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AM fungal contribution to sunflower (*Helianthus annuus* L.) in phytoremediation of Ni treated soils

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Abstract

The main objective of this study was to examine the contribution of arbuscular mycorrhizal (AM) colonization on nickel (Ni) uptake and Ni tolerance in sunflower (*Helianthus annuus* L.) at a vegetative or reproductive stage of development. The combined effect of AM colonization, Ni input, and nitrogen (N) fertilization on N-assimilation in sunflower plants was also investigated. Furthermore, concerns over the transfer of heavy metals (HMs) to higher trophic levels led us to investigate whether the AM colonization and accumulation of Ni within plant tissues would induce synthesis of secondary defense compounds. It was hypothesized that AM colonization increases Ni content and plant Ni tolerance, the activities of N-assimilating enzymes (nitrate reductase, NR; glutamine synthetase, GS; and glutamine dehydrogenase, GDH), and induces the accumulation of sesquiterpene lactones (STLs), in sunflower grown under soil Ni conditions. It was also hypothesized that N-type fertilization affects ammonium assimilation as the activities of GS and GDH would be enhanced in plants supplied with an NH_4^+ as compared to a NO_3^- fertilizer. To verify these hypotheses, three greenhouse experiments were performed with sunflower cv."Lemon Queen", with or without the AM fungus, *Glomus intraradices* Schenck & Smith, and treated with (1) 0 or 100 mg Ni kg^{-1} dry soil (DS) at the reproductive stage, and supplied with NO_3^- or NH_4^+ fertilizer; (2) 0, 100, 200 or 400 mg Ni kg^{-1}, at the reproductive stage and supplied with a complete NH_4NO_3 fertilization; and (3) 0, 200 or 400 mg Ni kg^{-1}, at the vegetative stage and supplied with a complete NH_4NO_3 fertilizer. The overall results indicated that AM colonization significantly enhances Ni content in sunflower plants, exposed to a moderate soil Ni level of 100 mg Ni kg^{-1}, at the reproductive stage. Furthermore, at 100 mg Ni kg^{-1}, the AM plants had a significantly higher shoot Ni extracted %, suggesting that the AM symbiosis contributed to Ni uptake and its translocation from roots to shoots. The AM contribution to plant Ni content and Ni extracted % were significantly higher in plants supplied with NO_3^- than with NH_4^+. Moreover, the plant biomass and shoot height were significantly higher in plants supplied with NO_3^- than with NH_4^+. In late Ni exposed sunflower, the

AM colonization significantly increased the Ni extracted % at 400 mg Ni kg^{-1}, yet also resulted in a biomass reduction of 45% as compared to only 14% at 100 mg Ni kg^{-1}. Furthermore, a soil [Ni] of 400 mg Ni kg^{-1} was toxic to sunflower directly seeded in Ni treated soils, as all seedlings died within four weeks after sowing. The mineral concentrations were enhanced in AM plants, especially at lower soil Ni treatments. It is therefore concluded that the AM contribution to Ni uptake was optimal at 100 mg Ni kg^{-1}. The AM colonization also contributed to enhance the activities of N-assimilating enzymes, especially under NH_4^+ fertilization. Moreover, our results showed that the effects of HM stress and N fertilization were linked, as the activities of NR, GS, and GDH were significantly enhanced in plants under NH_4^+ and at 100 mg Ni kg^{-1}. These results suggest that the combined treatments of soil Ni input and NH_4^+ nutrition enhance N assimilation via concurrent activities of the GS/GOGAT and GDH pathways. We also observed that both soil Ni input and AM colonization lead to an accumulation of STLs in sunflower leaves. In addition, the combination of AM colonization and soil Ni input would result in a synergistic effect to maximize defense properties while minimizing energy expenditure. These findings support the hypothesis that the AM symbiosis contributes to enhanced Ni uptake and Ni plant tolerance. It is therefore concluded that sunflower, especially in association with AM fungi, shows promise as a "candidate" species in phytoremediation strategies.

Résumé

Notre objectif principal visait à étudier la contribution de la colonisation arbusculaire mycorhizienne (AM) sur l'absorption du nickel (Ni) et de sa tolérance chez le tournesol (*Helianthus annuus* L.), et ce au stade végétatif ou reproductif de son développement. Les effets combinés de la colonisation AM, d'intrant de Ni ainsi que de la fertilisation en azote (N) sur son assimilation ont aussi été étudiés. Par ailleurs, les risques que des métaux lourds puissent être transférés à des niveaux trophiques supérieurs ont suscité la poursuite de nos recherches sur l'impact de la colonisation AM et de l'accumulation de Ni chez le tournesol sur la synthèse de composés secondaires de défense. Notre première hypothèse stipule que la colonisation AM augmente la teneur et la tolérance au Ni, l'activité des enzymes assimilatrices de N (NR, GS et GDH), et induit l'accumulation des sesquiterpènes lactones (STLs) chez le tournesol avec intrant de Ni dans le sol. Notre seconde hypothèse propose que le type de fertilisation en N affecte l'assimilation de l'ammonium via des activités accrues de la GS et de la GDH chez les plantes de tournesol fertilisées plutôt avec le NH_4^+ qu'avec le NO_3^-. Pour tester ces deux hypothèses, trois expériences en serre ont été réalisées avec le tournesol, cv "Lemon Queen", avec ou sans inoculation du champignon AM, le *Glomus intraradices* Schenck & Smith, en fonction des traitements suivants : (1) 0 ou 100 mg Ni kg^{-1} de sol sec, au stade reproductif, avec fertilisation de type NO_3^- ou NH_4^+; (2) 0, 100, 200 ou 400 [Ni], au stade reproductif avec fertilisation NH_4NO_3; et (3) 0, 200 ou 400 [Ni], au stade végétatif avec fertilisation NH_4NO_3. Dans l'ensemble, nos résultats ont montré que la colonisation AM a significativement contribué à augmenter la teneur en Ni dans le tournesol au stade reproductif, à 100 mg Ni kg^{-1} dans le sol. À cette même concentration, les plantes AM avaient un pourcentage d'extraction du Ni significativement plus élevé dans les parties aériennes, suggérant ainsi que la symbiose AM a contribué à l'absorption du Ni, puis à son transport depuis les racines, et ce de façon plus significative chez les plantes fertilisées avec le NO_3^-. De plus, la biomasse et la hauteur des plantes étaient significativement plus élevées sous fertilisation NO_3^- que NH_4^+. Au stade reproductif, la colonisation AM

a significativement augmenté le pourcentage d'extraction du Ni à 400 mg Ni kg^{-1} du sol, réduisant cependant 45% de la biomasse des plantes comparativement à seulement 14% à 100 mg Ni kg^{-1}. De plus, le niveau de toxicité du Ni, chez le tournesol au stade végétatif, a été évalué à 400 mg Ni kg^{-1}, car toutes les plantules ont dépéri quatre semaines au plus après l'ensemencement. Les concentrations des minéraux étaient plus élevées chez les plantes AM, et ce particulièrement pour les traitements moins élevés en Ni. Nous concluons que la colonisation AM a été optimale dans l'absorption du Ni avec l'ajout de 100 mg Ni kg^{-1} dans le sol et a contribué à l'augmentation des activités des enzymes assimilatrices de N, particulièrement avec le NH_4^+ comme fertilisant. De plus, nos résultats suggèrent que les effets de stress causés par le Ni ou autres métaux lourds d'une part, et la fertilisation en N d'autre part, sont étroitement liés, en ce sens que les activités de NR, GS et GDH étaient significativement accrues dans les plantes sous 100 mg Ni kg^{-1} et NH_4^+. Ces résultats ont montré que les intrants de Ni et de NH_4^+ ont conduit à une assimilation accrue en N, via les activités concurrentes de la GDH et du cycle GS/GOGAT. Nous avons aussi montré que l'ajout de Ni et la colonisation AM ont conduit à l'accumulation des STLs dans les feuilles du tournesol, suggérant ainsi un effet de synergie dans la maximisation des modes de défense tout en minimisant les dépenses d'énergie. Ces résultats appuient l'hypothèse que la symbiose AM contribue à une absorption accrue du Ni ainsi qu'à sa tolérance. Le tournesol, particulièrement en symbiose AM, peut être considéré comme un 'candidat' prometteur dans les stratégies de phytoremédiation.

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List of Abbreviations

AM	Arbuscular mycorrhiza
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Carbon
Chl	Chlorophyll
Cys	Cysteine
dH_2O	Distilled water
DAS	Days after sowing
DM	Dry mass
DMSO	Dimethylsulfoxyde
DPP	3,3-dimethylallylpyrophosphate
DS	Dry soil
DTT	Dithiothreitol
ECM	Ectomycorrhiza
EDTA	Ethylenediamine-tetra-acetic-acid
F	Fertilizer
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FPP	Farnesyl pyrophosphate
GDH	Glutamate dehydrogenase
GH	Gutamylhydroxamate
Glu	Glutamate
Gly	Glycine
GOGAT	Glutamate synthase
GPP	Geranyl pyrophosphate
GS	Glutamine synthetase
His	Histidine
HEPES	N-2-hydroxypiperazine-N-2-ethanesulfonic acid
HM	Heavy metal
HPLC	High pressure liquid chromatography
IPP	Isopentyl pyrophosphate
K_m	Michaelis-Menten constant
LANS	Long Ashton Nutrient Solution
M–	Treatment without AM
M+	Treatment with AM
MIE	Mycorrhizal inoculation effect
MT	Metallothionein
MW	Molecular weight
N	Nitrogen

NADH	Nicotinamide adenine dinucleotide (reduced form)
NED	N-1-naphthylethylene-diamine-dihydrochloride
NH_4^+	Ammonium
Ni	Nickel
NiR	Nitrite reductase
NO_3^-	Nitrate
NR	Nitrate reductase
OD	Optical density
P	Phosphorus
PC	Phytochelatin
PVLG	Polyvinyl-alcohol-lactic acid-glycerol
SE	Standard error
STL	Sesquiterpene lactone
TCA	Tricholoroacetic acid
TCD	Thermal conductivity detector

Chapter 1

General Introduction

Anthropogenic sources of contaminants, including mining and smelting of metalliferous ores, municipal wastes, fertilizer and pesticide uses, contribute to the growing problem of environmental pollution. Current physico-chemical remediation methods are costly and environmentally disruptive as they remove beneficial fungi and microorganisms from soils. Phytoremediation, the use of plants to remediate soils from organic and inorganic contaminants, is currently being developed and targeted for commerialization as this method is more environmentally friendly and less costly than conventional methods. High biomass, metal tolerant plant species such as sunflower, *Helianthus annuus* L., has been proposed for phytoremediation practices (Chen *et al.* 2001, Lesage *et al.* 2005, Mei *et al.* 2002). Furthermore, the use of mycorrhizal fungi in phytoremediation strategies is being investigated as their symbioses with roots in metalliferous soils have been shown to enhance tolerance and accumulation of metals in host plants (Killhan and Firestone 1983, Li *et al.* 1991, Turnau and Mesjasz-Przybylowicz 2003). However, only a few studies have looked into the potential of mycorrhizal symbiosis and sunflower in phytoremediation (Davies *et al.* 2001, 2002). To our knowledge, this is the first study conducted on the impact of mycorrhizal fungi on the uptake and tolerance of Ni in sunflower.

1.1 Mycorrhizal fungi

The term symbiosis was first defined by de Bary in 1887, as "the living together of differently named organisms" (Raina *et al.* 2000). Depending on the fitness of the two

symbionts living independently or in association, this relationship can either be mutualistic (in which all the organisms involved derive some benefits) or parasitic (in which one organism benefits to the detriment of the other) (Raina *et al.* 2000). However, this relationship is not static, and changes in environmental conditions can lead to changes in the symbiosis; i.e. a relationship that starts out as mutualistic may become parasitic or *vice versa* (Raina *et al.* 2000).

1.1.1 Evolution of mycorrhizal fungi

Colonization of land by the ancestors of vascular plants has long been speculated to have been facilitated by symbiotic organisms such as mycorrhizal fungi (Brundrett 2002, Raina *et al.* 2000, Smith and Read 1997). Fossil records dating as early as the Early Devionian, 410-360 million years ago, from the Rhynie chert flora revealed both spores and arbuscules, within the protosteles of the primitive vascular plant *Aglaophyton major* (Kerp 2002, Raina *et al.* 2000, Smith and Read 1997). These structures are characteristic of modern members of endomycorrhizal fungi of the phylum Zygomycetes, order Glomales (Smith and Read 1997). These fossils provide examples of primitive plant-fungus associations and insight into the significance of their existence (Brundrett 2002, Smith and Read 1997).

1.1.2 Types of mycorrhizal fungi

Mycorrhizal fungi are chemoheterotrophic as they derive their carbon compounds from either nonliving organic material or living tissues, with concomitant exchange of absorbed nutrients to the host plant (Raina *et al.* 2002, Smith and Read 1997). This biotrophy, whereby only minimal host tissue damage occurs from the bilateral transfer of nutrients, forms the basis of symbiosis involving fungi and plant roots (Raina *et al.* 2002). Mycorrhizal fungi are ubiquitous organisms that associate with as many as 90%

of plant species; hence the name mycorrhiza from the Greek words *mukês* and *rhiza*, meaning fungus and root, respectively (Raina *et al.* 2002, Smith and Read 1997).

The benefits to the host plant derived from mycorrhizal associations include an increase in plant growth by increasing plant access to relatively immobile minerals such as P, improving soil texture by binding soil particles into stable aggregates that resist wind and water erosion, protection from root pathogens (nematodes and fungi), tolerance to adverse conditions such as temperature, high salinity, high or low pH, increase in rhizospheric microflora, enhanced uptake of water and tolerance to toxic heavy metals (Gaur and Adholeya 2004, Gupta *et al.* 2000). The mycorrhizal fungi benefit from this symbiosis by receiving 4% to 20% of photosynthates (Smith and Read 1997).

