfertilized or NH_4-fertilized plants at the lower inoculum level. Similarly to DA analysis, CCA did not reveal any significant modification of AMF ribotypes in soils ($F=0.517$, $P=0.198$) (Fig. 3B).

Table 1: Characteristics of soils harvested from a forage field experiment treated for 12 yrs with no fertilization (FertCtrl), dairy manure slurry (FertOrga), or NH_4-fertilizer (FertInor).

Parameter	Soil analysis		
	FertCtrl	FertOrga	FertInor
pH	6.1	6.3	5.8
CEC (mEq per 100 g)	19.9	22.7	20.3
Organic matter (%)	7.2	7.8	6.6
P[a] (mg kg^{-1})	134.7	217.2	141.8
K[a] (mg kg^{-1})	39.2	281	38.3
Mg[a] (mg kg^{-1})	94.5	280	79.4
Ca[a] (mg kg^{-1})	1271.1	1735	923.2
Al[a] (mg kg^{-1})	1870	1690	1920
Saturation P (%)	7.2	12.9	7.4
Saturation K (%)	0.5	3.2	0.5
Saturation Mg (%)	4	10.3	3.3
Saturation Ca (%)	31.4	38.2	22.8
Saturation K+Mg+Ca (%)	36.4	51.7	26.5

[a] Mehlich-3 extractions

Table 2: Effects of the N-fertilization and mycorrhizal inoculation treatments on plant biomass, mycorrhizal root colonization percentages, and AMF diversity and species richness, based on factorial ANOVA.

	P-values[1]		
	N-fertilization	AMF inoculation	Fert*inoc interaction
Dry shoot weight	>0.001	>0.001	0.550
Dry root weight	>0.001	0.009	0.736
Total dry plant weight	>0.001	>0.001	0.647
Mycorrhizal root colonization	>0.001	>0.001	>0.001
AM diversity index in roots	0.047	0.932	0.062
AM species richness in roots	0.070	0.783	0.070

[1] ns: not significant, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$; (n =6).

Table 3: Effect of N-fertilization and mycorrhizal inoculation treatments on sunflower plant biomass and root colonization percentages.

Mycorrhizal treatment	Dry shoot weight			
	Fertilization treatment			
	FertCtrl	FertOrga	FertInor	Mean
MycCtrl	69.7	103.4	62.1	78.3Y
MycLow	71.1	103.1	64.9	79.6Y
MycHigh	31.0	59.1	30.2	40.1X
Mean	57.2a	88.5b	52.4a	
	Dry root weight			
	Fertilization treatment			
	FertCtrl	FertOrga	FertInor	Mean
MycCtrl	3.9	6.7	3.3	4.6X
MycLow	3.4	6.4	3.6	4.5X
MycHigh	2.2	5.9	2.3	3.5X
Mean	3.1a	6.3b	3.1a	
	Total dry plant weight			
	Fertilization treatment			
	FertCtrl	FertOrga	FertInor	Mean
MycCtrl	73.6	110.1	65.4	83.0Y
MycLow	74.5	109.6	68.6	84.2Y
MycHigh	33.1	65.1	32.6	43.6X
Mean	60.4a	94.9b	55.5a	
	Root colonization percentages			
	Fertilization treatment			
	FertCtrl	FertOrga	FertInor	Mean
MycCtrl	0.95aX	0.88aX	00aX	0.61
MycLow	21.7aY	61.5bY	25.9aY	36.3
MycHigh	53.1aZ	67.8bY	70.5bZ	63.8
Mean	25.2	43.4	32.1	

[a] Means within rows followed by the same small letter are not significantly different by one-way ANOVA ($P<0.05$).

[b] Means within columns followed by the same capital letter are not significantly different by one-way ANOVA ($P<0.05$).

[c] FertCtrl: no N-fertilization; FertOrga: dairy manure slurry; FertInor: NH_4-fertilizer; MycHigh: high level of native AM inoculum; MycLow: low AM inoculum level; MycCtrl: no AMF inoculation.

Table 4: Arbuscular mycorrhizal fungi taxa identified from roots and rhizospere soil of sunflower plants submitted to different N-fertilization and AMF inoculum treatments, and their detection frequency, as revealed by DGGE analysis.

Band [a]	Most affiliated reference isolate from GenBank (% BLAST sequence similarity)	Accession numbers	Detection frequency (%) [c]								
			Roots						Soils		
			FertCtrl/ MycHigh	FertOrga/ MycHigh	FertInor/ MycHigh	FertCtrl/ MycLow	FertOrga/ MycLow	FertInor/ MycLow	FertCtrl/ MycHigh	FertOrga/ MycHigh	FertInor/ MycHigh
B1 [b]	*G. etunicatum* (87)	AJ852598, FJ831640, EU340319	0	16	0	66.7	16.7	100	0	0	0
B2 [b]	*G. intraradices*\ *G. irregulare* (99)	EU232660, EU232659, FJ009617, FJ009617	16.7	50	33.3	0	83.3	16.7	0	25	25
B3 [b]	*Glomus* sp (99)	GQ140610	0	0	16.7	16.7	33.3	0	0	0	0
B4 [b]	*Glomus* sp (99)	GQ140610	0	0	16.7	16.7	0	0	0	0	0
B5	*Glomus* sp (99)	HM122275, EU573765	33.3	33.3	0	0	0	0	0	0	0
B6	*Glomus* sp (99)	HM122275, EU573765	0	16.7	0	0	0	0	0	0	0
B7	*Glomus* sp (99)	EU368274, FM956703, FM956686	0	50	66.7	0	0	0	50	100	100
B8	*Glomus* sp (99)	FM956703	33.3	50	83.3	16.7	0	0	50	100	100
B9 [b]	*Glomus* sp (96)	EF041097, EF041096, EF041095	66.7	16.7	16.7	83	16.7	100	50	0	0
B10 [b]	*Glomus* sp (97)	EF041097	66.7	16.7	16.7	66	16.7	100	50	0	0
B11 [b]	*Acaulospora* sp (99)	EU368247, EU123381	33.3	0	0		0	0	0	0	0
B12 [b]	*Acaulospora* sp (84)	AF485885	0	0	0	0%	0	0	0	0	0

[a] band positions are labelled in Fig 1.

[b] bands were found in AMF inoculum.

[c] unfertilized /high-inoculum (FertCtrl/MycHigh), unfertilized/low-inoculum (FertCtrl/MycLow), manure-fertilization/high-inoculum (FertOrga/MycHigh), manure-fertilization/low-inoculum (FertOrga/MycLow), NH_4-fertilization/high-inoculum (FertInor/MycHigh), NH_4-fertilization/low-inoculum (FertInor/MycLow)

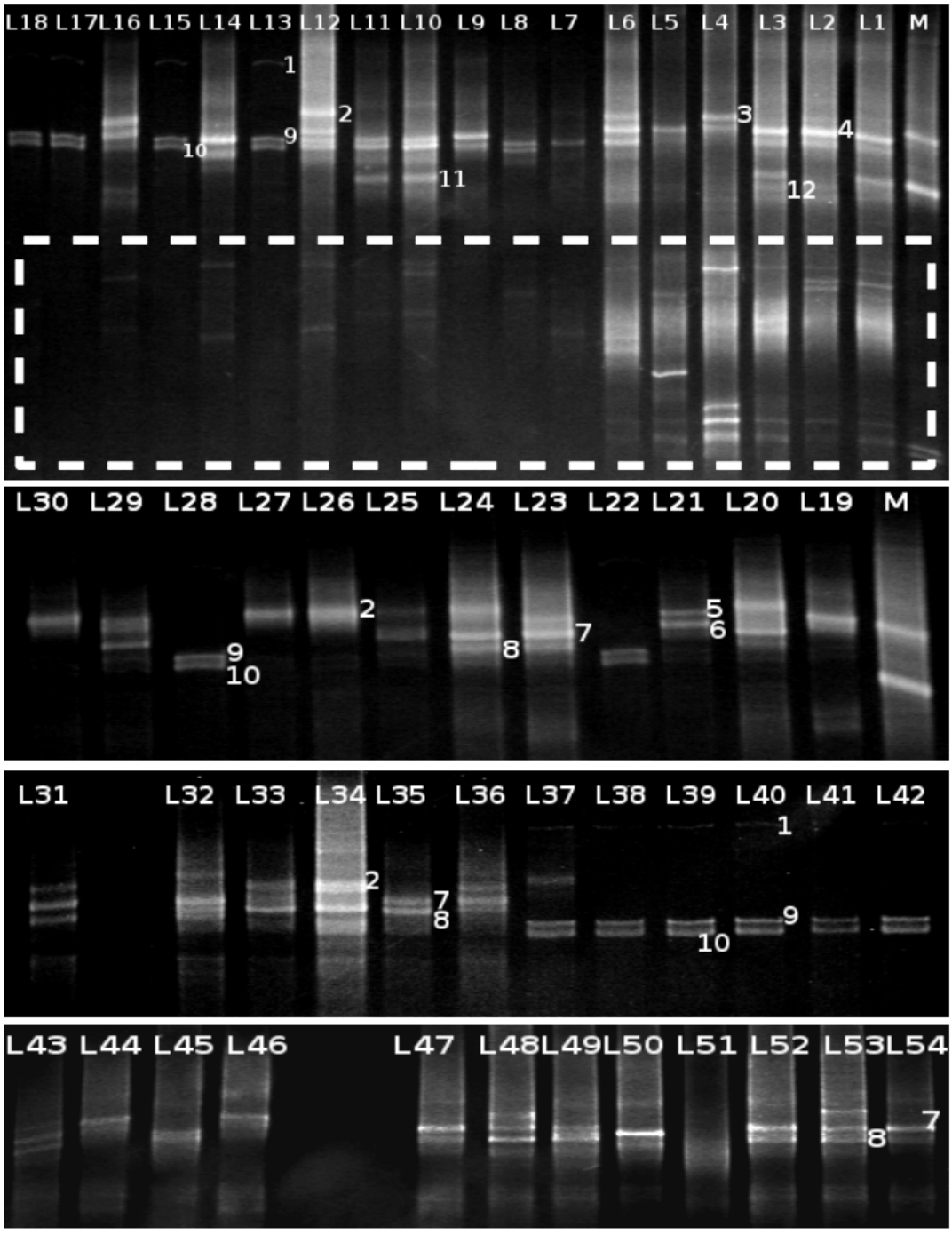

Figure 1. DGGE patterns of partial 18S rRNA gene amplified from roots and soil samples of sunflower plants grown in different N-fertilization and mycorrhizal inoculation treatments. Six replicates were analysed from each treatment. M, marker; lanes L1 to L42 are from root samples and L43 to L54 are from soil samples. Lanes: L1 to L6 are the grass roots used as part of the AMF inoculum; L7 to L12 and L43 to L46 are samples from the

FertCtrl/MycHigh; L13 to L18 are samples of FertCtrl/MycLow; L19 to L24 and L47 to L50 are samples of FertOrga/MycHigh; L25 to L30 are samples of FertOrga/MycLow; L31 to L36 and L51 to L 54 are samples of FertInor/MycHigh; L37 to L42 are samples of FertInor/MycLow. Samples from the uninoculated treatments are not shown since no AMF were detected. Bands numbering refers to AMF ribotypes identification given in Table 4. White box denote the bands corresponding to non-AMF ribotypes. Unfertilized / high-inoculum (FertCtrl/MycHigh), unfertilized / low-inoculum (FertCtrl/MycLow), manure-fertilization / high-inoculum (FertOrga/MycHigh), manure-fertilization /low-inoculum (FertOrga/MycLow), NH_4-fertilization /high-inoculum (FertInor/MycHigh), NH_4-fertilization /low-inoculum (FertInor/MycLow).

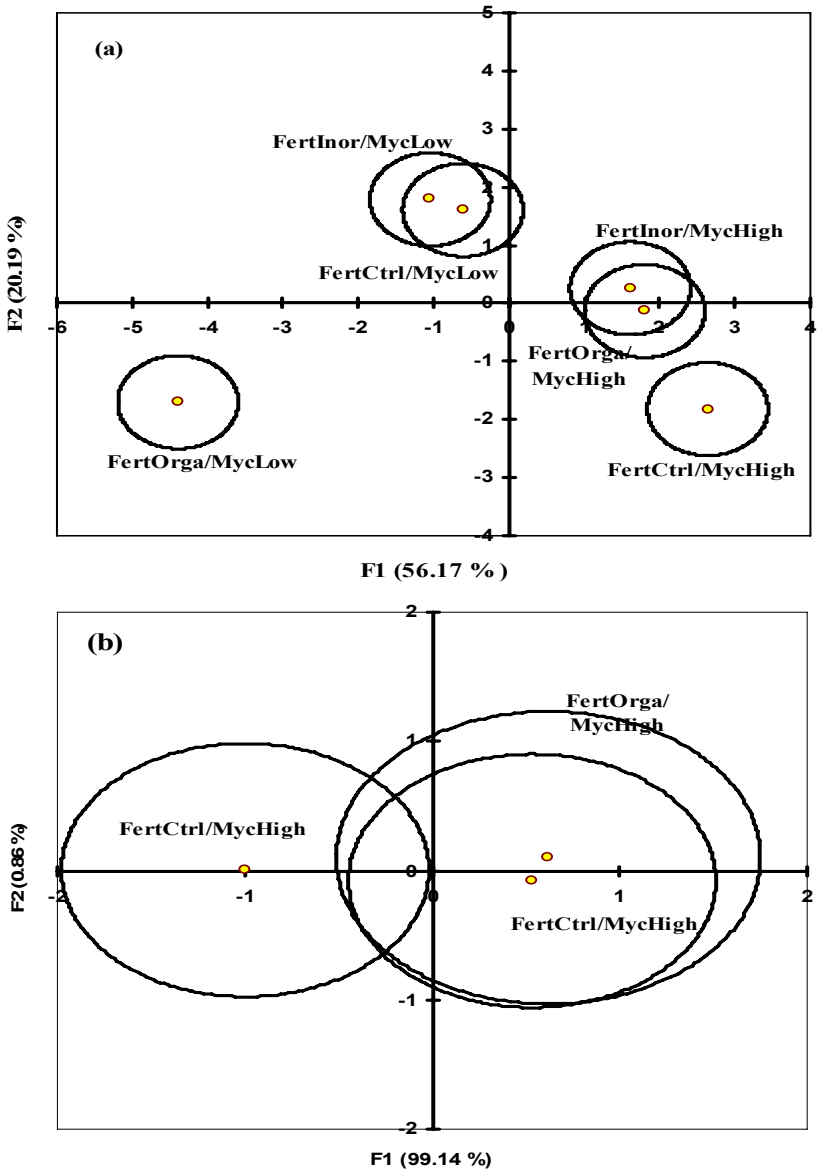

Figure 2. Discriminant analysis (DA) showing the relationship between AMF ribotypes and the different treatments. A. DA of AMF community structure within roots samples. B. DA of AMF community structure within soil samples. Unfertilized /high-inoculum (FertCtrl/MycHigh), unfertilized /low-inoculum (FertCtrl/MycLow), manure-fertilization /high-inoculum (FertOrga/MycHigh), manure-fertilization /low-inoculum (FertOrga /MycLow), NH_4-fertilization /high-inoculum (FertInor/MycHigh), NH_4-fertilization /low-inoculum (FertInor/MycLow).

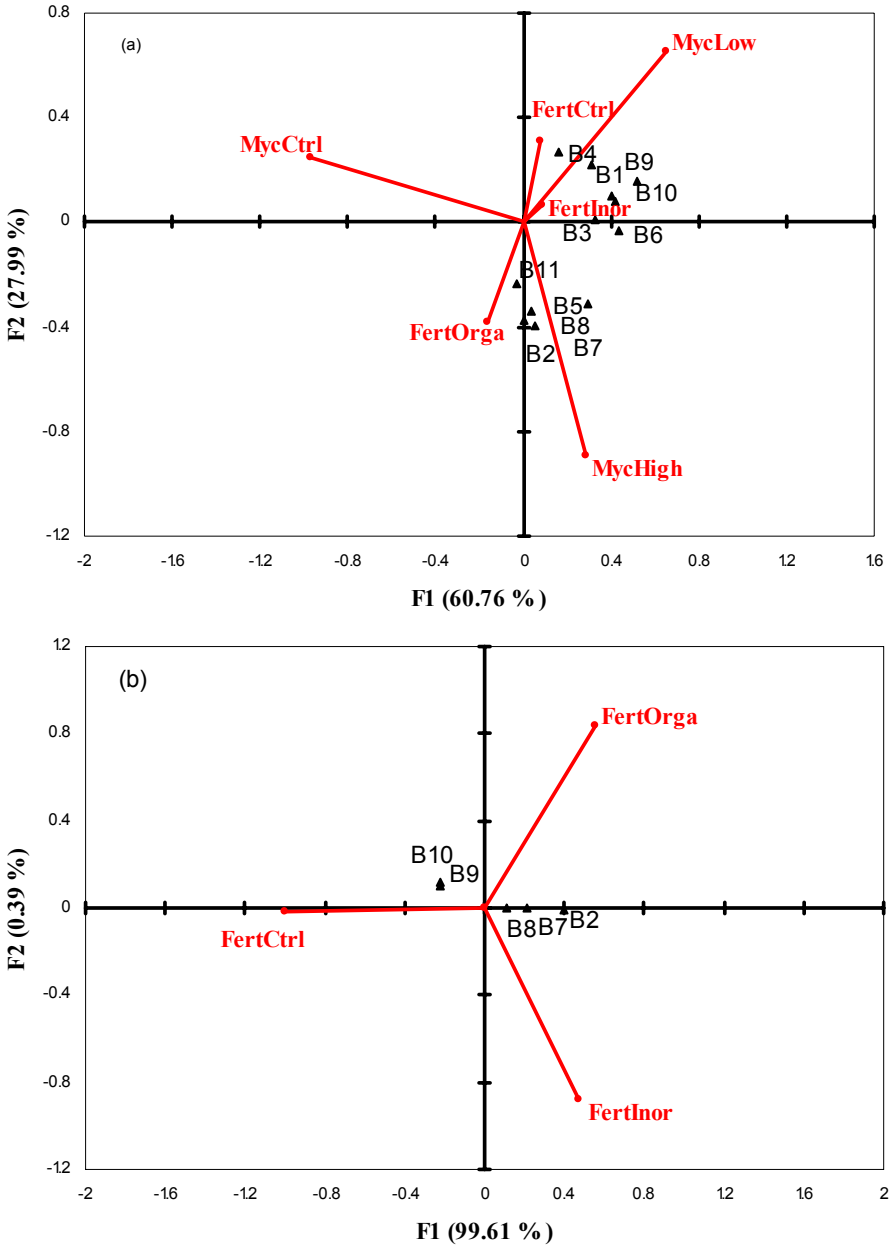

Figure 3. Canonical correspondence alaysis (CCA) biplot of species, mycorrhizal, and N-fertilization treatments showing the relationship between AMF ribotypes and the different treatmnets. A. CCA of AMF community structure within roots samples. B. CCA of AMF community structure within soil samples. FertCtrl: no N-fertilization; FertOrga: dairy manure slurry; FertInor: NH_4-fertilizer; MycHigh: high level of native AM inoculum; MycLow: low AM inoculum level; MycCtrl: no AMF inoculation.

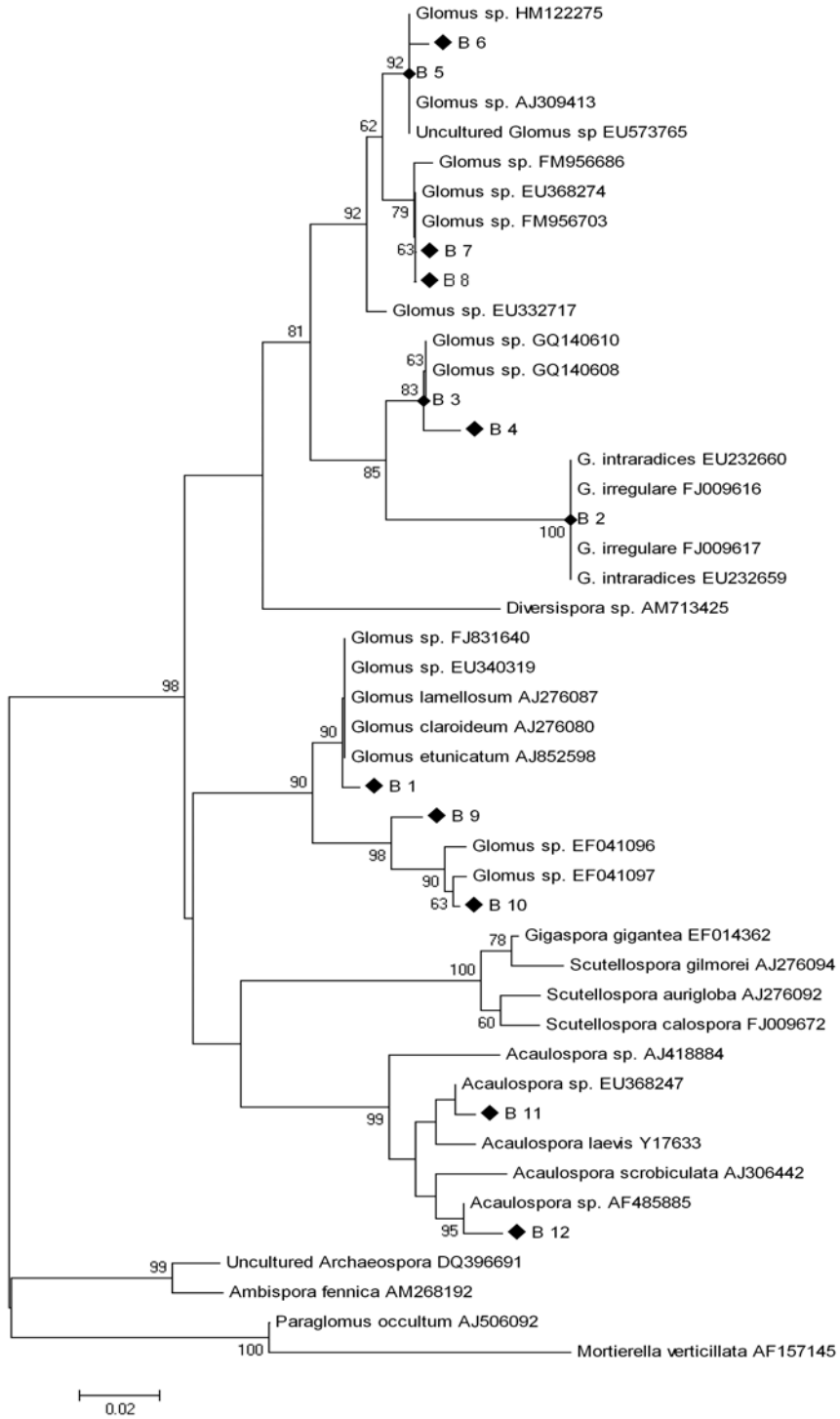

Figure 4. Phylogenetic tree of partial 18S rRNA ribotypes obtained from PCR-DGGE bands, and the closest relative species obtained from NCBI database. ♦ Refer to ribotypes

recovered in this study whose numbers are given in Table 5. GenBank accession numbers are shown after species name. The matrices of tree reconstruction were determined using a Maximum Composite likelihood model of the neighbor joining method. Bootstrap values (1000 replicates) greater than 60% were listed. The number of substitutions per sequence is shown on the scale. *Mortierella verticillata* was used as outgroup.