The three basic functional components of the mycorrhizal symbiosis are: (1) the fungal mycelium that explores large volumes of soil and helps in retrieving mineral nutrients; (2) the fungus-plant interface where the nutrient transfer occurs; and (3) the fungal tissues which produce and store carbohydrate (Raina *et al.* 2000). There are two main types of mycorrhizae (Figure 1.1): (i) endotrophic or endomycorrhiza, which has no fungal sheath around the root but has intercellular penetration of the host by the fungus and (ii) ectotrophic or ectomycorrhiza (Raina *et al.* 2000). Ectomycorrhizal fungi are almost exclusively of the phyla Basidiomycetes and Ascomycetes, most of which form symbioses with woody perennials from the Pinaceae (boreal forests), the Fagaceae (northern temperate forests), and the Myrtaceae family (temperate and subtropical regions of the souther hemisphere) (Gupta *et al.* 2000, Smith and Read 1997). The ectomycorrhizal root is characterized by a fungal sheath mantle which encloses the hyphae that grow between the epidermal and cortical root cells called the Hartig net (Smith and Read 1997).

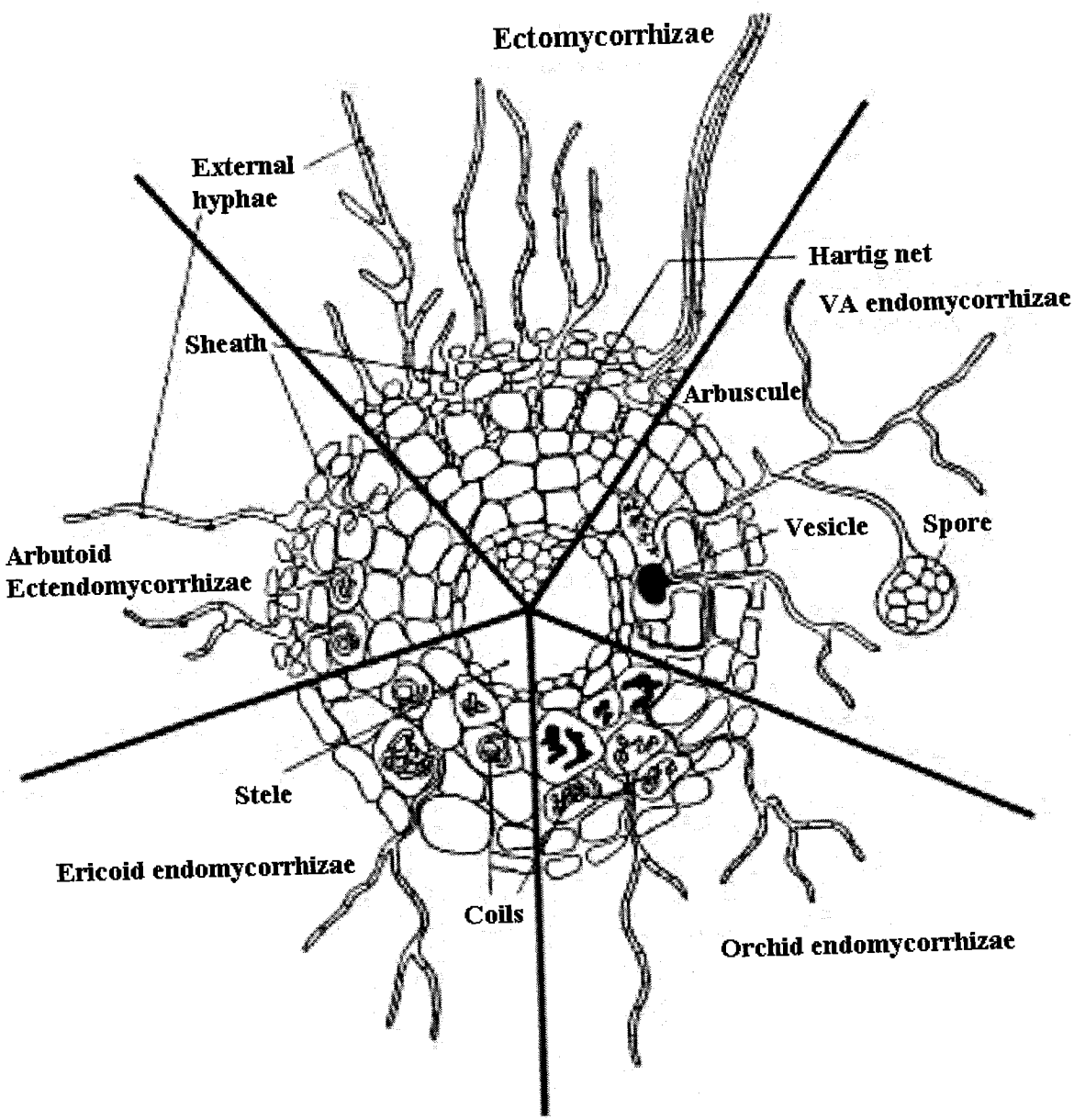
Other types of mycorrhizae include (Figure 1.1): (i) ectendomycorrhiza, (ii) arbutoid

ectendomycorrhiza, (iii) monotropoid mycorrhiza, (iv) ericoid endomycorrhiza and (v) orchid endomycorrhiza (Raina *et al.* 2000). Ectendomycorrhizae share many of the same characteristics as ectomycorrhizae, but differ in that penetration of root cells from the Hartig net or from the hyphae occurs (Smith and Read 1997). Arbutoid ectendomycorrhizae are characterized by intracellular penetration of the epidermal cells by Hartig net or by mantle (Gupta *et al.* 2000, Smith and Read 1997). Ericoid mycorrhizae are unique in that the role of the mycobiont is not to aid in increasing the absorptive surface area, but to release enzymes and other exudates into the substrate to make recalcitrant substances available to the plant (Gupta *et al.* 2000, Smith and Read 1997). Orchid endomycorrhizal association is not usually considered to be mutually beneficial, as the mycorrhizal fungi provide a supply of carbohydrate to the orchid seedling, referred to as a protocorm, thereby inducing germination (Smith and Read 1997).

Arbuscular mycorrhizae

Endomycorrhizae are most commonly referred to as arbuscular mycorrhizae (AM) as the arbuscule formation is distinctive to this type (Smith and Read 1997). The AM fungi belong to phylum Zygomycetes of the order Glomales, subdivided in two suborders, the Glomineae and Gigasporineae (Gupta *et al.* 2000). The AM inoculum used in this study, *Glomus intraradices*, belong to the Glomineae suborder, Glomaceae (Gupta *et al.* 2000).

Figure 1.1 Diagram of mycorrhizal types. Reprinted from Trends in Ecology and Evolution, Vol. 13, Selosse and Le Tacon. The land flora: a phototroph-fungus partnership? pp. 15-20. Copyright (1998) with permission from Elsevier Science.


Ectomycorrhizae
External hyphae
Hartig net
VA endomycorrhizae
Sheath
Arbuscule
Vesicle
Spore
Arbutoid Ectendomycorrhizae
Stele
Ericoid endomycorrhizae
Coils
Orchid endomycorrhizae

Colonization of roots by AM fungi can arise from three sources: spores, colonized root fragments and hyphae - collectively termed propagules (Smith and Read 1997). The AM fungi, the most complex group of mycorrhizae, form extra- and intraradical structures: (i) intracelullar hyphae forming coils in the outer layers of cortical parenchyma; (ii) the intercellular hyphae; (iii) the intracellular hyphae with numerous ramifications (arbuscules); and (vi) the inter or intracellular hypertrophied hyphae (vesicles) (Raina *et al.* 2000).

The arbuscules, short-lived ($\sim$ 7 days) and highly branched structures, are formed in the inner cortex and are believed to be the site for nutrient exchange between the fungus and root, although it is uncertain whether or not they are involved in carbohydrate transfer to the fungus (Smith and Read 1997). Vesiclular formation generally occurs in most endomycorrhizal fungal species; *Scutellospora* and *Gigaspora* species do not develop vesicles but instead produce auxiliary cells on the extraradical mycelium (Smith and Read 1997). Vesicles may be spherical, oval or lobed and develop in either inter- or intracellular positions in the cortex (Raina *et al.* 2000, Smith and Read 1997). They serve as the endophytic storage organs and are rich in lipids (Raina *et al.* 2000). Colonization of roots reaches a maximum at about 8 weeks, when the mycorrhizal fungus may contribute as much as 20% of root dry mass (Smith and Read 1997).

1.2 Nitrogen metabolism

1.2.1 Nitrogen-assimilating enzymes

Nitrogen (N) is one of the most limiting nutrients in soil, owing in part to the large demand by plants for optimal growth (2 to 5% of plant dry mass) (Marschner 1995). Plants can assimilate inorganic forms such as nitrate (NO_3^-) and ammonium (NH_4^+), and organic forms such as urea (Crawford 1995).

1.2.2 Nitrate assimilation

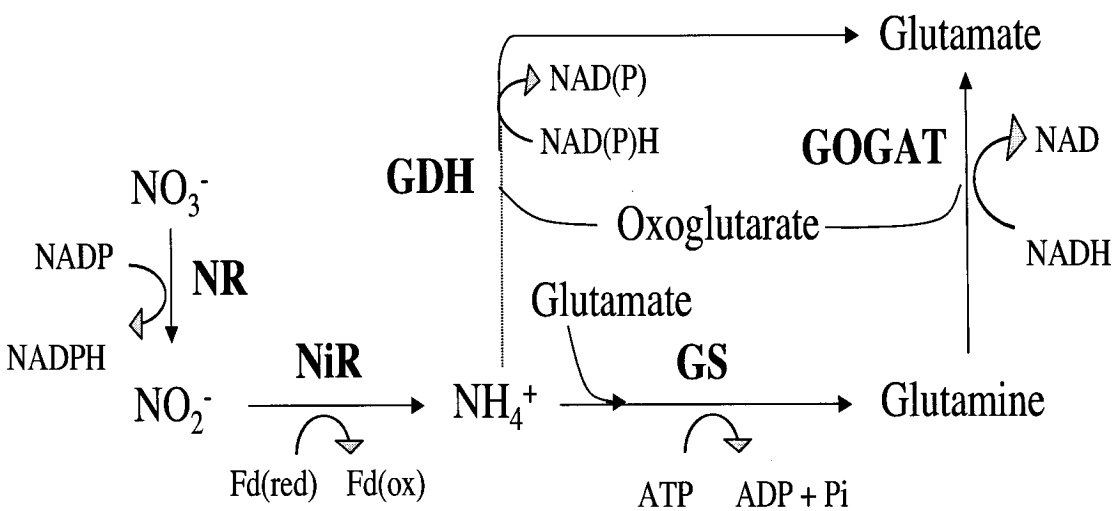
The assimilation of nitrate proceeds rapidly, as NO_3^- must first be reduced to NH_4^+ within plants to become available for amino acid synthesis. Roots and shoots are capable of nitrate assimilation but its assimilation depends on various factors, including the level of nitrate supply, the plant species, or age (Marschner 1995). In most plants, reduction takes place in the leaves as it demands less energy (Campbell 1988, Marschner 1995). Nitrate is assimilated to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR) (Figure 1.2). Nitrite enters the chloroplasts where it is further reduced to NH_4^+ by nitrite reductase (NiR) (Inokuchi *et al.* 2002).

Nitrate reductase (NR, E.C.1.6.6.1)

Nitrate reductase (NR), a substrate induced cytoplasmic enzyme, is labile, both *in vivo* and *in vitro*, and difficult to purify; as such, the reduction of nitrate to nitrite is considered the rate limiting step in the N assimilation pathway (Campbell 1988, Naik *et al.* 1982). Thus, NR activity, NR protein amount and NR mRNA accumulation increase in response to nitrate (Campbell 1988, Hoff *et al.* 1992, Naik *et al.* 1982). However, its activity is low relative to other enzymes in the pathway (NiR, GS, GOGAT, GDH) (Naik *et al.* 1982).

Figure 1.2 Schematic diagram of nitrogen assimilation pathway in higher plants. *Abbreviations:* NR, nitrate reductase (E.C.1.6.6.1), NiR, nitrite reductase (E.C.1.7.7.1), GS, glutamine synthetase (E.C.6.3.1.2), GOGAT, glutamate dehydrogenase (E.C.1.4.1.14), GDH, glutamate dehydrogenase (E.C.1.4.1.2).

(from Subramanian 1997, printed with permission from C. Charest, University of Ottawa).


Glutamate
NAD(P)
NAD(P)H
GDH
GOGAT
NAD
NO_3^-
Oxoglutarate
NADP
NR
NADH
Glutamate
NADPH
NiR
GS
NO_2^-
NH_4^+
Glutamine
Fd(red)
Fd(ox)
ATP
ADP + Pi

NR is a homodimer or homotetramer composed of two identical subunits of 110 kDa (Crawford 1995). Each NR subunit contains three prosthetic groups, a flavin adenine dinucleotide (FAD), a heme (cytochrome b_{557}), and a molybdenum cofactor, each of which represents a redox center (Campbell 1988, Hoff *et al.* 1992). Most higher plants and algae contain a NADH-specific NR (E.C.1.6.6.1). Two other isoforms of NR have also been shown: a NAD(P)H-bispecific NR (E.C.1.6.6.2) found in a limited number of higher plants, and a NADPH-specific (E.C.1.6.6.3) isoform (Hoff *et al.* 1992).

Nitrite reductase (NiR, E.C.1.7.7.1)

Similar to NR, NiR is a substrate inducible enzyme which catalyzes the ferredoxin-dependent reduction of nitrate to ammonium (Ahmad and Hellebust 1991, Inokuchi *et al.* 2002, Oaks and Hirel 1985). The NADPH-dependent NiR is a monomer of 60-64 kDa containing a 4Fe-4S cluster at the active site (Crawford 1995, Inokuchi *et al.* 2002). NiR is localized in the plastids of roots and leaf chloroplasts of C_3 plants, and in chloroplasts of mesophyll cells of C_4 plants (Inokuchi *et al.* 2002).

1.2.3 Ammonium assimilation

The assimilation of NH_4^+ occurs predominantly in the roots with only a small proportion translocated to the shoots (Crawford 1995, Inokuchi *et al.* 2002). Prior to 1970, it was assumed that NH_4^+ was directly incorporated into glutamate via glutamate dehydrogenase (GDH). However, GDH has a relatively low affinity for NH_4^+ (K_m 7 to 100 mM) and functions both in the aminating and deaminating directions, but the *in vivo* aminating activity has yet to be demonstrated (Lea *et al.* 1990, Lea and Miflin 2003, Miflin and Habash 2002, Smith *et al.* 1985). The discovery of NAD(P)H glutamate synthase (GOGAT) in bacteria (Tempest *et al.* 1970) and later ferrodoxin-dependent GOGAT in plants (Lea and Miflin 1974), established an alternative pathway, the glutamate synthase

cycle, whereby NH_4^+ was assimilated via the combined activities of GS and GOGAT (Lea *et al.* 1990, Miflin and Habash 2002). Although, there has recently been a revival of interest in the role of GDH, the glutamate synthase cycle is considered to be the main mechanism of NH_4^+ assimiliation in higher plants (Lea *et al.* 1990, Miflin and Habash 2002, Oaks 1994).