Discussion

In this study, N-fertilization in the form of manure or NH_4-fertilizer had a significant effect on AM fungal community structure, mycorrhizal root colonization extent, and plant growth. However, the fertilizer type had no effect on AMF species diversity indices and ribotype richness. When sunflower plants were inoculated either with the higher or lower AMF inoculum dose, N-fertilization modified the AMF community structure in roots; additionally, shift of root-colonizing AMF ribotypes under N-fertilization resulted in significant changes of plant biomass production. Inoculation with MycHigh inoculum dose increased the number of AMF ribotypes in plant roots grown in manure or NH_4-fertilizer, but not for the control no-fertilizer treatment as compared to inoculation with the MycLow inoculum dose. Moreover, inoculation with the higher or lower AMF inoculum dose had a significant effect on mycorrhizal root colonization extent and plant growth.

Identification of AMF ribotypes

Our results showed the predominance of *Glomus* ribotypes both in roots and rhizosphere soil, with ten different AMF ribotypes belonging to *Glomus* species and two to *Acaulospora* species. The predominance of *Glomus* species have also been reported in other studies performed in various habitats, such as geothermal soils (Appoloni et al., 2008), tropical forest soil (Wubet et al., 2004), agricultural soils (Daniell et al., 2001), phosphate contaminated soils (Renker et al., 2005), trace metal contaminated soils (Vallino et al., 2006; Yang et al., 2010), and N-fertilized soil (Bhadalung et al., 2005; Jumpponen et al., 2005; Porras-Alfaro et al., 2007). Since *Glomus* species have the ability to proliferate by mycelial fragments and mycorrhizal root fragments, they are much better fit than other AMF species that rely on spore germination to colonize roots, such as *Gigaspora* species. Predominance of *Glomus* species may not only be due to their better hyphal extension but also to a higher sporulation rate (Daniell et al., 2001). No AMF ribotype corresponding to the *Diversisporaceae*, *Gigasporaceae*, *Archaeosporaceae* or *Paraglomaceae* was detected. This may be due to their absence or rarity, which would have prevent their detection using

PCR-DGGE since this approach is known to mainly detect the dominant taxa; on the other hand, primer unspecificity for these taxa also may have biased detection of *Archaeosporaceae* and *Paraglomaceae* (Toljander et al., 2008).

N-fertilization and AMF diversity

The analyses revealed that plants inoculated with the lower AMF dose and grown in manure-fertilized soil had an AMF community structure in roots significantly different than those either fertilized with NH_4 or unfertilized. The abundance of *Glomus intraradices/irregulare* (B2) in the manure/low inoculum treatment could explain by itself the difference in AMF community structure between this treatment and the other treatments. Manure improved soil fertility by increasing P, K and Mg, as well as soil pH, which might have created more suitable conditions for proliferation of some AMF species such as *G. intraradices/irregulare* which colonized sunflower roots. On the other hand, changes in soil properties caused by manure application also could have created unfavourable conditions or have been detrimental to sporulation, root colonization, and survival of more sensitive AMF species, which may have favored an agressive species such as *G. intraradices*. Wang et al. (2009) suggested that manure amendment improved soil properties and that this promoted the proliferation of *Glomus mosseae* while inhibiting the proliferation of *Scutellospora pellucida*. Our results showed not only the abundance of *G. intraradices/irregulare* ribotypes in manure fertilized soil, but also the occurrence of this species in all other treatments, except in the unfertilized/low inoculum treatment. This is consistent with other studies, where the widespread distribution of *G. intraradices* in maize plants grown in calcium nitrate, green manure, farmyard manure, and sewage sludge treated soil was observed (Toljander et al., 2008). The global distribution of *G. intraradices* was also reported in arable, tropical, grassland, and semiarid fields, suggesting the high genetic, phenotypic, and functional variability of *G. intraradices* (Koch et al., 2006; Öpik et al., 2006; Porras-Alfaro et al., 2007).

In the present study, our results showed that NH_4-fertilization did not induce a shift in AMF community structure, with *Glomus* ribotypes B1, B9, and B10 more frequently

found in both NH_4 and unfertilized treatments at the lower inoculation level. The similarity in other soil nutrient (P, K, and Mg) concentrations in both control and NH_4-fertilized soils might have more influence than N on the root-colonizing AMF community. However, NH_4-fertilization reduced the number of AMF ribotypes compared to the FertCtrl treatment, but not significanty. Variable results were recorded concerning the effect of N-fertilization on AMF community structure; for instance, Santos et al. (2006) recorded that N-fertilization reduced AMF diversity and found a negative relationship between soil nitrogen and the frequency of AMF ribotypes in roots of *Festuca pratensis* and *Achillea millefolium*. Contrarily, Porras-Alfaro et al. (2007) reported that N-fertilization resulted in a higher diversity and change in AMF community in the roots of *Bouteloua gracilis*. Jumpponen et al. (2005) noted that mineral N-fertilization did not change AMF root colonization in tallgrass prairie, but that N-amendment shifted AMF community structure. These conflicting results of the effect of N-fertilization on AMF community structure might result from different N-fertilizer forms and doses that differently influenced soil pH, nutrient, or trace element concentration in soils.

A clear separation between high-inoculum manure and high-inoculum NH_4 treatments from high-inoculum unfertilized plants based on AMF community profiles in roots was found. While at the higher inoculum dose *Glomus* ribotypes B7 and B8 were more frequently found in manure and NH_4-fertilized plants, *Glomus* ribotypes B9 and B10 were more abundant in roots harvested from unfertilized plants. The similarity in root-colonizing AM community in manure and NH_4 treatments could be explained by *Glomus* ribotypes B7 and B8 that were the most frequent AM fungal ribotypes in long-term manure and NH_4-fertilized soils. These indigenous AMF taxa showed a higher ability to colonize roots under N-fertilization than the other AMF taxa that were detected in the mycorrhizal inoculum used in this study.

AMF inoculation and plant growth

Our results showed that N-fertilization changed root-colonizing AMF community structure, which in turn affect plant biomass production and the extent of root length

colonization. We also found that inoculation with the highest AMF inoculum dose increased the number of AMF ribotypes relation to the lowest dose for NH_4-fertilization treatment but not for the unfertilized treatment. Thus, there was a difference in root-colonizing AMF community between the higher and lower inoculum dose and this variation in AMF community significantly modifyied plant growth under N-fertilization treatments. In addition, under control and NH_4-fertilization, we showed that unmycorrhized or mycorrhized plants inoculated with the low inoculum dose produced a greater biomass than mycorrhized plants inoculated with the higher dose. Additionally, a clear difference in root colonization percentage was found between different mycorrhizal inoculation doses under control and NH_4-fertilization, where inoculation with the higher dose caused a two-fold increase in root colonization compared to plants inoculated with the lower dose. Our results are consistent with other studies, where different AMF species showed different effect on plant growth and N uptake in N-fertilized soil (Hawkins and George, 2001; Guo et al., 2006; Tu et al., 2006). van der Heijden et al. (1998) also reported that change in AMF community structure affected plant community composition and plant growth.

In contrast, under manure fertilization, the percentage of colonized root length was similar in both high and low mycorrhizal inoculation levels. However, plants in the manure/MycLow treatment produced a greater biomass than those in the manure/MycHigh treatment. As we recorded a difference in root-colonizing AMF community structure between these treatments, this might explain the positive effect of inoculation with the lower dose compared to the higher dose on plant growth response. Also, the MycHigh inoculation increased the number of AMF ribotypes more than MycLow. Most of the AMF ribotypes that were detected in manure/MycLow treatment were also found in manure/MycHigh treatment. Therefore, our results showed that variation in root-colonizing AMF community significantly shifted plant growth in response to N-fertilization. This suggests that the AMF species in the grass roots used as AMF inoculum were more effective than those in the soil but were less effective when the soil was notfumigated. A differential effect of different mycorrhizal inocula on plant growth and P uptake in organic manure treatment was also showed previously (Verma and Arya, 1998). These authors

found that two different mycorrhizal inocula induced similar root colonization of plantlets, but that one of them was more effective than the other to increase plant biomass and P uptake. AMF-plant symbiotic interaction influence the growth response of both partners and carbon-phosphorus transfer between the fungus and the plant (Fitter et al., 2004; Munkvold et al., 2004; Koch et al., 2006).

Under the low mycorrhizal inoculum dose, manure significantly increased root colonization and plant growth compared to Ctrl and NH_4-fertilization. It was previously observed that manure fertilization enhanced AMF root colonization and promoted the growth of AM fungal hyphae in soil, but did not increased spores density (Gryndler et al., 2005; Gryndler et al., 2006). Manure application also was shown to increase growth, yield, and root colonization of wheat plants inoculated with *Glomus fasciculatum* (Groaker and Sreenivasa, 1994). Joner (2000) found that plants receiving manure had higher growth than those amended with mineral fertilization in pasteurized soil, but produced lower growth rate under unpasteurized treatments, suggesting that pasteurization affected the mineralization of the organic nutrients of the manure, in particular N, and consequently affected plant growth. The positive effect of manure here may be due to the increased soil nutrients in manure-fertilized soils compared to control and NH_4-fertilization, which contributed to the enhanced plant productivity. On the other hand, under the higher inoculum dose, both manure and NH_4-fertilized plants had greater root colonization than plants grown in the unfertilized control. Although similar percentage of root length bearing mycorrhizal colonization and AMF community structure were found in manure and NH_4 treaments, manure fertilized plants had higher productivity than NH_4-fertilized plants. The reason for this may be related to the higher soil fertility (higher P, K and Mg) caused by manure than by NH_4-fertilization. We suggests that similar root-colonizing AMF communities under different N-fertilizer forms, which normally show different concentrations other nutrient may explain the different effects of these fungi on plant growth response.

Conclusion

Indigenous AM fungi colonized plant roots in long-term manure or NH_4-fertilized soils, but a variation in root-colonizing AMF community structure was noted between manure and NH_4 fertilization. *G. intraradices/irregulare* ribotype B2 was more associated to manure, while other ribotypes (B1, B9, and B10) were more affiliated with NH_4-fertilization. In addition, NH_4-fertilization showed a tendency to reduce AMF ribotype number while manure had the inverse effect, though not significanty. Variation in root-colonizing AMF community changed plant growth response to N-fertilization. The main goal of N-fertilization application is to increase soil fertility and plant productivity. However, the intensive use of N-fertilization result in the loss and leaching of nutrients, P pollution, and fertilizer-associated trace metal pollution, as well as with a modification of AMF community structure, which may affect plant productivity positively or negatively. Our results showed that under our conditions, the manure-treated soil produced the greatest increase in plant biomass under low-AMF inoculum level or without inoculation, and that *G. intraradices/irregulare* was the most frequent AM fungal taxon under these conditions. More effort is required to understand the plant-AMF-fertilizer interactions to improve the efficient use of N-fertilization and to minimize the detrimental effects of long-term fertilization application on the environment.

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CHAPTER V

Effect of arbuscular mycorrhizal fungi on trace metals uptake by sunflower plants grown on cadmium contaminated soil

This chapter will be prepared for submission to the international Journal.

Also, it was presented in the following conference:

- Hassan SE, M Hijri, M St-Arnaud 2010 Heavy metal contamination lower the biodiversity and modify the community structure of arbuscular mycorrhizal fungi in soil and plant roots. pp. 6 in Proceedings of the 3rd Montreal Plant Meeting, March 27, Univ. Concordia, Montréal, Canada.

Preface

In the previous chapters, the biodiversity of arbuscular mycorrhizal fungi (AMF) was screened from metal contaminated urban and agricultural areas. The results showed that several AMF strains could naturally thrive in these polluted environments. In particular, some AMF strains were found to be preferentially associated with these adverse environments, suggesting that these AMF could be suited for phytoremediation purposes. *Glomus irregulare* and *Glomus mosseae*, due to their predominance in non contaminated and metal contaminated areas, they were selected to develop phytoremediation experiment. To date little is known about the ecological impact of AMF inoculation on cadmium (Cd) phytoremediation because 1) Cd is a non-essential metal found in many natural and agricultural sites and it is highly toxic in comparison with other trace metal, and was classified as a class 1 human carcinogen by the international agency for research on cancer, and 2) it is unknown whether AMF species is more suited for uptake or immobilize Cd from contaminated soils. In this issue, this chapter provides the impact of *Glomus irregulare* and *Glomus mosseae* inoculation on Cd phytoremediation.

Abstract

Greenhouse trial was conducted to investigate the role of the arbuscular mycorrhizal fungi (AMF) *Glomus irregulare* and *G. mosseae* on cadmium (Cd) uptake by sunflower plants grown in soil complemented with three Cd concentrations (0.75, 10, and 30 mg kg^{-1}). Plants were harvested after 10 weeks, and mycorrhizal root colonization and plant biomass as well as Cd, Zn, and Cu trace metals (TM) concentrations in roots and shoots were determined. We found that root mycorrhizal colonization rates were not significantly affected by Cd treatments. At high Cd concentration, the total dry plant biomass of non-inoculated plants decreased by 20% compared to non-inoculated plants grown in soil with low Cd concentration. However, for mycorrhizal plants, Cd concentrations in soil had no significant on plant biomass. Independently of the Cd concentration in soil, *G. irregulare* had no significant effect on plant biomass compared to non-inoculated plants, while inoculation with *G. mosseae* significantly reduced plant biomass. At low soil Cd

concentration (0.75 mg kg^{-1}), *G. irregulare*-inoculated plants had significantly higher shoot Cd and Zn concentrations than plants inoculated with *G. mosseae* and non-inoculated plants. In addition, inoculation with *G. irregulare* or *G. mosseae* significantly increased shoot Cu concentration compared to non-inoculated plants. At 10 mg kg^{-1} of Cd concentration in soil, no significant difference in shoot TM concentrations was found between plants inoculated with *G. irregulare* and non-inoculated plants. While, At 30 mg kg^{-1} of Cd concentration in soil, *G. irregulare* caused a significant increase shoot Cd concentration compared to *G. mosseae* and control plants. Moreover, *G. irregulare*-inoculated plants had significantly higher shoot Cd biological exchange factor (BCFs) values than plants inoculated with *G. mosseae* and non-inoculated plants. On the other hand, at the highest soil Cd concentrations, *G. mosseae*-inoculated plants had significantly lower shoot Cd and Zn concentrations and BCFs values than plants inoculated with *G. irregulare* and non-inoculated plants. The results suggest that *G. irregulare* tolerate high Cd concentration in soil possibly through a Cd transportation mechanism from soil to aboveground plant tissues, while *G. mosseae* contribute to reduce shoot Cd and Zn concentrations potentially through Cd and Zn immobilization in soil. These results also indicated that these AMF strains mediate different tolerance strategies to alleviate TM toxicity in their host plants. We conclude that *G. irregulare* and *G. mosseae* might respectively be used for phytoextration (Cd) and phytosatibilization (Cd and Zn) of TM in phytoremediation strategies.

Introduction

Trace metals (TM) pollution of soils represents an important environmental problem, and cadmium (Cd) is a non-essential metal found in many natural and agricultural sites, resulting mainly from industrial and agricultural activities. Cd is potentially toxic when it accumulates in soil and translocates in the food chain through contaminated plant tissues. Cd is highly toxic in comparison with other TM (Duffus 2002) and was classified as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC 1993). Therefore, Cd constitutes a serious concern for the environment and is an important soil pollutant.

In recent years, a great effort has been paid to novel techniques of TM phytoremediation, in which plants are used to take up or immobilize TM from contaminated soils. Because phytoremediation is an *in situ*, inexpensive, effective, and an accepted eco-friendly approach, it is a promising method for environmental cleanup of TM contaminated soils (De Coninck and Karam 2008). Hyperaccumulator plants can accumulate high concentrations of TM in their tissues but unfortunately they produce low biomass, which limits the efficiency of phytoremediation (Kramer 2005). Alternatively, other crops such as sunflower, maize, and fast growing trees such as willow and poplar can be used because of their high biomass production, as well as their capacity to accumulate TM (Kramer 2005; Lingua et al., 2008). Using plants capable of forming symbiotic relationship with soil microorganisms that mobilize TE is another way to improve the phytoremediation efficiency (Khan 2005).

Arbuscular mycorrhizal fungi (AMF) are root inhabiting symbionts found in most plant species and in most terrestrial habitats (Smith and Read 2008), including heavily TM contaminated soils (Zarei et al., 2008; Hassan et al., 2011). AMF generally improve mineral nutritions uptake to the host plant, in particular phosphorus, facilitate water uptake and reduce root pathogen damages (St-Arnaud and Vujanovic 2007). AMF also affect TM uptake, and were shown to alleviate TM toxicity to the host plant (Hildebrandt et al., 2007). However, conflicting results have been reported concerning the effect of AMF on TM uptake or immobilization by plants. For instance, AMF were shown to enhance TM

accumulation in aboveground tissues (Davies et al., 2002; Citterio et al., 2005) or to reduce metal concentration in host plant tissues (Shen et al., 2006; Li et al., 2009). Based on a meta-analysis of AMF feedback on TM plant uptake, Audet & Charest (Audet and Charest 2007) have proposed that AMF colonization increases TM accumulation in plant tissues at low soil TM concentration, but reduce soil TM bioavailability through metal-binding with fungal structures at high soil TM concentrations, resulting in lower TM uptake in mycorrhized plants than in non-mycorrhized plants. Thus, the role of AMF in TM immobilization and translocation would depend on host plant species, TM concentration and speciation, and AMF species (Audet and Charest 2008; Lingua et al., 2008).

Various AMF species or isolates have shown variable effects on TM uptake or sequestration in different environmental conditions. For example, AMF isolates from metal-polluted soils exhibited higher resistance to TM toxicity compared to those found in non-polluted soils (Gonzalez-Chavez et al., 2002). Different AMF species were also shown to differentially affect translocation and accumulation of TM in their host plants (Bai et al., 2008; Liang et al., 2009). Then, AMF might use different mechanisms to alleviate TM toxicity and their contribution might change depending on the environment.

The aims of the present study were therefore (1) to compare the effect of the AMF species *Glomus irregulare* and *G. mosseae* on the growth of sunflower plants in soil contaminated with Cd (0, 25 and 100 mg kg^{-1} Cd added), and (2) to assess the interaction between AMF taxa and Cd concentration on TM (Cd, Zn, Cu) translocation from soil to roots and shoots, and accumulation in plant tissues.

Materials and methods

Experimental design

A greenhouse experiment was conducted in a completely randomized design with a 3 x 3 factorial scheme and the following factors: three Cd concentrations added to soils (0, 25, and 100 mg L^{-1}) and three AMF species inoculations (*G. irregulare*, *G. mosseae*, control non-inoculated plants). Five replicates were conducted for each treatment for a total of 45 pots.

Soil preparation

A sandy loam soil was collected from a field within the Montréal Botanical Garden (Montréal, QC, Canada). The soil pH, cation exchange capacity (CEC), organic matter, and soil granulometric analyses are shown in Table 1. Soil bioavailablity of P, K, Mg, Al and Ca are measured after Mechlich-3 extraction, and TM concentration (Cd, Cu, Zn) after HNO_3 digestion for 5h at 120 ºC, using an inductively coupled plasma mass spectrophotometer (IPC-MS). Blanks, standardized reference soil and replicates were optimized for these analyses. The initial Cd, Zn, and Cu concentrations were above the contamination threshold defined for agricultural (Cd) or residential (Zn, Cu) soils by provincial regulations (Beaulien and Drouin 1999).

The soil was air-dried, sieved to less than 2 mm, mixed with quartz sand at a soil:sand ratio of 2:1 and autoclaved twice for 1h at 120 ºC. Then, the soil was supplemented with 0, 25, and 100 mg L^{-1} of Cd solutions by adding 50 ml/kg of an aqueous $CdSO_4$ solution. The soil was let to rest for 2 weeks after the Cd solutions were thoroughly mixed in, to allow metal stabilization. The measured Cd concentrations in the spiked soil were assessed after HNO_3 digestion and IPC-MS to 0.75, 10, and 30 mg kg^{-1}, respectively.

Arbuscular mycorrhizal fungi inoculant preparation

Ri T-DNA-transformed *Daucus carota* L. roots colonized with *Glomus irregulare* (isolate DAOM-234328) were grown in minimal (M) medium containing 0.4 % (w/v)

gellan gum (Gel Gro, ICN Biochemical) for six months in the dark at 26 ºC (Fortin et al., 2002). The spores were collected by dissolving the gel in sodium citrate buffer (Doner and Bécard 1991) and suspending it in sterile water. *Glomus mosseae* (Nicol. & Gerd.) Gerdemann and Trappe (BEG 12) was propagated *in vivo* using pot culture with leek plants grown in a sandy loam soil for 6 months in a greenhouse. *Glomus mosseae* spores were isolated by wet sieving and decanting and purified by centrifugation at 2000 rpm for 2 min in a density gradient with a 50% sucrose layer at the bottom. Spores were collected from the gradient interface and suspended in sterile water.

The experimental set up and cultural condition

Seeds of sunflower (*Helianthus annuus* L) Pacino Gold cultivar were surface sterilized in 2.5% sodium hypochloride for 15 min and rinsed with sterilized Milli-Q water. Seeds were grown in pots containing 100 g soil, and after emergence, one seedling was transplanted per 25 cm pot containing 3 kg of Cd-treated soil. Each seedling was inoculated with 10 ml of spore suspension of *G. irregulare* or *G. mosseae*, containing approximately 1000 spores, poured on the seedling roots. The control received sterile water. Pots were not fertilized during the growing period. Plants were watered as needed and the pots were placed in individual saucers to allow the reabsorption of irrigation water and avoid TE leaching. Sunflower plants were grown for 10 weeks in a greenhouse with 16 h daylight (20-22ºC).

Throughout the growing period, two treatments against powdery mildew with Phyton-27® were applied at a rate of 2 ml L^{-1} and 55 ml sprayed per plant. During these treatments, the soil was covered in each pot to prevent the fungicide reaching the soil.