Glutamine synthetase (GS, E.C.6.3.1.2)

GS catalyzes the formation of glutamine from ammonium and glutamate with the simultaneous hydrolysis of ATP with Mg^{2+} as a cofactor (Ahmad and Hellebust 1991, Oaks and Hirel 1985). GS is localized in the chloroplasts and cytosol in leaves and the cytosol in roots (Miflin and Habash 2002, Oaks and Hirel 1985). The activity of GS is usually higher in leaf than root (Lea *et al.* 1990).

GS occurs in two major octameric forms, with the plastidic form (GS2) being the most predominant (Inokuchi *et al.* 2002, Lea *et al.* 1990, Miflin and Habash 2002). Cytosolic GS (GS1) is present mainly in non-green tissues such as seeds, roots, flowers, nodules, and the phloem companion cells in leaves (Inokuchi *et al.* 2002, Miflin and Habash 2002, Oaks 1993). The two isoforms differ in that GS1 is not induced by light and is less labile than GS2 (Ahmad and Hellebust 1991, Miflin and Habash 2002, Oaks 1994). The GS2 isoform is composed of 43 kDA polypeptides encoded by a single gene, while the GS1 isoform is encoded by 4-6 genes and is composed of two 39 kDa polypeptides, designated as α- and β subunits (Inokuchi *et al.* 2002, Lea *et al.* 1990).

Glutamate synthase (GOGAT, E.C.1.4.7.1)

GOGAT catalyzes the reductant-dependent conversion of glutamine and 2-oxoglutarate to two molecules of glutamate using ferredoxin (E.C.1.4.7.1), NADPH (E.C. 1.4.1.13),

or NADH (E.C.1.4.1.14) as a coenzyme (Inokuchi *et al.* 2002, Lea and Miflin 2003). The major isozyme, Fd-GOGAT, is monomeric (145-168 kDa) and contains one flavin mononucleotide (FMN), one FAD, and one 3Fe-4S cluster (Inokuchi *et al.* 2002, Lea and Miflin 2003), and is located in leaf chloroplasts or root plastids (Inokuchi *et al.* 2002, Lea and Miflin 2003).

Glutamate dehydrogenase (GDH, E.C.1.4.1.2)

GDH catalyzes the reversible reaction of the amination of 2-oxoglutarate (synthetic reaction) to glutamate in the presence of NADH or NAD(P)H and NH_4^+, as well as the deamination of glutamate (catabolic reaction) (Inokuchi *et al.* 2002, Oaks and Hirel 1985). GDH has numerous isozymes ranging from 208 to 280 kDa, each composed of six subunits of about 42-46 kDa (Inokuchi *et al.* 2002). NADH-GDH (E.C.1.4.1.2) is localized in the mitochondria in both leaves and roots and functions primarily in the deaminating direction (Ahmad and Hellebust 1991), while NADPH-GDH (E.C. 1.4.1.4) is found in leaf chloroplasts (Oaks and Hirel 1985). GDH plays a complementary role to the GOGAT cycle in the aminating direction or as a shunt to the GOGAT cycle through the oxidation of glutamate and providing carbohydrates to the tricarboxylic acid cycle (Lea and Miflin 2003, Miflin and Habash 2002, Oaks 1994, Robinson *et al.* 1991).

1.2.4 Role of AM fungi in N metabolism

The P uptake by AM fungal hyphae has been well established (Smith and Read 1997); however, much less is known about N uptake (Breuninger *et al.* 2004, Toussaint *et al.* 2004). Enhanced NH_4^+ assimilation via an increase in GS activity in mycorrhizal roots of red clover was correlated with an increased P contribution by extraradical hyphae (Smith *et al.* 1985). Cliquet and Stewart (1993) reported increased NR and GS activities in AM maize roots. In compartmented pot systems, the uptake and transport of slowly diffusing ^{15}N-labelled NO_3^- ions under drought conditions were shown to be enhanced in

AM lettuce (Tobar *et al.* 1994) and AM maize plants resulting in enhanced GS activity (Subramanian and Charest 1998, 1999). Other studies using compartmented *in vitro* systems provided evidence for significant uptake and transfer of ^{15}N-labelled glycine and glutamine (Hawkins *et al.* 2000) and $^{15}NH_4^+$ and $^{15}NO_3^-$ (Toussaint *et al.* 2004) by AM fungi to Ri T-DNA carrot roots. Govindarajulu *et al.* (2005) reported that $^{15}NH_4^+$ and $^{15}NO_3^-$ can be taken up by the AM fungus outside the roots and incorporated as arginine before being transferred to the host root. Therefore, in undisturbed soils the external mycelium of AM fungi play an important role in the turnover of inorganic and organic N by competing efficiently with the other soil microorganisms (Subramanian and Charest 1998, 1999).

1.3 Phytoremediation

It is still uncertain when the first observations of plants capable of accumulating high concentrations of metals were first documented (Reeves and Baker 2000). This rare phenomenon received very little attention until the discovery by Minguzzi and Vergnano (1948) of nickel (Ni) accumulation in *Alyssum bertolonii* Desvaux, growing on Italian serpentine soils. Their finding sparked renewed interest in the use of plants for the cleanup of heavy metal (HM) contamination in soils and waters (Cunningham 1995, Ensley 2000). Termed *phytoremediation*, this approach provides a relatively low cost alternative to conventional physical and chemical methods of organic and inorganic contaminant removal (Cunningham 1995).

1.3.1 Physico-chemical and phytoremediation strategies

Current soil remediation methods are based primarily on civil-engineering techniques that, whether *in situ* or *ex situ*, fall into one of these two categories: (1) site decon-

tamination techniques, where the contaminant is removed from the substrate, or (2) site stabilization techniques, where the risk posed by the contaminant is reduced by reducing exposure (Vangronsveld and Cunningham 1998). The selection of any of these remediation techniques depends on: (i) the size, location and history of the site; (ii) the soil characteristics (e.g. structure, texture, pH); (iii) the type, physical and chemical state of the contaminants; (iv) the degree of pollution (i.e. contaminant concentration and distribution); (v) the desired final land use; (vi) the technical and financial means available; and (vii) the environmental, legal, geographical, and social issues (Vangronsveld and Cunningham 1998). Engineers, government officials, and industry heads have to carefully consider all these aspects in order to determine which technique(s) would be the most efficient for completely removing the contaminant(s) while also remaining cost-effective and which pose(s) the least environmental disruption (Ensley 2000, Vangronsveld and Cunningham 1998).

As industrialization continues to grow and with the increasing concerns on the environmental impact of these remediation techniques, the implementation of phytoremediation strategies with current physico-chemical methods present potential solutions. Aside from the economical and environmental advantages, phytoremediation provides a broad range application for removal of metals and radionucleides, poses minimal environmental disturbance, reduces secondary airborne or waterborne wastes, is aesthetically pleasant, and has garnered an increased public acceptance (Cunningham 1995, Glass 2000). However, several drawbacks of phytoremediation, impeding its large-scale field application, include: the slow time frame for contaminant removal, seasonal dependence, the incomplete removal or reduction of contaminants, the application only to surface (top 1 m) soils, and the limitation of solubility or availability of the contaminant (Cunningham 1995, Glass 2000). As such, phytoremediation is still not widely accepted as an efficient remediation alternative (Vangronsveld and Cunningham 1998). However, promising laboratory investigations have substantially contributed to the development of phytoreme-

diation technology (Kumar *et al.* 1995), and field-scale trials are currently in progress (Vangronsveld and Cunningham 1998). The use of hyperaccumulators has been suggested, as these highly specialized plants have been found to take up large amounts of metals into their tissues.

1.3.2 Hyperaccumulators

The term "hyperaccumulator" was first described by Brooks *et al.* (1977), but later redefined by Reeves (1992) for Ni concentrations of at least 1000 mg kg^{-1} in the plant's dry matter of any aboveground tissue in at least one specimen growing in its natural habitat. Hyperaccumulation for Co, Cu, Cr, and Pb have also been defined for plants containing over 1000 mg kg^{-1} in the dry matter and for Mn and Zn, the criterion being 10, 000 mg kg^{-1} (Baker and Brooks 1989).

The use of hyperaccumulators for phytoremediation of HM contaminated soils has been met with criticism largely due to the feasibility of its application in field-based trials. Many hyperaccumulators are rare and endemic to the metalliferous soil they inhabit, and as such, very little information exists about agronomic characteristics, breeding capability, and the potential hazards involved in the introduction of a new species in an environment (Cunningham 1995, Reeves and Baker 2000). In addition, hyperaccumulator species are usually low biomass plants with a high single-element specificity (Reeves and Baker 2000). These dilemmas pose new challenges for the implementation of phytoremediation strategies for the removal of excess metal(s) from soils.

1.3.3 Mycorrhizal fungi in phytoremediation strategies

With the advancement of technologies, researchers have proposed new strategies for ameliorating phytoremediation. The use of chelators as amendments and the devel-

opment of transgenic hyperaccumulating and nonhyperaccumulating plants have been investigated (Gaur and Adholeya 2004, Prasad 2003). However, these strategies have also raised concerns of chelator leaching and the introduction of genetically modified plants into the environment (Krämer and Chardonnens 2001, Prasad 2003). Another strategy being pursued is the use of mycorrhizal fungi to enhance metal uptake by plants (Audet and Charest 2006, Davies *et al.* 2001, Gaur and Adholeya 2004, Prasad 2003).

The extraradical hyphae have been shown to contribute to mineral uptake and transfer to host plants by providing a greater rhizospheric zone, especially under drought conditions or in poor soils (Azcón *et al.* 2004, Smith and Read 1997). In metalliferous soils, AM fungi confer an enhanced tolerance to host plants through (i) immobilization of metals in the fungal biomass, (ii) reduced or selective transfer of metals, and (iii) dilution effect as a result of an increase in plant biomass (Gaur and Adholeya 2004, Prasad 2003). The hyphae are also less sensitive than roots to HM toxicity; thus, hyphal growth and nutrient uptake may be maintained when roots are impaired (Gaur and Adholeya 2004). Chelation of metals by such compounds as siderophores and metallothioneins (MT) released by fungi or other rhizosphere microbes in addition to sequestration by plant-derived phytochelatins (PC) may increase the uptake (Gaur and Adholeya 2004). Furthermore, AM fungi can affect rhizosphere conditions (i.e. pH, microbial communities and root-exudation patterns), thereby accelerating the revegetation of severely degraded lands such as coal mines or waste sites containing high levels of HMs (Gaur and Adholeya 2004).

1.4 Sunflower

Sunflower, *Helianthus annuus* L. (Figure 1.3), of the Asteraceae family (Foster and Duke 2000), gets its name from the Greek *helios* (sun) and *anthos* (flower) because it is

heliotrophic; that is, it always turns to face the sun (Harris 2003). Sunflower is native to southern Canada, western United States, and Mexico; the species has now spread to both tropical and temperate countries (Foster and Hobbs 2002).

Sunflower is an erect, robust herbaceous annual with a deep tap root system (3 m in depth) and a large lateral spread of surface roots (Duke 1983). It is characterized by coarse, rough stems (sometimes branching) that can reach heights of up to 3 m (Foster and Duke 2000). The rough-hairy leaves, irregularly toothed and ovate, are first opposite but gradually develop a spiral phyllotaxy of alternate leaves; the lamina has 3 main veins 10 to 30 cm long and 5 to 20 cm wide (Duke 1983). The large flower head is 10 to 40 cm in diameter and contains two types of flowers: the yellow ray flowers (broad-based petals that ring the outer edge of the flower head) and the reddish-brown disk flowers (tubular central flowers) are spirally arranged and produce the achenes (Duke 1983). *Helianthus annuus* is a polymorphic species comprising numerous wild and cultivated varieties (Duke 1983).

The common sunflower is believed to have been domesticated from wild sunflower since 1000 B.C. (Myers and Minor 2005). Native American tribes such as the Apache, Navajo, Hopi, and Mohave used sunflower as a foodstuff before the cultivation of corn and as a source of oil (Moerman 1998). Sunflower extracts were used as dyes for basket materials and as body paint in ceremonies (Moerman 1998, Stevens 2005). Teas from the flowers, leaves and roots were used for lung ailments and malaria, high fevers and rheumatism (Foster and Duke 2000, Moerman 1998). Sunflower leaves were also used as an astringent, poultice on snakebites and spider bites, sores and swellings (Foster and Duke 2000, Foster and Hobbs 2002, Moerman 1998).