Plant tissue analyses

Shoots and roots were separately harvested after 10 weeks of transplantion. Roots were washed with tap water to remove soil particles and subsamples were stored in 50% ethanol for mycorrhizal colonization assessment. The fresh weight of shoots and roots are measured before oven dried for 48 h at 60ºC, and weighed to determine shoot and root dry

weights. Dried tissues (0.2 g) were separately ground and digested in HNO_3. Samples were then pooled (replicates 1 and 2, 3 and 4, 5) to obtain three replicates per treatment, and Cd, Zn, and Cu concentration was measured by ICP-MS. Total TM content of shoots and roots were estimated by multiplying tissue TM concentration (mg kg^{-1}) by tissue dry weight (kg). Biological concentration factor (BCF) was calculated for each TM as the ratio of TM concentration in plant tissue to TM concentration in soil.

Mycorrhizal root colonization extent was estimated after clearing in 10 % KOH (10 min at 100 ºC) and staining with Schaeffer black ink in a 5% vinegar solution for 3 min at 100 ºC (Vierheilig et al., 1998), using the gridline intercept method (Giovannetti and Mosse 1980). The percentage of root intercepts harboring AMF structures was estimated under the compound microscope from at least 100 fields per sample.

Statistical analyses

The effect of Cd concentration and mycorrhizal inoculation on plant dry biomass, mycorrhizal root colonization percentages, shoot and root TM concentration, TM content and BCF value were analyzed by two-way analysis of variance (ANOVA). Within each Cd concentration, *a posteriori* comparisons between mycorrhizal inoculation treatments was conducted by the Tukey's HSD test ($P \leq 0.05$). All statistical analyses were performed using SPSS software v. 17 (SPSS Inc., Chicago, Illinois).

Results

Plant biomass and mycorrhizal root colonization

The results of the factorial ANOVA of the effect of Cd and mycorrhizal inoculation treatments and their interaction on plant biomass and root colonization are summerized in Table 2. Mycorrhizal treatments had significant effect on plant biomass. High Cd concentrations in soil tend to reduce total plant biomass weight, but not signifigant ($P = 0.059$). The effect of mycorrhizal inoculation on plant dry weight under different soil Cd concentration is shown in Table 3. Inoculation with *G. irregulare* had no effect on total plant dry biomass. *G. mosseae* inoculated plants had significantly lower total plant dry biomass than control and *G. irregulare* inoculated plants. Root dry weights were not affected by mycorrhizal inoculations at all Cd concentrations.

Cd and mycorrhizal treatments did not affect mycorrhizal root colonization percentages (Table 2 and 3). No mycorrhizal root colonization occurred in non-inoculated control plants. The mycorrhizal root colonization percentages ranged from 38% to 43%.

Cadmium (Cd) accumulation in plant tissues

The results of factorial ANOVA of the effect of Cd and mycorrhizal treatments and their combination on TM concentration, content and BCF are summerized in Table 4. At the lowest Cd concentration in soil (0.75 mg kg^{-1}), shoot Cd concentrations measured in *G. mosseae*-inoculated plants were significantly lower than in those inoculated with *G. irregulare* and in control plants. *Glomus irregulare*-inoculated plants caused a significant increase of shoot Cd concentration compared to control plants (Table 5). No difference was found between mycorrhized and control plants in their shoot Cd content; however, *G. mosseae*-inoculated plants had significantly lower shoot Cd content than *G. irregulare*-inoculated plants (Table 6). At moderate Cd concentration in soil (10 mg kg^{-1}), *G. mosseae*-inoculated plants had significantly lower shoot Cd concentration and content than *G. irregulare*-inoculated and control plants, contrary to *G. irregulare* which did not modified shoot Cd concentration and content compared to control plants (Table 5 and 6). At the

highest Cd concentration in soil (30 mg kg^{-1}), *G. mosseae* inoculation significantly lowered shoot Cd concentration and content compared to *G. irregulare*-inoculated and non inoculated plants (Table 5 and 6). While *G. irregulare*-inoculated plant significantly inceased shoot Cd concentration compared to *G. mosseae*-inoculated and non inoculated plants. However, *G. irregulare*-inoculated and non inoculated plants had similar shoot Cd contents.

G. irregulare-inoculated, *G. mosseae*-inoculated, and non inoculated plants had similar root Cd concentrations, while *G. mosseae* caused a significant decrease in root Cd and total plant Cd content compared to *G. irregulare* and non inoculated plants (Table 5 and 6).

G. irregulare-inoculated plants had higher shoot Cd BCF than *G. mosseae*-inoculated and non inoculated plants, while *G. mosseae* had a significant decrease shoot Cd BCF compared to *G. irregulare* and non inoculated plants (Table 7). Mycorrhizal inoculated and non inoculated plants had similar root Cd BCF, while *G. irregulare* caused a significant increase in root Cd BCF compared to *G. mosseae* (Table 7).

Copper and Zinc accumulation in plant tissues

No significant differences in Cu concentration, Cu content, and Cu BCF values were found in plant tissues between mycorrhized and control plants. At the lowest soil Cd concentration (0.75 mg kg^{-1}), *G. mosseae* inoculation did not modify Zn shoot concentration compared to control plants, while *G. irregulare*-inoculated plants had a significantly greater shoot Zn concentration than *G. mosseae*-inoculated and control plants. At 10 and 30 mg kg^{-1} Cd concentration in soil, *G. mosseae* inoculation significantly reduced shoot Zn concentration compared to *G. irregulare*-inoculated and control plants (Table 5).

No effect of mycorrhizal inoculation or Cd concentration treatments was found on root Zn concentration and root Zn content (Table 5 and 6). *G. mosseae* inoculation caused a significant decrease in shoot Zn content and total Zn plant content compared to *G. irregulare* inoculation and control plants.

At the lowest soil Cd concentration (0.75 mg kg^{-1}), *G. irregulare*-inoculated plants had a significantly greater shoot Zn BCF value than *G. mosseae*-inoculated and non inoculated plants. While, *G. mosseae* inoculation did not modify shoot Zn BCF compared to control plants (Table 7). At the highest Cd concentration in soil (10 and 30 mg kg^{-1}), *G. mosseae*-inoculated plants had a significantly lower shoot Zn BCF than *G. irregulare*-inoculated and non inoculated plants. No effect of mycorrhizal inoculation was found on root Zn BCF (Table 7).

Table 1: Soil analyses, TM concentration and soil characteristics.

Soil parameters	Measurements
pH	7.4
CEC (mEq per 100 g)	18.2
Organic matter (%)	3.9
Particle size distribution (%)	
Clay (%)	11.3
Silt (%)	9.0
Sand (%)	79.8
P[a] (mg kg^{-1})	193.1
K[a] (mg kg^{-1})	164.6
Mg[a] (mg kg^{-1})	187.3
Ca[a] (mg kg^{-1})	3046.2
Al[a] (mg kg^{-1})	374.2
Saturation P (%)	23.1
Saturation K (%)	2.3
Saturation Mg (%)	8.6
Saturation Ca (%)	83.7
TM concentration[b] (mg kg^{-1})	
Cd	0.75
Cu	91
Zn	286

[a] Mehlich-3 extractions
[b] HNO3 extractions

Table 2: Factorial ANOVA of the treatment effects and their interactions on plant biomass and root mycorrhizal colonization percentages.

	P-values		
	Cd treatment	AMF treatment	Cd*AMF treatments
Dry shoot weight	0.059	< 0.001	0.296
Dry root weight	0.032	0.007	0.531
Dry plant weight	0.033	< 0.001	0.316
Mycorrhizal root colonization	0.068	0.066	0.848

Table 3: Effect of mycorrhizal inoculation and soil Cd concentrations on the biomass of sunflower plants grown in Cd contaminated soil.

Cd treatment [abc]	Dry shoot weight			
	AMF treatment			
	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	9.25	8.36	6.33	7.99X
10 mg kg^{-1}	8.79	8.10	6.48	7.79X
30 mg kg^{-1}	7.42	8.18	5.87	7.16X
Mean	8.49a	8.22a	6.23b	
	Dry root weight			
	AMF treatment			
	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	1.99	1.73	1.51	1.75X
10 mg kg^{-1}	2.07	1.66	1.51	1.74X
30 mg kg^{-1}	1.47	1.64	1.11	1.41X
Mean	1.85a	1.68ab	1.38b	
	Dry plant weight			
	AMF treatment			
	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	11.25	10.10	7.87	9.73X
10 mg kg^{-1}	10.86	9.76	7.99	9.54X
30 mg kg^{-1}	8.90	9.83	6.99	8.57X
Mean	10.34a	9.90a	7.61b	
	Root colonization percentage			
	AMF treatment			
	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	0	38.4	40.2	39.3X
10 mg kg^{-1}	0	40.0	41.0	40.5X
30 mg kg^{-1}	0	40.8	43.0	41.9X
Mean		39.7a	41.5a	

[a] Means values for each AMF treatment within rows followed by the same small letter are not significantly different by one-way ANOVA ($P<0.05$) ($n=15$).

[b] Means values for each Cd treatment within the columns followed by the same capital letter are not significantly different by one-way ANOVA ($P<0.05$) ($n=15$).

[c] Ctrl, non-inoculated plants; Gi, *G. irregulare*-inoculated plants; Gm, *G. mosseae*-inoculated plants.

Table 4: Factorial ANOVA of the treatment effects and their interactions on TM concentration, content and biological accumulation factor in sunflower shoots and roots.

		P-values [a,b]		
		Cd treatments	AMF treatments	Cd*AMF treatments
Cd Concentration	shoot	> 0.001	> 0.001	> 0.001
	root	> 0.001	0.036	0.077
Cu Concentration	shoot	0.260	0.004	0.038
	root	> 0.001	0.011	0.815
Zn Concentration	shoot	0.015	> 0.001	0.024
	root	0.723	0.368	0.502
Cd Content	shoot	> 0.001	> 0.001	0.009
	root	> 0.001	0.050	0.105
	plant	> 0.001	> 0.001	0.146
Cu Content	shoot	0.986	0.164	0.853
	root	0.138	0.181	0.586
	plant	0.731	0.134	0.905
Zn Content	shoot	0.402	0.001	0.783
	root	0.080	0.201	0.839
	plant	0.314	0.003	0.844
Cd BCF	shoot	0.013	> 0.001	0.077
	root	0.014	0.002	0.721
Cu BCF	shoot	0.260	0.004	0.038
	root	> 0.001	0.011	0.815
Zn BCF	shoot	0.015	> 0.001	0.024
	root	0.368	0.723	0.502

[a] TM content = dry biomass × metal concentration in tissue .
[b] BCF, biological concentration factor = tissue concentration / soil concentration.

Table 5: Effect of AMF and soil Cd teatments on TM concentration (mg kg^{-1}) in shoots and roots of sunflower plants grown in Cd contaminated soil.

Cd treatment [abc]	Shoot Cd concentration				Root Cd concentration			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	0.63aX	1.02bX	0.40cX	0.68	0.76	0.87	0.46	0.70X
10 mg kg^{-1}	8.8aY	10.73aY	3.50bY	7.67	8.74	10.79	7.40	8.98Y
30 mg kg^{-1}	25.11aZ	31.62bZ	12.17cZ	22.96	40.32	48.17	26.08	38.19Z
Mean	11.51	14.45	5.35		16.61a	19.94a	11.32a	
	Shoot Cu concentration				Root Cu concentration			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	99.7aX	156.4[bX]	157.6[bX]	137.96	48.13	58.78	58.53	55.15X
10 mg kg^{-1}	137.3aY	148.7aX	141.0aX	142.39	61.61	81.22	80.21	74.34Y
30 mg kg^{-1}	144.1aY	160.5aX	147.4aX	150.71	71.12	95.16	83.31	83.20Y
Mean	127.07	155.25	148.75		60.29a	78.38a	74.02a	
	Shoot Zn concentration				Root Zn concentration			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	109.0aX	150.8[bX]	96.8aX	118.88	50.08	55.65	61.34	55.69X
10 mg kg^{-1}	155.1aY	159.8aX	88.6bX	134.54	58.29	64.39	56.94	59.87X
30 mg kg^{-1}	119.7aX	147.0aX	83.2bX	116.70	55.81	52.74	54.24	54.26X
Mean	127.99	152.56	89.56		54.73a	57.59a	57.51a	

[a] Means values for each inoculation treatment within rows followed by the same small letter are not significantly different by one-way ANOVA ($P<0.05$) ($n=9$).

[b] Means values for each Cd treatment within the columns followed by the same capital letter are not significantly different by one-way ANOVA ($P<0.05$) ($n=9$).

[c] Ctrl, non-inoculated plants; Gi, *G. irregulare*-inoculated plants; Gm, *G. mosseae*-inoculated plants.

Table 6: Effect of AMF and soil Cd treatments on TM content (mg per plant) of sunflower plants grown in Cd contaminated soil.

Cd treatment [abcd]	Shoot Cd content				Root Cd content			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	9.65[abX]	14.59[aX]	4.31[bX]	9.51	2.60	2.42	1.16	2.05X
10 mg kg^{-1}	132.04[aY]	146.96[aY]	38.44[bY]	105.81	31.69	30.81	18.54	27.01Y
30 mg kg^{-1}	314.39[aZ]	437.34[aZ]	120.38[bZ]	290.70	102.08	137.21	69.24	96.18Z
Mean	152.02	199.62	54.38		45.45a	56.81a	22.98b	

	Plant Cd content				Shoot Cu content			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	12.24	17.00	6.78	11.57X	1539.5	2215.1	1716.6	1823.7X
10 mg kg^{-1}	163.73	177.78	56.98	132.83Y	2032.7	2038.1	1537.5	1869.5X
30 mg kg^{-1}	416.47	574.56	169.64	386.88Z	1803.4	2218.1	1474.6	1832.0X
Mean	197.48a	256.44a	77.36b		1791.9a	2157.1a	1576.2a	

	Root Cu content				Plant Cu content			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	162.4	163.3	147.3	157.7X	2793.2	3614.1	2880.1	3095.8X
10 mg kg^{-1}	216.0	229.0	198.3	214.4X	3642.9	3804.8	2947.2	3465.0X
30 mg kg^{-1}	178.8	267.8	153.4	200.0X	3249.6	4269.8	2718.3	3412.6X
Mean	185.7a	220.0a	166.3a		3228.6a	3896.2a	2848.6a	

	Shoot Zn content				Root Zn content			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	1703.6	2126.5	1036.9	1622.3X	167.9	158.0	153.6	159.8X
10 mg kg^{-1}	2297.3	2175.8	976.6	1816.6X	204.0	179.7	144.9	176.2X
30 mg kg^{-1}	1496.7	2039.8	819.1	1451.9X	135.5	147.6	99.3	127.5X
Mean	1832.6a	2114.0a	944.2b		169.1a	161.8a	132.6a	

	Plant Zn content			
	AMF treatment			
	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	3019.3	3479.4	2046.9	2848.5X
10 mg kg^{-1}	3907.6	3679.4	1972.6	3186.5X
30 mg kg^{-1}	2612.5	3335.4	1598.2	2515.4X
Mean	3179.8a	3498.1a	1872.5b	

[a] Means values for each inoculation treatment within rows followed by the same small letter are not significantly different by one-way ANOVA ($P<0.05$) (n =9).

[b] Means values for each Cd treatment within the columns followed by the same capital letter are not significantly different by one-way ANOVA ($P<0.05$) (n =9).

[c] TM content = dry biomass × metal concentration in tissue.

[d] Ctrl, non-inoculated plants; Gi, *G. irregulare* inoculated plants; Gm, *G. mosseae* inoculated plants.

Table 7: Effect of AMF and soil Cd treatments on biological concentration factor (BCF) of TM in shoots and roots of sunflower plants grown on Cd contaminated soil.

Cd t reatment [abcde]	Shoot Cd BCF				Root Cd BCF			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	0.83	**1.36**	0.53	0.91X	**1.01**	**1.16**	0.62	0.92X
10 mg kg^{-1}	0.88	**1.07**	0.34	0.76X	0.87	**1.07**	0.74	0.89X
30 mg kg^{-1}	0.83	**1.05**	0.40	0.76X	**1.34**	**1.6**	0.86	1.27X
Mean	0.85a	1.16b	0.42c		1.07ab	1.28a	0.74b	
	Shoot Cu BCF				Root Cu BCF			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	0.99a	**1.56b**	**1.57b**	1.37	0.48	0.58	0.58	0.55a
10 mg kg^{-1}	**1.37a**	**1.48a**	**1.41a**	1.42	0.62	0.81	0.8	0.74b
30 mg kg^{-1}	**1.44a**	**1.6a**	**1.4a**	1.50	0.71	0.95	0.83	0.83b
Mean	1.27	1.55	1.48		0.60a	0.78a	0.74a	
	Shoot Zn BCF				Root Zn BCF			
	AMF treatment				AMF treatment			
	Ctrl	Gi	Gm	Mean	Ctrl	Gi	Gm	Mean
0.75 mg kg^{-1}	0.36aX	0.5bX	0.32aX	0.39	0.17	0.19	0.2	0.18X
10 mg kg^{-1}	0.52aY	0.53aX	0.3bX	0.44	0.19	0.21	0.18	0.19X
30 mg kg^{-1}	0.39aX	0.49aX	0.27bX	0.38	0.18	0.17	0.18	0.18X
Mean	0.42	0.50	0.29		0.18a	0.19a	0.19a	

[a] Means values for each inoculation treatment within rows followed by the same small letter are not significantly different by one-way ANOVA ($P<0.05$) (n =9).

[b] Means values for each Cd treatment within the columns followed by the same capital letter are not significantly different by one-way ANOVA ($P<0.05$) (n =9).

[c] Biological concentration factor = tissue concentration / soil concentration.

[d] Ctrl, non-inoculated plants; Gi, *G. irregulare*-inoculated plants; Gm, *G. mosseae* inoculated plants.

[e] Values in bold are active bioaccumulation (> 1).

Discussion

Effect of Cd concentration and AMF inoculation on root colonization and plant biomass

High concentrations of TM in soil are toxic to plants, bacteria and fungi (Vivas et al., 2003). It has also been reported that high Cd concentrations in soil inhibited mycorrhizal colonization (Weissenhorn and Leyval 1995; Vivas et al., 2003). However, our study clearly showed that different Cd concentrations in soil had no effect on colonization by AMF *G. irregulare* and *G. mosseae* within the range of soil Cd concentrations studied. Although the *G. irregulare* and *G. mosseae* strains used in this study were not isolated from TM contaminated soils, they efficiently colonized plants grown in a Cd contaminated soils. Our results suggest the Cd tolerance of these AMF species and their capacity to colonize roots under high Cd stress in soil. Our data are in concordance with (Rivera-Becerril et al., 2002; de Andrade et al., 2008), who showed that mycorrhizal colonization and the fungal biomass produced by AMF were not affected by Cd contamination.

Biomass production can reflect the toxic effect that TM may cause in plants (de Andrade et al., 2008). In the present study, no visual phytotoxicity symptoms were observed in Cd treated plants. However, plants showed a slower growth under high Cd concentration in soil. Biomass of non-inoculated plants decreased in the highest soil Cd concentration but not in *G. irregulare* or *G. mosseae*-inoculated plants. Vassilev et al. (2002) showed that Cd inhibit the biosynthesis of photosynthetic pigments and proposed that this effect was responsible for the growth reduction caused by cadmium. Mycorrhized plants were showed to have higher photosynthetic pigments content in the presence of Cd than non-mycorrhized plants (Kapoor et al., 2007; Andrade et al., 2009). A positive effect of AMF on plant growth under high metal concentration was previously reported (Janousková et al., 2006). Others found no influence of AMF inoculation on plant biomass production under Cd stress (Chen et al., 2004; Janousková et al., 2007). Contrarily, Citterio et al., (2005) showed that *G. mosseae* negatively affected hemp biomass under Cd contamination. Relationships between plant and AMF are considered mutualistic (Smith

and Read 2008). However, neutral or negative plant growth responses to AMF have sometimes been found (Johnson et al., 1997; Citterio et al., 2005). It have been suggested that in some circumstances, it may be energetically more economical for the plant to take up nutrients directly from soil than to uptake through the AMF hyphal network (Jakobsen et al., 2002). The effect of mycorrhizal colonization on plant growth can be explained by the ratio of the net cost (carbon allocation from plant to fungus) to the net benefit (nutrients transfer from fungus to plant). The effect of mycorrhizal colonization on plant growth will be positive when the net benefit is greater than the net cost; while, the relationship will be negative when the net cost is greater than the net benefit (Johnson et al., 1997). This can be explained by genetic or environmental factors that determine mycorrhizal-plant association benefits (Citterio et al., 2005).

Role of AMF in trace metals uptake

We showed that Cd accumulated in high concentration in sunflower shoots, where it over passed the phytotoxicity concentration defined as 5-30 mg kg^{-1} (Kabata-Pendias 2001). Here, we also found that the sunflower plants acted as Cd accumulator, which is in accordance with previous reports (Davies et al., 2002; de Andrade et al., 2008). AMF may reduce or increase metal absorption, depending on the plant and AMF species involved and on the metal concentration and speciation in soil (Audet and Charest 2008; Lingua et al., 2008). In our study, there was no difference in TM (Cd, Zn, and Cu) concentrations in roots of mycorrhized and non-mycorrhized plants. However, in the shoot tissues and under the lowest soil Cd concentration, a significant difference in TM concentration was found, where shoot Cd concentration was decreased in *G. mosseae*-inoculated plants compared to *G. irregulare*-inoculated and non-inoculated plants; in addition, *G. irregulare* caused a significant increase in shoot Cd concentration. This suggests that at low Cd concentration, *G. irregulare* can tolerate Cd stress through an increase Cd transfer from the plant roots to shoots, while *G. mosseae* potentialy increase Cd immobilization in soil. Furthermore, *G. irregulare*-inoculated plants had greater shoot Zn concertation than *G. mosseae*-inoculated and non-inoculated plants. *Glomus irregulare* and *G. mosseae*-inoculated plants had also

greater shoot Cu concentration than non-inoculated plants. Our results support the hypothesis of Audet and Charest (2007), who proposed based on a meta-analysis that mycorrhized plants enhance TM uptake compared to plants without AMF colonisation at low soil TM concentration.

At moderate and high Cd concentration in soil (10 and 30 mg kg^{-1}), although similar concentrations of Cd, Zn and Cu were found in roots of mycorrhized and non-mycorrhized plants, *G. mosseae*-inoculated plants had lower Cd and Zn shoot concentrations than *G. irregulare*-inoculated and non-inoculated plants. On the other hand, *G. irregulare*-inoculated had a greater shoot Cd concentration than non-inoculated plants, but they had similar shoot Zn concentration. This suggests again that *G. mosseae* had higher capacity to retain Cd and Zn in soil and to reduce Cd and Zn concentrations in plant shoots than *G. irregulare*. Our results agree with Li et al. (2009) who found that *Astragalus sinicus* plants inoculated with *G. mosseae* had lower shoot Cd concentrations than non-mycorrhized plants. *G. mosseae* also reduced shoot Cd concentration in *Zea mays* and *Trifolium repens*. Similarly, Janousková et al. (2007) found that different AMF isolates can decrease shoot Cd concentration.