In Canada, sunflower has been commercially grown since the early 1940s, mostly in the southern areas of Manitoba and Saskatchewan (Lalonde 2003). Canada is the 10^{th}

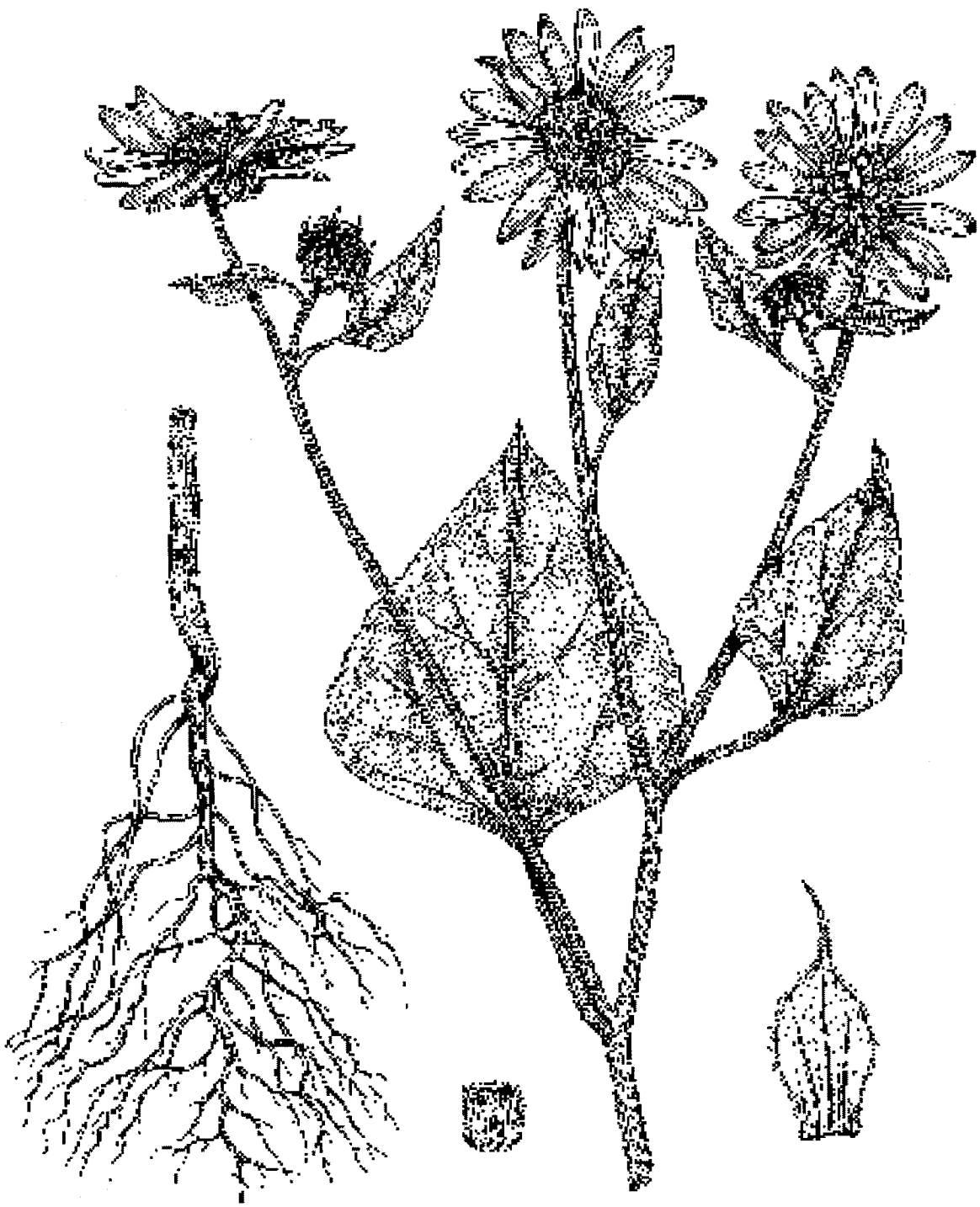
largest exporter and the 24^{th} largest producer of sunflower seeds in the world (Lalonde 2003). Nutritional values of sunflower include: healthy unsaturated fats, proteins and fibers, Vitamin E, selenium, copper, zinc, folate, iron, and phytochemicals (choline, lignan, phenolic acids, betaine, and arginine; Duke 1983).

1.4.1 Sunflower in phytoremediation strategies

Studies have reported that sunflower may be a viable canididate for phytoremediation strategies (Davies *et al.* 2001, 2002). Soybean and sunflower were selected after screening for Cr tolerance and survival among ten candidate crop species (Mei *et al.* 2002). Turgut *et al.* (2004) determined that *H. annuus* cv. dwarf sunspot amended with 0.1 g kg^{-1} EDTA as chelator, resulted in an optimal combination for Cd, Cr and Ni uptake as compared to higher concentrations of EDTA (0.3 g kg^{-1}), and/or the teddy bear sunflower cultivar. In the cv. Toma, Cd, V and Zn were predominantly found in the leaves than in the seeds; while Cu and Ni exhibited the opposite behaviour (Lombi *et al.* 1998). Other studies involving sunflower as a test species for phytoremediation purposes, have determined its effectiveness under HM input such as Cd (Pankovć *et al.* 2000), Cr (Davies *et al.* 2001, 2002), Ni (Gerendás and Sattelmacher 1997, Zornoza *et al.* 1999), Se (Ximénez-Embún *et al.* 2004), Al (Saber *et al.* 1999), Zn (Saber *et al.* 1999), and Pb (Kastori *et al.* 1996, Parent 2004). Only two of these studies, Davies *et al.* (2001, 2002) and Parent (2004), have included mycorrhizal fungi as a factor affecting HM tolerance in sunflower, specifically Cr and Pb tolerance, respectively. However, to our knowledge, no other studies have been performed to determine the feasibility of AM sunflower for other metal uptake.

Figure 1.3 Diagram of sunflower, *Helianthus annuus* L., including the large and ovate leaves, deep tap root system and the large flower head displaying two types of flowers: the ray flowers and the disk flowers.

(cited 2006: $< http://pestplants.okstate.edu/ >$)



1.5 Nickel

1.5.1 Nickel in plants

Only recently has nickel been considered an essential micronutrient in plant growth and production when it was demonstrated that barley plants cannot complete their life cycle without adequate Ni content (Brown *et al.* 1987). Nickel is a component of urease (Dixon *et al.* 1975), and may have essential functions in grain maturation, plant senescence, N metabolism and Fe uptake (Brown *et al.* 1987). Nickel has also been found in the active site of the Co-hydrogenase, methyl-coenzyme M reductase, and the Ni-superoxide dismutase and glyoxalase I (Watt and Ludden 1999).

1.5.2 Distribution, binding and translocation of nickel in plants

The uptake and transport of Ni and other HMs are of the utmost importance in strategies of phytoextraction. Mechanisms of transport and plant tolerance are considered to be plant and metal specific. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations (Clemens 2001). Nieboer and Richardson (1980) proposed that HMs have binding preferences and tend to seek out ligands containing either oxygen, nitrogen, or sulfur. In plants, these compound corresponds to phytochelatins (PC), metallothioneins (MT), and organic or amino acids.

Chelation of Ni to citrate (Lee *et al.* 1977, 1978) or to histidine (Krämer *et al.* 1996, 2000), is considered one of the primary detoxification strategies of plants for Ni stress (Clemens 2001). Other organic acids which have been reported to bind to Ni are malate and oxalate (Bhatia *et al.* 2005). Chelation of Ni to either amino or organic acids has been suggested to be highly pH-dependent; at acidic pH, organic acids are better chelators of Ni than amino acids, and as the pH becomes more alkaline, the

affinity of Ni to amino acids increases (Bhatia *et al.* 2005). Furthermore, these metal-ion complexes are thought to prevent the accumulation of toxic HM concentrations in the cytoplasm and organelles (Krämer *et al.* 2000, Leopold *et al.* 1999). The chelate enables transport through the cytoplasm with subsequent sequestration in the vacuole (Krämer *et al.* 2000). The PCs are small, cysteine-rich, metal-binding polypeptides of the general structure $(\gamma\text{-Glu-Cys})_n$-Gly with n = 2-11 (Clemens 2001, Leopold *et al.* 1999). The MTs are ubiquitous low-MW, cysteine-rich proteins, which bind metal ions in metal-thiolate clusters (Clemens 2001). Although PCs and MTs have been shown to be involved in Zn, Pb and Cd detoxification process, they have not been shown to be involved in the detoxification nor in the mechanism of hyperaccumulation of Ni because phytochelatin synthase, which synthesizes $(\gamma\text{-Glu-Cys})_n$-Gly, is not activated by Ni (Sagner *et al.* 1998).

1.6 Nickel hyperaccumulation as a defense against herbivores and pathogens

Many hypotheses concerning the selective advantage of metal hyperaccumulation in plants have been proposed (Pollard 2000). Boyd and Martens (1998) suggested that metal hyperaccumulation represents a form of chemical or "elemental" defense against herbivores and pathogens. These elemental defenses differ from plant organic (secondary compounds) defenses, in that elemental defenses involve a toxic element taken up from the soil and that cannot be chemically degraded by herbivores (Boyd and Martens 1998). Elemental defenses are thought to protect hyperaccumulator plants from herbivory or pathogens either through acute toxicity or as a deterrent to herbivores (Boyd and Martens 1998).

Peterson *et al.* (2003) showed that grasshoppers, spiders and other invertebrate herbivores contained high Ni concentrations in their tissues after feeding on the Ni hy-

peraccumulator *Alyssum pintodasilvae* collected from Portuguese serpentine soil. Boyd *et al.* (2002) reported that the hyperaccumulator *Senecio coronatus* is defended against mollusks via both elevated Ni toxicity and partial deterrence from the herbivore. Boyd *et al.* (2002) argued that the lack of damage to the leaves indicates that snails may be able to directly assess leaf Ni concentrations, and thus avoid feeding on the toxic tissues. However, elemental defenses against herbivory may depend on the herbivore species, in addition to both the plant species and the metal involved (Behmer *et al.* 2005). Furthermore, as phytoremediation technology is being developed for commercialization, the risk of transmission of toxic metals from plant tissues to herbivores and thus into higher trophic levels should be considered (Behmer *et al.* 2005, Peterson *et al.* 2003).

1.6.1 Elemental and organic defenses

A rationale for the elemental defense hypothesis suggests that the costs involved may be less than producing organic defenses (Martens and Boyd 1994). Thus, hyperaccumulator plants could invest a greater proportion of C and N to primary production than secondary metabolite synthesis as compared to non-hyperaccumulators (Martens and Boyd 1994). McNaughton and Tarrants (1983) reported that silica in African savanna grasses could act as an elemental defense as its content was increased after exposure to simulated herbivory. Glucosinate levels in the Ni hyperaccumulator *Streptanthus polygaloides* were found to be higher than in the non-hyperaccumulator *S. insignus*, suggesting that the intrinsically high Ni content in the hyperaccumulator species provided an increased defense against herbivory (Davis *et al.* (2000). Although interest in organic based defenses in hyperaccumulating plant species have increased, there exists very little information regarding the secondary metabolite production in plants growing on metalliferous soils (Davies and Boyd 2000).

1.6.2 Accumulation of secondary metabolites in response to mycorrhizal colonization

Isoprenoid secondary metabolites are constitutive metabolites and can also be selectively synthesized in response to plant development, stress, infection, or mycorrhizal colonization. In AM colonized roots of *Medicago truncatula*, elevated levels of phenylalanine ammonia-lyase and chalcone synthase transcripts were detected by *in situ* hybridization, specifically in the cells containing arbuscules (Harrison and Dixon 1993, 1994). Maier *et al.* (1997) reported that *Glomus intraradices* induces the accumulation of sesquiterpenoid cyclohexenone glycosides in roots of barley, with blumenin also being found to accumulate in oat, rye and wheat. Maier *et al.* (1997) suggested that formation of AMs might initiate a cascade of metabolic events, among which the accumulation of the cyclohexenone derivatives could be part of some stress-induced side reactions.

1.6.3 Sesquiterpene lactones

Sesquiterpene lactones (STLs) form one of the largest groups of cytotoxic and antitumour compounds of plant origin (Picman 1986). The STLs are a subclass of terpenoid secondary compounds, comprised of 3 isoprene units (C_5H_8; Dey and Harborne 1991). The monoterpenes and sesquiterpenes, frequently volatile, are the major components of the essential oils in plant essences and fragrances (Dey and Harborne 1991, Neerman 2003). The majority of STLs occur among members of the Asteraceae, where they are characteristic constituents of this family (Picman 1986). Most STLs are present in the shoots (mainly in flowering heads and leaf trichomes) where they constitute up to 5% of dry mass; roots have usually no or very small quantities of STLs (Picman 1986). The STLs (Appendix A.1) are formed by condensation of isopentyl pyrophosphate (IPP) with 3,3-dimethyallylpyrophosphate (DPP) to give geranyl pyrophosphate (GPP) (Dey and Harborne 1991). Further addition of an IPP forms the farnesyl pyrophosphate (FPP); both steps are catalyzed by FPP synthetase (Dey and Harborne 1991). Biogenetic

transformation of FPP provides the germacradiene skeleton from which all other lactone skeletal types are formed (Dey and Harborne 1991).

In sunflower, the germacranolides alantolactone and isoalantolactone (Appendix A.2) have been reported to possess antibacterial, antifungal and antifeedent properties (Dey and Harborne 1991, Neerman 2003, Picman 1986, Picman *et al.* 1993). Although present in minimal concentrations in sunflower, the germacranolide parthenolide (Appendix A.2), a prominent STL of *Tanacetum parthenium* (feverfew), possesses both antibacterial and antifungal as well as anti-tumorigenic properties (Awang *et al.* 1991, Dey and Harborne 1991, Picman *et al.* 1986). The cytotoxic and anti-tumour activities of these STLs are due to the α-methylene-γ-lactone moiety that can alkylate biological nucleophiles, in particular the sulfhydryl groups of proteins, leading to a variety of cell, tissue, and organ lesions in mammals (Dey and Harborne 1991, Gershenzon and Croteau 1991, Picman 1986). Other STLs that have been isolated from sunflower include leptocarpin and 15-hydroxyleptocarpin (Picman 1986), and argophyllin and niveusin (Appendix A.2; Melek *et al.* 1985, Spring *et al.* 1989).

1.7 Hypotheses and Objectives

This study aimed to examine the physiology of sunflower, *Helianthus annuus* L. cv "Lemon Queen," in association with the AM fungal inoculum, *Glomus intraradices* Schenck & Smith, under (1) two types of inorganic nitrogen fertilizers and (2) soil nickel treatments. The potential benefits of AM colonization in alleviating HM toxicity in sunflower and its contribution in phytoremediation strategies were examined.

The first study (Chapter 2) aimed to determine the effect of AM symbiosis on plant physiology under conditions of N and HM stresses. It was hypothesized that AM colonization (1) enhances the activities of N-assimilating enzymes in sunflower supplied with an NH_4^+- as compared to a NO_3^--type fertilizer; and (2) increases Ni content and tolerance in sunflower grown in soil Ni treatments supplied with these two N fertilizers.

In the second study (Chapter 3), the AM fungal contribution to Ni tolerance was further investigated under increased soil [Ni], and by analyzing the AM contribution in sunflower exposed to Ni at a vegetative or reproductive stage of development. It was hypothesized that the AM colonization increases tolerance and uptake of Ni in sunflower exposed at different developmental stages and under increasing Ni input.

Finally (Chapter 4), we examined the effect of AM symbiosis on plant defense-like responses in addition to the elemental defense hypothesis for Ni accumulation in sunflower. The hypotheses are that (1) Ni accumulation in sunflower results in a decrease in sesquiterpene lactones (STLs) production; and (2) AM colonization induces an accumulation of STLs in sunflower.

Chapter 2

Effect of AM fungi, inorganic N and soil Ni input on the physiology and N metabolism in sunflower

2.1 Introduction

Nitrogen (N) is one of the most limiting nutrients in spite of increased fertilizer input to enhance crop growth. Excessive N application leads to detrimental effects on the environment as well as on human and animal health (Lasa *et al.* 2000, 2001). Plants acquire N from fertilizer, manure, and/or mineralization of organic matter and assimilate inorganic forms such as nitrate (NO_3^-), the preferred form, and ammonium (NH_4^+), or organic forms such as urea (Crawford 1995, Marschner 1995). Excess NO_3^- contaminates groundwater via leaching and gaseous losses as N_2O leads to the deterioration of the ozone layer (Lasa *et al.* 2000, 2001). Ammonium, on the other hand, is not readily subject to leaching and denitrification losses (Lasa *et al.* 2000, 2001).

Once taken up, NO_3^- is either stored in the vacuole or reduced to nitrite by nitrate reductase (NR), then to NH_4^+ via nitrite reductase (NiR) (Crawford 1995). There has been much debate over NH_4^+ assimilation pathways (Miflin and Habash 2002, Oaks 1993). Prior to 1970, the main pathway of NH_4^+ assimilation was considered to be through the reversible reaction by glutamate dehydrogenase (GDH) (Lea and Miflin 2003). However, it is generally accepted that NH_4^+assimilation occurs via two enzymes, glutamine synthetase and glutamate synthase (GS/GOGAT) cycle (Lea and Miflin 2003, Oaks 1993). Yet, there remains some questions as to the exact role of GDH in N metabolism (Ahmad *et al.* 1990, Lea and Miflin 2003, Toussaint *et al.* 2004).

The association of mycorrhizal fungi with plants, considered as beneficial, plays a key role in plant N metabolism. Studies have shown that mycorrhizal colonization enhances the activities of NR, GS and GOGAT in host plants (Subramanian and Charest 1998), and that the extraradical hyphae increases N nutrition as NO_3^- (Subramanian and Charest 1999) or NH_4^+ (Toussaint *et al.* 2004) were taken up and transferred to host roots under drought stress or *in vitro* conditions, respectively. Furthermore, some evidence has shown that the hyphae possess their own GS/GOGAT system (Johansen *et al.* 1996, Subramanian and Charest 1998), that assimilation in AM plants occurs via concurrent activities of the GS/GOGAT and GDH pathways (Ahmad *et al.*, 1990, Martin *et al.*, 1998, Toussaint *et al.* 2004), and that GDH plays a key role in NH_4^+ assimilation, especially in AM transformed *in vitro* carrot roots (Toussaint *et al.* 2004).

Mycorrhizal fungi have been shown to protect plants from heavy metal (HM) stresses (Davies *et al.* 2001 and 2002, Guo *et al.* 1996, Killham and Firestone 1983). The extraradical hyphae can bind HMs, thereby restricting their translocation to shoots (Gaur and Adholeya 2004). Joner *et al.* (2000) reported that the AM mycelium contributes to selective HM uptake and transport within plant tissues. Davies *et al.* (2001, 2002) showed that AM colonization enhances sunflower biomass, tolerance to and accumulation of chromium. Rivera-Becerril *et al.* (2002) reported that AM colonization in pea roots reduces the negative effect of cadmium on shoot mass and photosynthetic rate. In addition, the AM fungi were shown to accelerate the revegetation of severely degraded lands such as coal mines or waste sites containing high HM levels (Gaur and Adholeya 2004).

With increasing concerns about soil and water contamination, phytoremediation technology has gained momentum. Phytoremediation, the use of plants to remove organic and/or inorganic contaminants (Chaney *et al.* 1997), provides a less costly and invasive, and a more environmentally friendly alternative to classic physico-chemical remediation

methods. A current strategy of phytoremediation is to improve the uptake and accumulation of contaminants by using crop plants tolerant to low contaminant levels and by introducing mycorrhizal fungi (Khan *et al.* 2000).

2.2 Hypotheses and Objectives

This research aimed to explore the effect of AM symbiosis on the physiology of sunflower plants under different conditions of N nutrition and HM input. From the study of Toussaint *et al.* (2004) who presented evidence that AM fungi preferentially take up NH_4^+ over NO_3^-, our first goal was to examine how N assimilating enzymes are affected in AM colonized sunflower supplied with two different N-type fertilizers. The second goal was to investigate the potential benefits of AM colonization of sunflower for enhancing plant tolerance, specifically in Ni contaminated soils. The combination of these two research goals are presented in this study.

Our first hypothesis is that the activities of ammonium assimilating enzymes are enhanced in AM colonized sunflower supplied with NH_4^+ as compared to NO_3^- fertilization. To test the first hypothesis, the specific objectives were to determine if AM colonized sunflower plants supplied with NH_4^+ fertilization increases: (1) N metabolism, by analyzing activities of key N assimilating enzymes: glutamine synthetase (GS), glutamate dehydrogenase (GDH) and nitrate reductase (NR); and (2) physiological parameters such as biomass, shoot height, Root/Shoot ratio, chlorophyll and mineral concentrations.

The second hypothesis is that AM colonization increases Ni content and tolerance in sunflower grown under soil Ni conditions and supplied with two different N fertilizers. To test the second hypothesis, the specific objectives were to determine if the AM symbiosis increases: (1) the content of Ni in plant organs; (2) the physiological parameters; and (3) the N metabolic parameters, as described above.

2.3 Materials and Methods

A factorial block design (1 plant sp. x 2 M x 2 F x 2 Ni) greenhouse experiment was performed including: plants of sunflower, *Helianthus annuus* L. cv Lemon Queen, grown with or without the AM fungus, *Glomus intraradices* Schenck & Smith, supplied with a NO_3^-- or NH_4^+-type fertilizer with or without Ni, 100 or 0 Ni mg kg^{-1} dry soil (DS) (applied after 9 weeks of growth). In total, 104 pots (13 pots per treatment with 1 plant/pot) were prepared.

2.3.1 Planting conditions

Sunflower seeds (OSC Seeds, Waterloo, ON, Canada) were surface sterilized twice with 25 mL of NaOCl (Chlorox bleach) and 75 mL dH_2O for 5 min, then rinsed twice with 100 mL dH_2O. Pots (7.6L) were also surface sterilized in 20% bleach overnight and then washed thoroughly before use. Three seeds were sown ~12 mm deep and ~24 mm apart in a sand:soil mixture (1:1, v/v) previously autoclaved for 20 min at 121^0C and 15PSI. Pots were filled with 1/3 of sand:soil mixture, then 1/3 of the inoculum or control substrate, and topped with the sand:soil mixture (in total 3.74 kg DM of sand:soil). The inoculum consisted of a substrate containing the AM fungal structures (15 propagules g^{-1} dry substrate) of *G. intraradices* (DAOM 181602), or a non-AM control substrate that contained filtrates of the rhizosphere microflora while excluding the fungal spores (Mycorhize Pro, Premier Tech, Rivière-du-Loup, QC, Canada).

2.3.2 Greenhouse conditions

Plants were grown in the greenhouse of the Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa, for 11 weeks under the following conditions: minimum nightly and maximum daily temperatures of $20^0/24^0$C, average

relative humidity of 43%, and a 16 h-photoperiod under high-pressure sodium (HPS) lamps (PL Light Systems, Beamsville, ON, Canada). The average light intensity (175 μmol s^{-1} m^{-2} per μA) was measured using a light meter attached to a quantum sensor (LiCor L.I.-250A and LiCor L.I.-190SA, Lincoln, NE, USA).

2.3.3 Soil characteristics and fertilizer components

A dry sand:soil sample was sent to the *Laboratoire de Chimie Organique et Inorganique* (LCOI , Sainte-Foy, QC, Canada) for analysis of soil characteristics prior to the experiment (Appendix A.1). After 2 weeks of growth, all the pots were thinned to 1 seedling of approximately equal size and vigour. Plants were fertilized with either a nitrate-type or an ammonium-type Long Ashton Nutrient Solution (LANS, Hewitt and Smith 1975), once a week (250 mL) for 2 weeks, and bi-weekly (500 mL) thereafter for 7 weeks. The LANS fertilizer composition is described in the Appendix A.2.

2.3.4 Nickel treatment

Nickel, in the form of $NiCl_2 \cdot 6H_2O$ (500 mL/dose), was applied in two doses equaling 100 mg kg^{-1} DS during weeks 9 and 10. Plants not receiving Ni were given a dH_2O equivalent dose amount.

2.3.5 Harvest

Plants were harvested on week 11. The roots were separated from shoots at the cotyledonary node from which the shoot height was measured to the top of the stem. Leaves and flowers were separated from stems and each plant organ was individually weighed for fresh mass. The roots were rinsed in water to remove any substrate, blotted with paper towels to remove excess water and then weighed for fresh mass. Three root

replicates from each treatment were put aside in dH_2O overnight at 4^0C and used to determine percent AM colonization 24 h after harvest. The stem, leaves, and flowers from these three replicates were individually packaged in paper bags and oven-dried at 70^0C for a minimum of 72 h, then weighed for dry masses. Dry tissues were ground to powder with mortar and pestle and sent to the LCOI for mineral analyses. The plant organs (stems, leaves, flowers, and roots) from the other ten replicates per treatment were shock frozen in liquid nitrogen and stored at -80^0C until freeze-drying for 72 h at -50^0C, vapor pressure 3.7 x 10^{-1} mbar (Uni-trap Model 10-100, Virtis Inc., Gardiner, N.Y., U.S.). The freeze-dried leaf and root material was used for the enzyme and protein assays.

2.3.6 Root colonization

The percent root colonization was determined according to Dalpé (1993). Roots were heated at 90^0C on a hot plate in 2.5% KOH for 20 min, then washed 2-3 times to remove excess KOH and lower the pH, acidified in 1% HCl for 45 min at room temperature, and stained with an Aniline Blue solution (0.5 g Aniline Blue, 500 mL glycerol, 450 mL dH_2O and 50 mL 1% HCl). Roots were heated again on a hot plate for 15 min at 90^0C, then placed in a discoloring solution (500 mL glycerol, 450 mL dH_2O and 50 mL 1% HCl). Ten 2 cm-root sections were lined up per slide. A polyvinyl-alcohol-lactic acid-glycerol solution (PVLG; 8.33g PVA, 50 mL dH_2O, 50 mL lactic acid, 50 mL glycerol) was used as a preservative and the slide was sealed with clear nail polish. Three replicates per treatment were used to determine AM root colonization. Fifty root sections (10/slide) were observed for each replicate.

Root segments were examined at 4X, 100X and 400X magnification under a compound microscope (Nikon Eclipse 800), in the laboratory of Dr. Dalpé (Agriculture and Agri-Food Canada) for the presence of AM structures, hyphae, vesicles, arbuscules or spores.

Mycorrhizal colonization was estimated as the percentage of the total root segments containing at least one of the fungal structures. Pictures were taken using a digital camera (Nikon Coolpix 950 MDC Lens 0.82-0.29X).

2.3.7 Chlorophyll extraction and determination

Chlorophyll (chl) was determined according to the method of Bruinsma (1963). Fresh leaf material (1 g) was immersed in 100 mL of 95% ethanol in a conical flask covered with aluminum foil and kept in the dark at 4^0C until complete leaf discoloration. The ODs were read at 645 and 663 nm, and chl concentrations determined as follows:

Chl$a$ = (OD_{663} X 12.7) - (OD_{645} X 2.7)

Chl$b$ = (OD_{645} X 22.9) - (OD_{663} X 4.7)

Chltot = Chl$a$ + Chl$b$

2.3.8 Enzyme activity and protein concentration

Nitrate reductase (NR, E.C. 1.6.6.1)

The NR activity was determined by the combined methods of Long and Oaks (1990) and Subramanian and Charest (1998). Freeze-dried roots (0.2 g) or leaves (0.3 g) were ground with mortar and pestle over ice, with sand, and 10 mL of extraction buffer, pH 8.5 (25 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 20 μM FAD, 1% BSA and 10 mM cysteine). The protease inhibitors leupeptin (10 μM, dissolved in the buffer) or chymostatin (10 μM, dissolved in DMSO) were added to the extraction buffer for leaf or root material, respectively, in order to stabilize NR. The extracts were centrifuged at 12 100 g for 25 min at 4^0C. The assay mixture contained 0.2 mL of N-2-hydroxypiperazine-N-2-ethanesulfonic acid (HEPES) buffer (0.65 M, pH 7.0), 0.2 mL of KNO_3 (0.1 M), 0.1 mL enzyme extract and dH_2O (final volume 1.4 mL). The reaction was started by the addition of 0.1 mL of NADH (3.6 mg mL^{-1}) solubilized in KH_2PO_4 (0.04 M, pH 7.0) .

This mixture was incubated at 28^0C for 25 min and the reaction stopped by adding 1 mL of 1% sulfanilamide in 1M HCl, and 1 mL of 0.01% N-1-naphthylethylene-diamine-dihydrochloride (NED) in dH_2O, to produce a pink color reaction with NO_2^- formation. The NR activity was read at 540 nm, 30 min after the reaction was stopped. The blank, made for each extract, contained all the compounds except the reaction stopping agents were added before the enzyme extract. The NR activity was expressed as μmol NO_2^- produced g^{-1} DM h^{-1}.

Glutamine synthetase (GS, E.C. 6.3.1.2) and glutamate dehydrogenase (GDH, E.C. 1.4.1.2)

Enzyme Extraction and Assays

Freeze-dried roots or leaves (0.2 g) were ground with mortar and pestle over ice, with sand, 2% PVP, and 10 mL of extraction buffer. The GS/GDH extraction buffer, pH 8.0, contained 25 mM Tris, 1 mM EDTA-diNa salt, 1 mM dithiothreitol (DTT), 1 mM reduced glutathione, 10 mM $MgSO_4$, 5 mM glutamate, and 0.01% Triton. After centrifugation at 12 100g for 25 min at 4^0C, the supernatants were used for the GS or GDH activities, and soluble protein assays.