In our study, *G. irregulare*-inoculated plants had shoot Cd BCF values greater than 1 revealing that *G. irregulare* caused active Cd transport from soil to shoot tissues whatever the Cd concentration in soil. Moreover, *G. irregulare* increased Cd transport from soil to shoot tissues higher than *G. mosseae* and than that found in non-inoculated plants, indicating that *G. irregulare*-inoculated plants had greater Cd transportation capacity from soil to shoot than those inoculated with *G. mosseae* or not colonized. We suggest that *G irregulare* is not efficient in avoiding Cd translocation to aboveground tissues. Thus *G. irregulare* may be a potential AMF candidate for Cd phytoextration. Our results are in agreement with those of de Andrade et al. (2008) who showed that *G. irregulare* inoculated plants had greater Cd accumulating capacity than non-mycorrhizal plants, suggesting that *G. irregulare* tolerated high Cd concentration in soil through a potential transfer from root to shoot. Enhanced Cd absorption in mycorrhizal plants was also found for other plants and AMF species. In our study, shoot Cd BCF of *G. irregulare*-inoculated plants was decreased

with increased Cd concentration in soil, supporting the hypothesis that the highest BCF values would be associated with low concentrations of TM in soil. In addition, our results showed that *G. irregulare*-inoculated plants had shoot Zn BCF values lower than 1, and similar to non-inoculated plants, indicating that *G. irregulare* did not affect Zn translocation from soil to aboveground tissues. Similarly, Bissonnette et al. (2010) showed that *G. irregulare*-inoculated and non-inoculated plants had similar shoot Zn BCFs, and Lingua et al. (2008) found that *G. intraradices* never affected Zn concentrations in poplar plant tissues.

In our study, when the soil Cd concentration was low, shoot Cd and Zn BCF of *G. mosseae*-inoculated plants were similar to non-inoculated plants and lower than in *G. irregulare*-inoculated plants, showing that *G. mosseae*-inoculated plants take up Cd and Zn at the same rate as non-inoculated plants. However, at moderate and high Cd concentration in soil, shoot Cd and Zn BCF of *G. mosseae*-inoculated plants were lower than in *G. irregulare*-inoculated and non-inoculated plants, suggesting that at high soil Cd concentration, Cd and Zn ions were bound to mycorrhizal structures of *G. mosseae*-inoculated roots preventing their partitioning to shoots. Here, *G. mosseae* differentially affected the Cd and Zn transportation to shoot, depending on Cd concentration level in soil, indicating that soil Cd concentration had an important effect on the role of AMF in TM uptake. Therefore, our study suggest that *G. mosseae* inoculation could alleviate Cd and Zn toxicity in host plants not only by reducing Cd and Zn concentration in shoots but also by decreasing Cd and Zn transfer from soil to aboveground tissues through an avoiding mechanism. It has been shown that AMF mycelium has a particular sorption capacity for TM (Gonzalez-Guerrero et al., 2008). Hence, we presume that the retention of Zn and Cd into soil as well as the restriction in the transfer these metals to shoot might be caused by the immobilization of these metals by the *G. mosseae* mycelium. The immobilization of Cd in soil might be due to intercellular sequestration in the AMF structures as it has been suggested by de Andrade et al. (2008). Hildebrandt et al. (2007) showed that the retention of toxic metals in mycorrhizal roots and the subsequent restriction of metal transfer to shoot, since AMF seem to filter out toxic metal by accumulating them in AMF mycelia.

Conclusion

At high soil Cd concentration, *G. mosseae* showed greater capacity than *G. irregulare* not only in Cd and Zn immobilization in soil but also in the reduction of shoot Zn and Cd concentrations compared to non-inoculated plants. Further, *G. mosseae* contributed to reduce Zn and Cd transfer from soil to aboveground tissues, suggesting the capacity of this species to alleviate metal toxicity in the host plant. We suggest the *G. mosseae* might be a suitable AMF candidate for phytostabilization processes and revegetation of TM polluted soils. On the other hand, *G. irregulare*-inoculated plants had higher shoot Cd concentration and shoot Cd BCF value than *G. mosseae*-inoculated and non-inoculated plants, suggesting that *G. irregulare* tolerate the excess stress of Cd in soil because of the higher transport of Cd from soil to aboveground plant tissues. This strongly suggests that *G. irregulare* might be a good candidate for Cd phytoextration processes. However, further investigations will be required to ascertain the role of AMF in TM uptake or immobilization, as well as the crucial function of AMF to alleviate TM toxicity in host plants.

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CHAPTER VI

General discussion and conclusion

General discussion

In this thesis, we focused on the analysis of AMF community structural differences in polluted and unpolluted sites. AMF populations that were more frequently abundant and tolerant to this harsh stress were recorded. In addition, the potential role of different AMF species in phytoremediation technology was investigated. In order to do so, we selected TM contaminated sites from industrial soil dumping and long-term N fertilized sites to investigate AMF community composition differences. The reasons motivating the choice of these kinds of pollution were because TM interfere with the food chain, disrupt the soil microbial diversity and activity, affect the quality of agricultural and polluted soils, and thereby cause a serious problem in many areas around the world. Also, TM is not biodegradable, and therefore accumulate in soils (Jarup, 2003) and reduce or even prevent the establishment of natural vegetation. On the other hand, the overuse of N-fertilization causes many detrimental effects in the environment. For examples, phosphorus (P) and nitrogen (N) pollution (Gyaneshwar et al., 2002; Sharpley et al., 2003) change the soil pH and salt concentration, contribute to the production of greenhouse gases, and then to global warming and acid rains, and reduce the biodiversity (Adesemoye and Kloepper, 2009). Moreover, the long-term use of N-fertilizer causes trace metal pollution of soil and water and increases TM concentration in plants (Long et al., 2004; Wångstrand et al., 2007; Qureshi et al., 2008; Rui et al., 2008).

VI.1. DGGE and cloning as culture-independent methods to assess AMF community structural differences in the environmental samples

In this work, we used cloning and DGGE approaches to measure AMF community structure within root and soil samples. As cloning each sample followed by sequencing a significant number of clones is highly discriminant but costly molecular approach to analyze the microbial diversity, a modified cloning-DGGE approach to allow the assessment of AMF community structure in a high number of samples. Total genomic DNA was extracted directly from root and soil samples. PCR followed by denaturing

gradient gel electrophoresis (PCR-DGGE), augmented by cloning and sequencing, as well as direct sequencing techniques, were all used to investigate AMF community structure. Using cloning method, nineteen AMF ribotypes were identified in root and soil samples. However, it should be noted that reference band positions from the clones, though useful for our study, did occasionally prove inconclusive to identify bands that migrate close together of bands that did not show up in the clone collection.

The initial investigations also showed that clones with similar nucleotide sequences migrated to the same position on DGGE; however, in some cases, clones with different sequences also migrated to a same position on DGGE gels. Thus, the similarity in electrophoretic mobility of bands in the DGGE of environmental samples with that of clones could generate misidentification of a few DGGE bands, and need to be regarded cautiously (Liang et al., 2008). Therefore, the identification of AMF populations in our study mainly was based on sequencing the original DGGE bands from all migration positions on the DGGE gels. Since our results supported that the molecular DGGE approach is a reliable, reproducible, fast, and cost effective culture-independent method to examine the community structure of multiple samples within a short period of time, the AMF community structure was analyzed based on the DGGE banding patterns, and then bands were excised from gels, their DNA reamplified by PCR, and sequenced to give more information about the community composition of samples in our work. However, it was previously estimated that any target DNA fragment that is less than 1% of the total DNA fragment pool will not be detected by DGGE; therefore, it is important to remember that only the dominant ribotypes in a community can be monitored by DGGE (Helgason et al., 1998). DGGE is the most effective to analyze samples with low diversity, being able to directly identificate each band-forming DNA fragment by sequencing. Previous studies have successfully achieved the comparison of complex microbial communities using this approach, such as for soil samples (Helgason et al., 1998). Our results showed that the DGGE method detected the dominant AMF ribotypes, which were represented by the highest number of clones detected in root or soil samples, while rare clones detected by the more discriminant cloning approach were not detected by DGGE. Although the DGGE

method does not allow the detection of the less abundant populations in the microbial community, this approach can still produce a realistic profile of the microbial diversity of environmental samples (Helgason et al., 1998; Liang et al., 2008).

VI.2. Trace metal pollution reduces AMF diversity and modifies community structure

Our results showed that trace metals reduced AMF diversity and caused AMF community structural differences in roots and rhizospheric soils of plantain plants when compared to those detected in uncontaminated soils. We found that the single plant species could harbour diverse AMF ribotypes, and also the presence of diverse AMF communities within TM contaminated areas, suggesting the ability of AMF ribotypes to tolerate metal stress and help their host to establish themselves metal polluted sites. I was suggested that metal stress induces the disappearance of less tolerant AMF species, it also promotes AMF species that are more tolerant. This was strongly supported by our data, where seven *Glomus* ribotypes were detected in the rhizospere of plantains growing on TM contaminated sites but not found in uncontaminated sites, suggesting not only the ability of these species to tolerate the toxic effects of TM, but also either their preference for these conditions or their lower competivity in the uncontaminated soils.

Our results supported the fact that *Glomus* species are frequently found in TM polluted sites, indicating that they are tolerant to polluted environments. Similarly, the dominance of *Glomus* species has also been found in other metal contaminated sites (Vallino et al., 2006; Khade and Adholeya, 2009; Sonjak et al., 2009; Yang et al., 2010). Also, predominance of *Glomus* species has been found in various habitats, such as geothermal soils (Appoloni et al., 2008), tropical forests (Wubet et al., 2004), agricultural soils (Daniell et al., 2001), and phosphate contaminated soils (Renker et al., 2005). Since *Glomus* species have the ability to propagate by mycelial fragments and mycorrhizal root fragments, and also have a higher sporulation rate, they can be better fit than other AMF species that require spore germination to establish new colonizations (Daniell et al., 2001; Pawlowska and Charvat, 2004).

In this study, ribotype of *G. mosseae* were the dominant AMF ribotypes in the rhizosphere of plantain growing on metal contaminated sites. *G. mosseae* was commonly found in soil contaminated with Zn and Pb (Turnau et al., 2001; Vallino et al., 2006; Zarei et al., 2008). Consequently, the dominance of *G. mosseae* in TM contaminated soils suggests a better tolerance of that species to TM pollution stress. The *G. irregulare* (formerly *G. intraradices*) ribotype was the most frequent AMF ribotype detected in plantain roots growing on uncontaminated and metal contaminated sites, suggesting it is tolerant to a wide range of TM concentrations and explaining its widespread distribution. The tolerance of *G. intraradices* to Zn, Pb, and Cd was examined by Pawlowska and Charvat (2004) who found that spore germination, internal and external hyphal extension, and sporulation of *G. intraradices* showed a higher tolerance to these TM than other AMF species. On the other hand, two AMF ribotypes identified as *Scutellospora calospora* and *S. gilmorei*, and three *Glomus* spp. ribotypes were only found in uncontaminated sites, suggesting a higher sensitivity of these AMF taxa to metal stress. The presence of diverse AMF in the roots and associated soil of plantain plants on TM contaminated land might be due to their capacity to tolerant polluted environments. These AMF seem to tolerate TM toxicity but also help the host plants in tolerate and establishing in TM contaminated soils.