The GS and GDH activities were determined from the combined methods of Robinson *et al.* (1991) and Toussaint *et al.* (2004). GS was determined by the synthetase assay. The reaction mixture, pH 7.6, contained 2 mM ATP, 2.6 mM MgSO4, 0.7 mM hydroxylamine, 8 mM L-glutamate, and 5 mM Tris-HCl. The reaction was initiated by the addition of 0.25 mL of enzyme extract to 0.75 mL of the reaction mixture. The reaction was terminated, after 30 min at room temperature, with the addition of 0.75 mL of ferric chloride reagent (4 mL $FeCl_3$ 10%, 1 mL trichloroacetic acid 24% and 0.5 mL HCl 6 M in 6.5 mL dH_2O). After centrifugation for 2 min at 2000 g, the OD was read at 540 nm. A blank was prepared for each extract in the same manner, with the exception that the $FeCl_3$ solution was added before the extract to prevent any reaction

to occur. The GS activity was expressed as γ-glutamylhydroxamate (GH) produced g^{-1} DM h^{-1} using a γ-GH standard curve (0 - 3 μmol) (Appendix A.3).

The GDH activity was determined by the rate of 2-oxoglutarate-dependent NADH oxidation. The reaction mixture, pH 8.2, contained 15 mM NH_4Cl, 1 mM $CaCl_2$, 0.3 mM NADH, 20 mM 2-oxoglutarate and 100 mM Tris-HCl. The reaction was started by the addition of 0.2 mL of the enzyme extract to 1 mL of the reaction mixture. The GDH activity was read at 340 nm and expressed as NADH oxidized g^{-1} DM h^{-1}. The blank, made for each extract, contained all of the above compounds except NADH.

Protein concentration

The soluble proteins were determined according to the micro-method of Bradford (1976) in all the extracts used for GS, GDH and NR activities. Each protein extract, 20 μL, was combined to 780 μL dH_2O and 200 μL Bio-Rad dye reagent and mixed by manual inversion. The ODs were read at 595 nm, and the protein concentrations were measured from a BSA standard curve (0 - 1.2 mg mL^{-1}) (Appendix A.4).

The specific activities of NR, GS and GDH were calculated by dividing their activities by the soluble protein concentrations.

2.3.9 Mineral analyses

Macro- and microelement analyses

Approximately 0.5 g (per replicate) of dry root, leaf or flower material, was ground using a mortar and pestle and sieved (2 mm) before being sent to LCOI for mineral analyses. A sulfuric acid (H_2SO_4) digestion on hot plate was carried out for P, K, Ca, Mg, Mn, Cu, Zn, Al, Fe and Mo; for Ni, this was done in concentrated HNO_3 and

30% H_2O_2. Following the digestion, all of these elements were analyzed using Atomic Plasma Emission Spectroscopy. The soil mineral analysis using Mehlich-3 method was also determined by LCOI.

The percentages of Ni extracted and Ni distribution for shoot, root and the whole plant were calculated from the following formulas:

$$\text{Ni extracted \%} = \frac{\text{total Ni content (mg Ni organ}^{-1}\text{ DM)}}{\text{total Ni content in soil (mg Ni DS)}} \text{ X } 100$$

$$\text{Ni distribution \%} = \frac{\text{organ Ni content (mg Ni organ}^{-1}\text{ DM)}}{\text{total Ni content (mg Ni plant}^{-1}\text{ DM)}} \text{ X } 100$$

Carbon and nitrogen analyses

Total C and N contents (%) were analyzed in the G.G. Hatch Isotope Laboratory, University of Ottawa. Approximately 2 mg of dried root or leaf material was sealed in a tin capsule and dropped into a combustion chamber of the Elemental Analyzer (EA 1100 CHNS, Isomass Scientific, Calgary, AB, Canada) at 1800^0C. The N_2 and CO_2 production was subsequently separated by Gas Chromatography and detected by a Thermal Conductivity Detector (TCD).

2.3.10 Statistical analyses

Two or three-way Analyses of Variance (ANOVAs) were performed on all parameters using S-Plus (S-Plus 6.2, Insightful Corp. 2003). Mean comparison tests were done using Tukey's studentized range test at the 5% level of significance. All the data were verified for the assumptions of normality and homoscedasticity. When needed, log or square-root transformations were performed. When the assumptions were not met, non-parametric Kruskall-Wallis analyses were done.

2.4 Results

2.4.1 Mycorrhizal colonization

The AM colonization levels for plants fertilized with NO_3^- or NH_4^+ were 31% and 50%, respectively, without Ni input and $\simeq$ 40% with the input of 100 mg Ni kg^{-1} for both fertilizers.

2.4.2 Physiological parameters

Biomass

The fertilizer ($P < 0.05$) and soil [Ni] ($P < 0.001$) treatments significantly affected flower DMs (Tables 2.1 and 2.2). The interactions of AM with soil [Ni] ($P < 0.001$) and of fertilizer with soil [Ni] ($P = 0.058$) were significant and marginally non-significant on flower DM. The tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.01$) was also significant on flower DM. According to the Tukey's test, the flower DM of treatment M-Ni- supplied with NO_3^- was significantly higher than the M+Ni-, M-Ni+ and M+Ni+ treatments by 1.9, 2.7, and 1.6 times, respectively. This same treatment (M-Ni-) was also significantly higher (by 1.7 to 2.3 times) than the flower DMs when supplied with NH_4^+. The flower DM of M-Ni+ supplied with NO_3^- was significantly lower than in M+Ni+ supplied with NO_3^- by 1.7 times, and of M-Ni- supplied with NH_4^+ by 1.6 times.

The fertilizer ($P < 0.001$) significantly affected leaf, stem, root and total plant DMs (Tables 2.1 and 2.2). The Tukey's test indicated that leaf, stem, root and total plant DMs were significantly higher (by 1.4 to 6.3 times) when supplied with NO_3^- as compared to NH_4^+.

The AM ($P = 0.097$) treatment resulted in a marginally non-significant effect on the

total plant DM, while its interaction with fertilizer ($P < 0.05$) was significant for root DM, and marginally non-significant ($P = 0.056$) for plant DM. According to the Tukey's test, the root DM of M+Ni- supplied with NO_3^- was significantly higher than the M-Ni-, M-Ni+ and M+Ni+ treatments by 1.8, 1.5, and 1.3 times, respectively. The plant DM of M+Ni- supplied with NO_3^- was also significantly higher by 1.2 times than at M-Ni+, and 2 times higher than the plant DMs when supplied with NH_4^+.

Shoot height and Root/Shoot (R/S) ratio

The fertilizer ($P < 0.001$) significantly affected the shoot height and R/S ratios (Tables 2.1 and 2.2). The soil [Ni] ($P < 0.01$), and the interactions of AM with fertilizer ($P < 0.001$), and of AM with soil [Ni] ($P < 0.05$) were significant on R/S ratios. According to the Tukey's test, the shoot heights of M+Ni- and M+Ni+ supplied with NO_3^- were significantly higher than M+Ni+ supplied with NH_4^+, by 1.2 times. Although not significant, the shoot heights tended to be higher for plants supplied with NO_3^- than NH_4^+. The Tukey's test indicated that the R/S ratios were significantly higher (by 2 to 4 times) when supplied with NO_3^- than NH_4^+. The R/S ratio of M-Ni- was significantly lower (by 2.7, 2.0, and 2.4 times, respectively) than M+Ni-, M-Ni+ and M+Ni+ treatments supplied with NO_3^-.

2.4.3 Chlorophyll concentration

The AM treatment significantly ($P < 0.05$) affected the concentration of chl*b* and was marginally non-significant on the concentrations of chl*a* ($P = 0.064$) and chl*tot* ($P = 0.057$) (Table 2.7). The soil [Ni] significantly ($P < 0.05$) affected the concentrations of chl*a* and chl*tot*, and was marginally non-significant ($P = 0.064$) on chl*b* concentration. The interaction of AM with soil [Ni] was significant ($P < 0.01$) for all the chl concentrations. The tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.05$) was significant for

chl*b* concentration, and marginally non-significant for the concentrations of chl*a* (P = 0.065) and chl*tot* (P = 0.057). According to the Tukey's test, the chl concentrations (*a*, *b* and *tot*) were significantly higher in M-Ni- supplied with NO_3^- than all other treatments. The chl concentrations were also significantly higher in M-Ni- supplied with NH_4^+ than in M+Ni- supplied with NO_3^-.

Table 2.1 Dry mass (DM, n = 5 to 8), shoot height (n = 11 to 13) and Root/Shoot ratio (R/S, n = 3 to 6) means and (SEs) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	DM (g)					Height (cm)	R/S ratio
	Flower	Leaf	Stem	Root	Total		
Nitrate							
M-Ni-	26.7^{a}	24.5^{a}	69.4^{ab}	29.3^{b}	150^{ab}	247^{ab}	0.14^{b}
	(3.29)	(0.97)	(5.77)	(4.56)	(4.78)	(7.30)	(0.03)
M+Ni-	14.2^{bc}	25.1^{a}	79.8^{a}	52.5^{a}	172^{a}	249^{a}	0.38^{a}
	(0.95)	(1.02)	(7.72)	(7.97)	(9.62)	(8.31)	(0.07)
M-Ni+	9.80^{c}	25.1^{a}	72.1^{a}	35.3^{b}	142^{b}	245^{ab}	0.28^{a}
	(0.93)	(1.93)	(4.49)	(2.45)	(8.18)	(3.53)	(0.02)
M+Ni+	16.6^{b}	23.5^{a}	74.0^{a}	39.1^{b}	153^{ab}	251^{a}	0.33^{a}
	(1.63)	(0.81)	(4.56)	(4.79)	(8.37)	(7.81)	(0.03)
Ammonium							
M-Ni-	15.6^{b}	15.3^{b}	49.8^{b}	10.7^{c}	91.4^{c}	225^{ab}	0.12^{b}
	(1.45)	(0.96)	(4.56)	(1.50)	(7.76)	(8.26)	(0.03)
M+Ni-	12.5^{bc}	14.1^{b}	49.9^{b}	8.28^{c}	84.8^{c}	232^{ab}	0.10^{b}
	(1.07)	(1.10)	(4.50)	(1.50)	(7.28)	(8.84)	(0.01)
M-Ni+	12.9^{bc}	14.2^{b}	48.5^{b}	10.1^{c}	85.7^{c}	234^{ab}	0.15^{b}
	(1.68)	(1.11)	(3.77)	(1.06)	(4.85)	(6.58)	(0.01)
M+Ni+	11.7^{bc}	15.1^{b}	52.7^{b}	8.89^{c}	88.4^{c}	215^{b}	0.10^{b}
	(1.00)	(1.02)	(3.29)	(0.33)	(5.00)	(7.75)	(0.01)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 2.2 ANOVA results for DM, shoot height and R/S ratio of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in two soil [Ni] treatments.

Parameters	DM					Height	R/S ratio
	Flower	Leaf	Stem	Root	Total		
F	*	***	***	***	***	***	***
M	NS	NS	NS	NS	MN	NS	NS
Ni	***	NS	NS	NS	NS	NS	**
F X M	NS	NS	NS	*	MN	NS	***
F X Ni	MN	NS	NS	NS	NS	NS	NS
M X Ni	***	NS	NS	NS	NS	NS	*
F X M X Ni	**	NS	NS	NS	NS	NS	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Table 2.3 Three-way ANOVA on flower and leaf (ranked data) dry masses.

Parameters	Flower				Leaf			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.11	0.11	6.93	1	18565.25	18565.25	180.09
M	1	0.02	0.02	1.62	1	0.98	0.01	0.92
Ni	1	0.24	0.24	15.83	1	59.76	0.58	0.45
F X M	1	0.09	0.09	0.56	1	0.01	0.00	0.99
F X Ni	1	0.06	0.06	3.74	1	52.62	0.51	0.48
M X Ni	1	0.30	0.30	19.53	1	16.63	0.16	0.69
F X M X Ni	1	0.18	0.18	11.65	1	125.76	1.22	0.27

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.4 Three-way ANOVA on stem and root (ranked data) dry masses.

Parameters	Stem				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	12072.44	12072.44	43.79	1	8897.72	8897.72	140.64
M	1	359.01	359.01	1.30	1	5.60	5.60	0.09
Ni	1	289.83	289.83	1.05	1	10.11	10.11	0.16
F X M	1	81.83	81.83	0.30	1	368.56	368.56	5.83
F X Ni	1	421.93	421.93	1.53	1	98.49	98.49	1.56
M X Ni	1	99.20	99.20	0.36	1	1.32	1.32	0.02
F X M X Ni	1	0.21	0.21	0.00	1	84.58	84.58	1.34

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.5 Three-way ANOVA on total dry mass.

Parameters	Total			
	df	SS	MS	F-value
F	1	55525.69	55525.69	158.77
M	1	1006.97	1006.97	2.88
Ni	1	661.24	661.24	1.89
F X M	1	1342.52	1342.52	3.84
F X Ni	1	719.97	719.97	2.06
M X Ni	1	107.13	107.13	0.31
F X M X Ni	1	662.98	662.98	1.90

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.6 Three-way ANOVA on ranked data for both height and R/S ratio.

Parameters	Height				R/S ratio			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	9023.96	9023.96	13.72	1	2385.00	2385.00	71.62
M	1	113.30	113.31	0.17	1	26.078	26.078	0.783
Ni	1	60.51	60.51	0.092	1	265.83	265.83	7.98
F X M	1	477.62	477.61	0.73	1	677.00	677.00	20.33
F X Ni	1	137.95	137.95	0.21	1	23.53	23.53	0.71
M X Ni	1	831.58	831.58	1.26	1	169.83	169.83	5.10
F X M X Ni	1	526.95	526.95	0.80	1	88.39	88.39	2.65

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.7 Leaf chlorophyll concentration (mg g^{-1} FM) means (n= 3) and (SEs) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Treatment	N-type	Chl*a*	Chl*b*	Chl*tot*
M-Ni-	NO_3^-	2.32^{a} (0.05)	0.89^{a} (0.05)	3.21^{a} (0.09)
	NH_4^+	1.82^{ab} (0.17)	0.68^{ab} (0.06)	2.51^{ab} (0.22)
M+Ni-	NO_3^-	0.95^{c} (0.02)	0.35^{c} (0.01)	1.30^{c} (0.03)
	NH_4^+	1.47^{bc} (0.13)	0.53^{bc} (0.05)	2.01^{bc} (0.18)
M-Ni+	NO_3^-	1.23^{bc} (0.18)	0.46^{bc} (0.05)	1.69^{bc} (0.23)
	NH_4^+	1.26^{bc} (0.03)	0.45^{bc} (0.02)	1.71^{bc} (0.04)
M+Ni+	NO_3^-	1.47^{bc} (0.06)	0.55^{bc} (0.03)	2.02^{bc} (0.08)
	NH_4^+	1.28^{bc} (0.24)	0.46^{bc} (0.09)	1.74^{bc} (0.34)
ANOVA: Fertilizer (F), AM fungi (M), Nickel (Ni)				
F		NS	NS	NS
M		MN	*	MN
Ni		*	MN	*
F X M		NS	NS	NS
F X Ni		NS	NS	NS
M X Ni		**	**	**
F X M X Ni		MN	*	MN

* $P < 0.05$; ** $P < 0.01$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 2.8 Three-way ANOVA on ranked data of chlorophyll *a* and *b* concentrations.

Parameters	Chl*a*				Chl*b*			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.09	0.09	0.00	1	0.01	0.01	0.00
M	1	92.24	92.24	3.95	1	125.19	125.19	5.63
Ni	1	116.48	116.48	4.98	1	88.48	88.48	3.98
F X M	1	41.42	41.42	1.77	1	36.48	36.48	1.64
F X Ni	1	44.01	44.01	1.88	1	52.24	52.24	2.35
M X Ni	1	335.54	335.54	14.36	1	328.32	328.32	14.77
F X M X Ni	1	92.24	92.24	3.95	1	104.01	104.01	4.68

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.9 Three-way ANOVA on total chlorophyll concentration.

Parameters	Chl*tot*			
	df	SS	MS	F-value
F	1	0.09	0.09	0.00
M	1	100.01	100.01	4.23
Ni	1	116.48	116.48	4.93
F X M	1	36.48	36.48	1.54
F X Ni	1	44.01	44.01	1.86
M X Ni	1	321.17	321.17	13.59
F X M X Ni	1	100.01	100.01	4.23

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

2.4.4 Nickel concentration and content

Flower Ni concentration and content

The AM ($P < 0.001$) and soil [Ni] ($P < 0.001$) significantly affected the flower [Ni] (Tables 2.10 and 2.11). The interactions of AM with fertilizer ($P < 0.001$) and with soil [Ni] ($P < 0.001$), as well as the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.001$) were all significant on the flower [Ni]. According to the Tukey's test, the [Ni] in flowers was significantly higher in M+N+ supplied with NH_4^+ than in the M-Ni+ and M+Ni+ treatments supplied with NO_3^- (by 1.2 times), and in M-Ni+ supplied with NH_4^+ (by 1.6 times). In addition, the Tukey's test indicated that flower [Ni] was significantly higher in AM than non-AM plants supplied with NH_4^+, but not in the plants supplied with NO_3^-.

The fertilizer ($P < 0.001$) and soil [Ni] ($P < 0.001$) significantly affected the flower Ni content (Tables 2.10 and 2.11). The interactions of fertilizer with soil [Ni] was significant ($P < 0.001$) on the Ni content. The interactions of fertilizer with AM ($P = 0.091$), as well as the tripartite interaction of fertilizer, AM and soil [Ni] ($P = 0.091$) were marginally non-significant on the Ni content. According to the Tukey's test, the Ni content was significantly higher (by 1.2 to 2.0 times) in the treatments supplied with NO_3^- than NH_4^+. Although not significant, the Ni content was lower in the AM (M+Ni+) than non-AM (M-Ni+) flowers supplied with NO_3^-, but higher when supplied with NH_4^+.

Leaf Ni concentration and content

The soil [Ni] had a significant ($P < 0.001$) effect on the leaf [Ni] (Tables 2.10 and 2.11). According to the Tukey's test, the leaf [Ni] was significantly higher (by 1.3 to 1.8 times) in treatments supplied with NO_3^- than NH_4^+.

The fertilizer ($P < 0.01$) and soil [Ni] ($P < 0.001$) significantly affected the leaf Ni

content (Tables 2.10 and 2.11). The interaction of AM with soil [Ni] ($P < 0.05$) was significant on the Ni content. The tripartite interaction of fertilizer, AM and soil [Ni] was marginally non-significant ($P = 0.093$) on the Ni content. According to the Tukey's test, the leaf Ni content was significantly (2-folds) higher in AM than non-AM plants supplied with NO_3^-, but was unchanged when supplied with NH_4^+. The Tukey's test also indicated that the Ni content tended to be higher (by 1.2 to 3.2 times) in plants supplied with NO_3^- than NH_4^+.

Root Ni concentration and content

The root [Ni] was significantly affected by the soil [Ni] ($P < 0.001$) treatment, with the AM ($P = 0.098$) treatment being marginally non-significant (Tables 2.10 and 2.11). The interaction of fertilizer with AM ($P < 0.001$) and the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.001$) were also significant on root [Ni]. According to the Tukey's test, the root [Ni] was significantly higher (by 1.6 times) in the AM than non-AM plants supplied with NO_3^-, but was lower (by 2.6 times) in the AM than non-AM plants supplied with NH_4^+. The root [Ni] was significantly higher in non-AM (M-Ni+) plants supplied with NH_4^+ than NO_3^-, but was significantly lower in AM (M+Ni+) plants.

The fertilizer ($P < 0.001$) and soil [Ni] ($P < 0.001$) significantly affected the root Ni content (Tables 2.10 and 2.11). The interactions of fertilizer with AM ($P < 0.05$) and with soil [Ni] ($P < 0.001$), as well as the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.05$) were significant on the Ni content. According to the Tukey's test, the root Ni content was significantly higher (by 2.7 to 8.7 times) when supplied with NO_3^- than NH_4^+. The root Ni content was also significantly higher (by 1.3 times) in AM than non-AM plants supplied with NO_3^-. Although not significant, the root Ni content tended to be lower (by 2.4 times) in AM than non-AM plants supplied with NH_4^+.

Total plant Ni content

The fertilizer ($P < 0.001$), AM ($P < 0.01$) and soil [Ni] ($P < 0.001$) significantly affected the plant Ni content (Tables 2.10 and 2.11). The interactions of AM with fertilizer ($P < 0.01$) and with soil [Ni] ($P < 0.01$), as well as the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.01$) were significant on the plant Ni content. According to the Tukey's test, the Ni content was significantly higher (by 2.4 to 4.7 times) with NO_3^- than NH_4^+. The Ni content was also significantly higher (by 1.3 times) in AM than non-AM plants supplied with NO_3^-. Although not significant, the Ni content tended to be lower (by 1.4 times) in AM than non-AM plants supplied with NH_4^+.

Ni extracted percentage (%)

The fertilizer significantly ($P < 0.01$) affected the shoot Ni extracted % (Table 2.16). According to the Tukey's test, it was significantly higher in shoots of plants supplied with NO_3^- than NH_4^+ (by 1.7 to 2.6 times), and tended to be higher in AM than non-AM plants for both N fertilizers (by 1.3 and 1.5 times, respectively).

The fertilizer ($P < 0.001$), and its interaction with AM ($P < 0.01$), significantly affected the root Ni extracted % (Table 2.16). According to the Tukey's test, it was significantly higher (by 2.7 to 8.9 times) with NO_3^- than NH_4^+. Although not significant, the root Ni extracted % tended to be higher (by 1.3 times) in AM than non-AM plants supplied with NO_3^-, but lower (by 2.4 times) with NH_4^+.

The total Ni extracted % was significantly and marginally non-significantly affected by the fertilizer ($P < 0.001$) and its interaction with AM ($P = 0.083$) treatments, respectively (Table 2.16). According to the Tukey's test, it was significantly higher (by 2.5 to 4.6 times) with NO_3^- than NH_4^+. The total Ni extracted % was also significantly higher (by 1.3 times) in AM than non-AM plants supplied with NO_3^-, but tended to be lower (by

1.4 times) with NH_4^+.

Plant Ni distribution

The plant organ ($P < 0.001$) and the tripartite interaction of fertilizer, AM and organ ($P < 0.001$) significantly affected the Ni distribution % in the control plants. According to the Tukey's test, the Ni content was distributed in this order: roots > leaves > flowers. In plants supplied with NO_3^-, the Ni distribution % was significantly higher (by 1.3 times) in roots of AM than non-AM plants, but tended to be lower (by 1.7 times) in leaves of AM than non-AM plants.

The fertilizer ($P < 0.01$), AM ($P < 0.05$), and soil [Ni] ($P < 0.001$) significantly affected the Ni distribution % in sunflower plants with the input of [100] soil Ni. The interactions of fertilizer with AM ($P = 0.06$), fertilizer with organ ($P < 0.001$) and AM with organ ($P < 0.001$) were significant on the Ni distribution %. The tripartite interaction of fertilizer, AM and organ was also significant ($P < 0.05$). According to the Tukey's test, the Ni content was distributed in this order: roots > leaves > flowers. The Tukey's test also indicated that the Ni distribution % of plants supplied with NH_4^+ was significantly lower (by 1.8 times) in roots of AM than non-AM plants, but significantly higher (by 2.5 times) in leaves of AM than non-AM plants. In addition, the R/S ratio of Ni content was 1.0:1.4 in AM plants, as compared to 2.7:1.0 in non-AM plants, supplied with NH_4^+. In plants supplied with NO_3^-, the R/S ratio of Ni content was 4:1, with no significant differences between AM and non-AM plants.

Table 2.10 Total Ni concentration (mg Ni g^{-1} (x 10^3) DM) and content (mg Ni $organ^{-1}$ or $plant^{-1}$ DM) means (n = 3 to 5) and (SEs) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	Ni concentration			Ni content			
	Flower	Leaf	Root	Flower	Leaf	Root	Total
Nitrate							
M-Ni-	*nd*[1]	2.00^{c} (0.58)	7.50^{c} (0.50)	*nd*	0.07^{c} (0.02)	0.10^{d} (0.00)	0.17^{d} (0.01)
M+Ni-	*nd*	1.33^{c} (0.33)	7.00^{c} (1.00)	*nd*	0.03^{c} (0.01)	0.10^{d} (0.01)	0.13^{d} (0.01)
M-Ni+	40.3^{b} (3.33)	54.9^{ab} (6.63)	172^{b} (15.1)	0.98^{a} (0.03)	1.15^{b} (0.14)	7.70^{b} (0.89)	9.83^{b} (0.88)
M+Ni+	43.0^{b} (2.02)	72.6^{a} (8.13)	276^{a} (31.6)	0.73^{ab} (0.09)	2.29^{a} (0.27)	10.3^{a} (1.15)	13.3^{a} (0.64)
Ammonium							
M-Ni-	*nd*	1.67^{c} (0.33)	8.67^{c} (2.73)	*nd*	0.02^{c} (0.00)	0.09^{d} (0.01)	0.11^{d} (0.01)
M+Ni-	*nd*	1.67^{c} (0.33)	10.3^{c} (1.20)	*nd*	0.03^{c} (0.01)	0.06^{d} (0.01)	0.09^{d} (0.01)
M-Ni+	30.5^{c} (2.96)	40.9^{b} (4.33)	341^{a} (27.0)	0.48^{c} (0.04)	0.71^{b} (0.13)	2.85^{c} (0.24)	4.04^{c} (0.18)
M+Ni+	50.3^{a} (2.01)	42.0^{b} (5.44)	131^{b} (11.1)	0.63^{bc} (0.10)	0.99^{b} (0.14)	1.19^{c} (0.02)	2.82^{c} (0.22)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[1] *nd*, not detectable.

Table 2.11 ANOVA results for Ni concentration and content for total plant and each organ of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in two soil [Ni] treatments.

Parameters	Ni concentration			Ni content			
	Flower	Leaf	Root	Flower	Leaf	Root	Total
F	NS	NS	NS	***	**	***	***
M	***	NS	MN	NS	NS	NS	**
Ni	***	***	***	***	***	***	***
F X M	***	NS	***	MN	NS	*	**
F X Ni	NS	NS	NS	***	NS	***	NS
M X Ni	***	NS	NS	NS	*	NS	**
F X M X Ni	***	NS	***	MN	MN	*	**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Table 2.12 Three-way ANOVA on ranked data of flower Ni concentration and content.

Parameters	Ni concentration				Ni content			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.01	0.01	0.92	1	42.67	42.67	16.92
M	1	27.54	27.54	32.61	1	0.00	0.00	0.00
Ni	1	741.42	741.42	878.00	1	864.00	864.00	342.74
F X M	1	18.13	18.13	21.47	1	8.17	8.17	3.24
F X Ni	1	0.01	0.01	0.01	1	42.67	42.67	16.93
M X Ni	1	27.54	27.54	32.61	1	0.00	0.00	0.00
F X M X Ni	1	18.13	18.13	21.47	1	8.17	8.17	3.24

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.13 Three-way ANOVA on ranked data of leaf Ni concentration and content.

Parameters	Ni concentration				Ni content			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	61.24	61.24	2.24	1	172.22	172.22	29.69
M	1	5.76	5.76	0.21	1	7 7.22	7.22	1.25
Ni	1	2309.28	2309.28	84.55	1	1037.00	1037.00	178.76
F X M	1	1.84	1.84	0.067	1	2.67	2.67	0.46
F X Ni	1	76.62	76.62	2.80	1	1.74	1.74	0.30
M X Ni	1	43.21	43.21	1.58	1	47.67	47.67	8.22
F X M X Ni	1	30.58	30.58	1.12	1	18.22	18.22	3.14

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.14 Three-way ANOVA on root Ni concentration and content.

Parameters	Ni concentration				Ni content			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.45	0.45	0.22	1	73.43	73.43	90.46
M	1	6.14	6.14	3.01	1	0.30	0.30	0.37
Ni	1	914.57	914.57	448.00	1	176.37	176.37	217.28
F X M	1	40.38	40.38	19.78	1	6.87	6.87	8.46
F X Ni	1	0.95	0.95	0.47	1	72.52	72.52	89.35
M X Ni	1	5.19	5.19	2.54	1	0.35	0.35	0.43
F X M X Ni	1	55.02	55.02	26.95	1	6.64	6.64	8.17

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.15 Three-way ANOVA on ranked data of total Ni content.