VI.3. The effect of long-term N-fertilization on AMF community structure

Long-term application of N-fertilization increase TM pollution for soil and underground water. Mineral N-fertilizers contain different trace metals and affect the soil metal concentration; for example, the long-term use of mineral N-fertilizer was shown to result in an increase in Cd concentration in soil and wheat grains (Wångstrand et al., 2007). Additionally, since organic manure may also contain different metals, its use can cause trace metal pollution of soil and water (Long et al., 2004; Qureshi et al., 2008). The effect of long-term nitrogen (N) fertilizations (manure and inorganic NH_4-fertilization) on AMF community structure and on the growth of sunflower plants was investigated.

Indigenous AM fungi were used as mycorrhizal inoculum since these fungi may have better adaptation to long-term N-fertilization than non-indigenous AM fungi (Bhadalung et al., 2005). Our results showed that the different forms of N-fertilization had a significant effect on AM fungal community structure, mycorrhizal colonization percentage, and plant growth; however, no significant effect on AMF species diversity indices and richness (number of AMF ribotypes) was found. Again, the dominance of *Glomus* specific species was observed in roots and soils of control and N-fertilization treatments.

The AMF community structure of sunflower roots grown in manure fertilized soils was different from that found in plants grown on NH_4-fertilized or control non-fertilized soils. *Glomus irregulare* ribotypes were more frequently found in manure fertilized soils, three distinct *Glomus* spp. ribotypes were most abundant in NH_4-fertilized and control treatments. Manure increased the concentration of P, K, and Mg in soils, as well as soil pH, which might favor proliferation of some AMF species such as *G. irregulare* to colonize sunflower roots under our experimental conditions. On the other hand, changes in soil properties caused by manure application could create unfavorable conditions that reduce root colonization and survival of other AMF ribotypes. Wang et al. (2009) suggested that manure application improved soil properties by increasing nutrient concentration, promoting the proliferation of *Glomus mosseae* and inhibiting that of *Scutellospora pellucida*.

Our results showed that AMF community structure was not different between NH_4-fertilization and control treatments, where the same *Glomus* ribotypes were more frequently found. The similarity in soil nutrients (P, K, and Mg) concentration in the control and NH_4-fertilized soils may be the cause of the similarity in root-colonizing AMF community structure. However, NH_4-fertilization reduced the number of AMF ribotypes compared to the control treatment. A negative effect of mineral N-fertilization on AMF community structure and on the AMF ribotype number in plant roots was also found in other studies (Santos et al., 2006; Toljander et al., 2008).

We found that changes in AMF community structure were associated with plant biomass production under control and N-fertilization treatments. Manure-fertilized plants (in which *G. irregulare* was abundant) produced a greater biomass than those hosting *Glomus* ribotypes B7 and B8. Also, NH_4-fertilized plants, in which *Glomus* ribotypes B9 and B10 were detected, had a higher biomass production than those harbouring *Glomus* ribotypes B7 and B8. Our results are consistant with other studies where different AMF species showed different effect on plant growth and N uptake in N-fertilized soils (Hawkins and George, 2001; Guo et al., 2006; Tu et al., 2006). van der Heijden et al. (1998) demonstrated that change in AMF community structure affected plant community composition and plant growth. Under similar circumstances, different AMF species varied in their functional traits such as scavenging and transfering of nutrients from soil to host plants, stabilization of soil particles, water uptake, and protecting the host roots against pathogens, as well as the amount of carbon taken up from the host plant (Jansa et al., 2005; Cavagnaro et al., 2005). Our results showed that plants inoculated with the poorer AMF inocula produced greater biomass than plants inoculated with the richer inocula. Similarly, Mickelson and Kaeppler (2005) observed that maize plants were inoculated with one AMF species, they produced greater biomass than those inoculated with six AMF species. Jansa et al. (2008) also found that inoculation with many AMF species reduced plant growth compared to inoculation with single AMF species. It may be assuming that it have been suggested that it is less costly for host plants to harbour single strain instead of multiple strains (Johnson 1993; Kiers et al., 2002; Egerton-warburton 2007; Johnson 2008).

VI.4. Differential effect of AMF on trace metals uptake under cadmium contamination stress

The effect of the AMF species *G. irregulare* and *G. mosseae* on growth and uptake of Cd, Zn, and Cu by sunflower plants grown on Cd contaminated soil was investigated. Cd is a nonessential element that is highly toxic for plants and mycorrhizal fungi. However, the mutalistic interactions between plants and AMF species have been proposed as an important factor in TM tolerance and uptake or immobilization of TE by plants (Gonzalez-

Chavez et al., 2002; Hall, 2002; Hildebrandt et al., 2007). High soil Cd concentrations have been shown to inhibit mycorrhizal root colonization (Weissenhorn and Leyval, 1995; Vivas et al., 2003). In contrast, our results showed that whatever the Cd concentration in the soil, there is no effect on root colonization by *G. irregulare* and *G. mosseae*, suggesting a high capacity of these strains to colonize plant roots under Cd stress. This agrees with other studies that found that mycorrhizal root colonization and the amount of fungal mycelium produced by AMF were not affected by Cd contamination (Rivera-Becerril et al., 2002; de Andrade et al., 2008).

In our study, *G. irregulare* slightly increased plant biomass production compared to non-inoculated or *G. mosseae*-inoculated plants at high soil Cd concentration and did not significantly affect plant biomass production at low and medium soil Cd concentrations. In contrast, *G. mosseae* inoculated plants had significantly lower biomass compared to non-inoculated or *G. irregulare*-inoculated plants. That could be because the inoculation with *G. irregulare* increased the uptake of other elements such as Cu and Zn while *G. mosseae* caused a significant reduction in the uptake of these metals. Plant biomass of non-inoculated plants lowered by 20% at the highest soil Cd concentration compared to those grown in the lowest soil Cd concentration. Whereas, there was no significant difference found in plant biomass of mycorrhized plants grown in different soil Cd concentration. Mycorrhized plants showed higher photosynthetic pigments contents in the presence of Cd than non mycorrhized plants (Kapoor et al., 2007; Andrade et al., 2009). The interaction between plants and AMF are mutualistic (Smith and Read, 2008); however, neutral or negative plant growth responses to AMF have been found (Johnson et al., 1997; Citterio et al., 2005). In some circumstances, it is less costly for the plant to take up nutrients directly from soil than to take it up from AM hyphae and to donate C to the AM fungi (Jakobsen et al., 2002; Citterio et al., 2005).

We found that sunflower plants accumulated high Cd concentration in shoot tissues, where the Cd concentration reached a higher level than the phytotoxicity concentration defined as 5-30 mg kg^{-1} for Cd (Kabata-Pendias, 2001). AMF reduced or increased Cd and Zn uptake, depending on the AMF species and metal concentration in the soil. At the

highest soil Cd concentrations, *G. irregulare*-inoculated plants had higher shoot Cd concentration than non-inoculated and *G. mosseae*-inoculated plants. The shoot Cd and Zn concentrations of *G. mosseae*-inoculated plants were significantly lower than in non-inoculated and *G. irregulare*-inoculated plants. This suggests that *G. mosseae* had higher capacity to retain Cd and Zn in the soil and to reduce Cd and Zn concentrations in plant shoots than *G. irregulare* and non mycorrhized plants when the soil had a high Cd concentration. Our results are in accordance with other studies, which found that *G. mosseae* reduced shoot Cd concentration in *Astragalus sinicus, Zea mays,* and *Trifolium repens* (Vivas et al., 2003; Chen et al., 2004; Li et al., 2009).

In this study, *G. irregulare* caused active Cd transport from soil to shoot tissues at any Cd concentration in soil, while *G. irregulare-inoculated* plants had shoot Cd BCFs values greater than 1. Moreover, our results showed that *G. irregulare* increased Cd transfer from soil to shoot tissues more than *G. mosseae* and non-inoculated plants. We suggest that *G. irregulare* tolerate the excess stress of high Cd concentration in soil through the transfer of more Cd from root to shoot, thereby becoming a suitable candidate for Cd phytoextration. Our results agree with those of de Andrade et al. (2008), who showed that *G. irregulare*-inoculated plants had greater Cd accumulating capacity than non-mycorrhized plants. Enhanced Cd absorption in mycorrhizaed plants was also found for other plant and AMF species (Rivera-Becerril et al., 2002; Hutchinson et al., 2004). In addition, the results showed that *G. irregulare* did not affect Zn translocation from soil to aboveground tissues.

In this study, when soil had low Cd concentration, shoot Cd and Zn BCF of *G. mosseae*-inoculated plants were similar to non-inoculated plants and lower than *G. irregulare*-inoculated plants. However, at moderate and high soil Cd concentration, shoot Cd and Zn BCF of *G. mosseae*-inoculated plants were lower than *G. irregulare*-inoculated and non-inoculated plants. Here, *G. mosseae* differentially affect the Cd and Zn transportation to shoots, depending on the Cd concentration in the soil, indicating that soil Cd concentration had an important effect on the role of AMF in TM uptake. We presume that the retention of Zn and Cd in the soil, as well as the restriction in the transfer of these

metals to shoots might be caused by the immobilization of these metals by the *G. mosseae* mycelium. It has been shown that AM fungal mycelium has a particular sorption capacity for trace metals (Joner et al., 2000; Gonzalez-Guerrero et al., 2008). Therefore, our study suggests that *G. mosseae* inoculation could alleviate Cd and Zn toxicity in host plant not only by reducing Cd and Zn concentration in shoots but also by decreasing Cd and Zn transfer from soil to aboveground tissues through an avoiding mechanism. Thus, we suggest that *G. mosseae* could inhibit Cd contamination through the food chain, and increase the effectiveness of phytostabilization and revegetation of metal polluted sites.

Conclusion

Trace-metal contamination and long-term N fertilization are ones of the environmental factors that influence and modify AMF community structure in the rhizosphere of plants. Although TM contamination reduced AMF diversity in rhizospheres, it did not completely inhibit their growth or the establishment of mycorrhizae. Furthermore, the presence of various AMF in the roots and associated soil of plants growing on TM contaminated sites suggests that AMF diversity contributes a critical functional component in disrupted environments. The ability of indigenous AM fungi to colonize roots in long-term manure or NH_4-fertilized soil was decumented, however variation of the AMF community structures was observed in both manure and NH_4 fertilization. NH_4-fertilization reduced the AMF ribotype number, whereas manure increased it. The main goal of N-fertilization application is to increase soil fertility and production; however, the intensive use of N-fertilization resulted in a lost of nutrients, leaching in nearby water and metal pollution, as well as modifying the AMF community structure that may positively or negatively affect plant productivity. The predominance of *G. mosseae* in TM polluted sites suggests the tolerance of this taxon to TM stress. *Glomus mosseae* showed a high capacity not only in Cd and Zn immobilization in the soil, but also in the reduction of shoot Zn and Cd concentrations. Further, *G. mosseae* contributed to reduce Zn and Cd transfer from soil to aboveground tissues, suggesting the high potential of this species to alleviate metal toxicity in host plants. Thus, this AM fungus may be a suitable candidate for

phytostibilization. *Glomus irregulare*-inoculated plants had higher shoot Cd BCF than *G. mosseae*-inoculated and non-inoculated plants, suggesting that *G. irregulare* tolerates the excess stress of Cd in soils through a mediation of the transport of more Cd from the soil to the aboveground plant tissues. Since the role of *G. irregulare* to the transfer of Cd from soil to shoot, this species might have more potential or Cd phytoextraction. However, further investigations will be required to ascertain the role of AMF in TM uptake or immobilization, as well as the crucial function of AMF in alleviating TM toxicity in host plants.

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