Parameters	Ni content			
	df	SS	MS	F-value
F	1	210.04	210.04	161.31
M	1	13.50	13.50	10.37
Ni	1	864.00	864.00	663.55
F X M	1	12.04	12.04	9.25
F X Ni	1	0.04	0.04	0.03
M X Ni	1	13.50	13.50	10.37
F X M X Ni	1	15.04	15.04	11.55

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.16 Ni extracted percentage (%) means (n = 3) and (SEs) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in 100 mg Ni kg^{-1} DS. ANOVA results are also presented.

Treatment	Ni extracted %[1]		
	Shoot[2]	Root	Total
Nitrate			
M-Ni+	0.57[a]	2.06[a]	2.63[b]
	(0.03)	(0.24)	(0.23)
M+Ni+	0.76[a]	2.75[a]	3.51[a]
	(0.14)	(0.31)	(0.17)
Ammonium			
M-Ni+	0.29[b]	0.76[b]	1.05[c]
	(0.04)	(0.06)	(0.05)
M+Ni+	0.45[ab]	0.31[b]	0.76[c]
	(0.05)	(0.01)	(0.06)
ANOVA: Fertilizer (F), AM fungi (M)			
F	**	***	***
M	NS	NS	MN
F X M	NS	**	**

** $P < 0.01$; *** $P < 0.001$; MN $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[1]Data for treatments without Ni are not shown since the Ni extracted % were below 0.001 %.

[2]Shoot Ni extracted % was calculated by combining the Ni content for flower and leaf.

Table 2.17 Three-way ANOVA on shoot (ranked data), root (ranked data) and total Ni extracted %.

Para-	Shoot				Root				Total			
meters	df	SS	MS	F-value	df	SS	MS	F-value	df	SS	MS	F-value
F	1	85.33	85.33	16.00	1	108.0	108.0	64.80	1	14.06	14.06	207.0
M	1	12.00	12.00	2.25	1	0.33	0.33	0.20	1	0.27	0.27	3.93
F X M	1	3.00	3.00	0.56	1	21.33	21.33	12.80	1	1.02	1.02	15.12

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi

Table 2.18 Ni distribution % means (n = 3) and (SEs) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg Ni kg^{-1} DS) soil treatments.

Plant organ	AM	soil Ni	
		- Ni	+ Ni
Nitrate			
Flower	M-	*nd*[1]	10^{d} (1.0)
	M+	*nd*	3.0^{e} (2.0)
Leaf	M-	41^{bc} (7.0)	12^{d} (2.0)
	M+	24^{c} (10)	19^{d} (3.0)
Root	M-	59^{b} (4.0)	78^{a} (2.0)
	M+	79^{a} (6.0)	78^{a} (5.0)
Ammonium			
Flower	M-	*nd*	12^{d} (1.0)
	M+	*nd*	22^{cd} (0.0)
Leaf	M-	20^{c} (4.0)	15^{d} (5.0)
	M+	29^{c} (6.0)	37^{bc} (4.0)
Root	M-	80^{a} (4.0)	72^{a} (4.0)
	M+	71^{a} (6.0)	41^{b} (2.0)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[1] *nd*, not detectable.

Table 2.19 ANOVA results for Ni distribution % for plant organs (O) of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in two soil [Ni] treatments.

Parameters	soil Ni	
	- Ni	+ Ni
F	NS	**
M	NS	*
O	***	***
F X M	NS	MN
F X O	NS	***
M X O	NS	***
F X M X O	***	*

* P < 0.05; ** P < 0.01; *** P < 0.001; MN, 0.05 < P < 0.1; NS, not significant

Table 2.20 Three-way ANOVA on Ni distribution %.

Parameters	− Ni				+ Ni			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.16	0.16	0.03	1	106.78	106.78	8.93
M	1	0.23	0.23	0.043	1	51.36	51.36	4.29
O	2	2904.30	1452.15	268.65	2	2692.63	1346.31	112.58
F X M	1	1.26	1.26	0.23	1	46.69	46.69	3.90
F X O	2	18.75	9.38	1.73	2	336.01	168.01	14.049
M X O	2	6.52	3.26	0.60	2	232.76	116.38	9.732
F X M X O	2	76.93	38.46	7.12	2	126.26	63.13	5.28

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, O = organ

2.4.5 Mineral concentrations and total C and N contents (%)

Leaf minerals

The fertilizer had significant ($P < 0.01$) effects on the concentrations of Ca, Mg, Mn and K ($P < 0.001$), and a marginally non-significant effect on P ($P = 0.07$) (Tables 2.21 and 2.23). According to the Tukey's test, the concentrations of P, Ca, Mg and Mn tended to be higher with NO_3^- than NH_4^+, but lower for K concentration.

The AM treatment had significant ($P < 0.05$) effects on the concentrations of Ca and Mg, and Fe ($P < 0.01$), and was marginally non-significant ($P = 0.062$) on Mn. According to the Tukey's test, the Fe concentration tended to be higher in AM (M+Ni-, M+Ni+) than non-AM (M-Ni-, M-Ni+) plants for both N fertilizers. The concentrations of Ca and Mg also tended to be higher in AM (M+Ni+) than non-AM (M-Ni+) plants for both N fertilizers. The Mn concentration tended to be lower in AM (M+Ni-) than non-AM (M-Ni-) plants without Ni, but tended to be higher in AM (M+Ni+) than non-AM (M-Ni+) plants with Ni for both N fertilizers. The soil [Ni] significantly ($P < 0.05$) affected the P concentration. According to the Tukey's test, treatment M-Ni- with NO_3^- was significantly higher than M+Ni- and M+Ni+ with NH_4^+, and higher than M-Ni+ for both N fertilizers.

The interaction of fertilizer with soil [Ni] resulted in significantly ($P < 0.001$) higher K concentrations in NO_3^--fed AM plants with Ni (M+Ni+) than M-Ni-, M+Ni-, M-Ni+; however, in NH_4^+-fed plants, this interaction led to lower K concentrations in plants with Ni (M-Ni+, M+Ni+) than M-Ni-.

Root minerals

The fertilizer had significant effects on the concentrations of P ($P < 0.05$), K ($P < 0.01$), Zn ($P < 0.001$), and marginally non-significant effects on the concentrations of Ca

(P = 0.059) and Cu (P = 0.06) (Tables 2.22 and 2.23). According to the Tukey's test, the concentrations of P, K, Cu and Zn tended to be higher in the roots of AM plants supplied with NH_4^+ than NO_3^-. The Ca concentration tended to be higher in M-Ni+ with NO_3^- than NH_4^+.

The AM treatment significantly (P < 0.01) affected the Cu concentration, and was marginally non-significant (P = 0.082) on the K concentration. According to the Tukey's test, these concentrations were significantly higher in NH_4^+-fed AM (M+Ni-) than non-AM (M-Ni-) plants.

The soil [Ni] had significant (P < 0.05) effects on the concentrations of Ca, Cu and Zn. According to the Tukey's test, the Ca concentration tended to be lower in treatments with (M-Ni+ and M+Ni+) than without (M-Ni- and M+Ni-) Ni, for both N fertilizers. This trend was also observed for Cu and Zn concentrations, but only with NH_4^+.

The interactions of fertilizer with AM were significant on the concentrations of K (P < 0.05), Cu (P < 0.01), and Zn (P < 0.01). The interaction of AM with soil [Ni] was significant (P < 0.01) on the K concentration, and marginally significant (P < 0.067) on the Zn concentration. The Tukey's test indicated that the concentrations of these minerals were significantly higher in NH_4^+-fed AM (M+Ni-) than non-AM (M-Ni-) plants. The Zn concentration in M+Ni- was significantly higher than M-Ni+ for plants supplied with NH_4^+. The tripartite interaction of fertilizer, AM and soil [Ni] marginally non-significantly (P = 0.072) increased the root Cu concentration in NH_4^+-fed AM (M+Ni-) than non-AM (M-Ni-) plants without Ni, but decreased it with Ni.

Total C and N (%)

The interaction of AM with soil [Ni] was marginally non-significant ($P = 0.087$) on the leaf C content (Table 2.33). Although not significant, the leaf C content tended to be higher in M+Ni+ with NH_4^+ than NO_3^-. The AM treatment ($P < 0.05$) and its interaction with fertilizer ($P < 0.05$) significantly affected the root N content (Table 2.33). The root N content tended to be higher in AM than non-AM plants supplied with NH_4^+.

Table 2.21 Mineral concentration (mg g^{-1} DM) means (n = 3) and (SEs) in leaves of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	N-type	P	K	Ca	Mg	Mn	Zn	Al	Fe
M-Ni-	NO_3^-	5.50^{a}	22.0^{c}	20.8^{ab}	9.26^{ab}	1.96^{ab}	0.09^{a}	*nd*[1]	0.12^{b}
		(0.40)	(3.68)	(3.38)	(1.63)	(0.95)	(0.02)		(0.01)
	NH_4^+	3.90^{ab}	46.2^{a}	18.2^{b}	6.62^{b}	1.03^{ab}	0.05^{a}	0.14^{a}	0.28^{b}
		(0.38)	(4.53)	(3.10)	(0.73)	(0.18)	(0.00)	(0.01)	(0.12)
M+Ni-	NO_3^-	3.83^{ab}	24.5^{c}	26.4^{ab}	9.46^{ab}	1.60^{ab}	0.09^{a}	0.15^{a}	0.52^{b}
		(0.63)	(1.60)	(4.48)	(1.25)	(0.29)	(0.03)	(0.01)	(0.35)
	NH_4^+	3.63^{b}	38.8^{ab}	17.6^{b}	6.08^{b}	0.80^{b}	0.11^{a}	0.16^{a}	0.82^{ab}
		(0.38)	(1.16)	(3.71)	(0.44)	(0.04)	(0.06)	(0.04)	(0.57)
M-Ni+	NO_3^-	3.13^{b}	28.9^{c}	19.7^{ab}	8.72^{ab}	1.06^{ab}	0.04^{a}	*nd*	0.20^{b}
		(0.09)	(2.87)	(3.58)	(1.18)	(0.15)	(0.00)		(0.02)
	NH_4^+	3.00^{b}	34.3^{bc}	12.1^{b}	6.68^{b}	0.80^{b}	0.06^{a}	0.13^{a}	0.25^{b}
		(0.30)	(2.34)	(0.58)	(1.21)	(0.16)	(0.02)	(0.00)	(0.07)
M+Ni+	NO_3^-	3.90^{ab}	32.1^{b}	35.6^{a}	13.6^{a}	2.72^{a}	0.12^{a}	0.23^{a}	2.40^{a}
		(0.23)	(3.08)	(5.15)	(1.56)	(0.53)	(0.04)	(0.06)	(0.54)
	NH_4^+	3.60^{b}	34.0^{bc}	20.3^{ab}	7.97^{ab}	1.48^{ab}	0.07^{a}	0.27^{a}	0.74^{ab}
		(0.51)	(1.21)	(2.02)	(0.81)	(0.32)	(0.02)	(0.04)	(0.42)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[1] *nd*, not detectable; Copper concentration data are not shown since the levels were below the detectable limit.

Table 2.22 Mineral concentration (mg g^{-1} DM) means (n = 3) and (SEs) in roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	N-type	P	K	Ca	Mg	Mn	Cu	Zn	Al	Fe
M-Ni-	NO_3^-	1.87^{a}	13.9^{b}	6.52^{ab}	2.43^{a}	0.13^{a}	0.03^{b}	0.06^{b}	1.06^{a}	1.06^{a}
		(0.27)	(2.82)	(1.06)	(0.36)	(0.03)	(0.00)	(0.00)	(0.16)	(0.21)
	NH_4^+	2.07^{a}	11.7^{b}	6.31^{ab}	2.69^{a}	0.23^{a}	0.04^{b}	0.07^{ab}	1.37^{a}	1.73^{a}
		(0.24)	(2.30)	(0.15)	(0.09)	(0.07)	(0.01)	(0.01)	(0.05)	(0.60)
M+Ni-	NO_3^-	1.97^{a}	14.3^{b}	6.81^{a}	2.70^{a}	0.18^{a}	0.05^{b}	0.05^{b}	1.01^{a}	0.65^{a}
		(0.32)	(0.79)	(0.18)	(0.40)	(0.04)	(0.01)	(0.00)	(0.32)	(0.06)
	NH_4^+	3.37^{a}	31.6^{a}	6.40^{ab}	1.86^{a}	0.15^{a}	0.10^{a}	0.09^{a}	0.64^{a}	0.54^{a}
		(0.73)	(4.82)	(0.47)	(0.33)	(0.02)	(0.02)	(0.00)	(0.05)	(0.01)
M-Ni+	NO_3^-	1.90^{a}	15.8^{b}	6.66^{ab}	2.55^{a}	0.12^{a}	0.04^{b}	0.05^{b}	1.02^{a}	1.02^{a}
		(0.40)	(2.62)	(0.62)	(0.24)	(0.01)	(0.00)	(0.01)	(0.53)	(0.56)
	NH_4^+	2.13^{a}	22.8^{ab}	4.45^{b}	2.12^{a}	0.13^{a}	0.02^{b}	0.05^{b}	0.88^{a}	0.96^{a}
		(0.19)	(3.17)	(0.52)	(0.50)	(0.02)	(0.01)	(0.00)	(0.30)	(0.40)
M+Ni+	NO_3^-	1.63^{a}	11.8^{b}	5.67^{ab}	2.29^{a}	0.14^{a}	0.03^{b}	0.05^{b}	1.55^{a}	1.68^{a}
		(0.32)	(1.32)	(0.17)	(0.21)	(0.01)	(0.00)	(0.00)	(0.84)	(1.15)
	NH_4^+	2.63^{a}	23.7^{ab}	5.09^{ab}	1.95^{a}	0.18^{a}	0.05^{b}	0.07^{ab}	0.81^{a}	0.78^{a}
		(0.27)	(4.33)	(0.76)	(0.35)	(0.03)	(0.01)	(0.00)	(0.19)	(0.18)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 2.23 ANOVA results for leaf and root mineral concentrations of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in two soil [Ni] treatments.

Parameters	P	K	Ca	Mg	Mn	Cu	Zn	Al	Fe
Leaf									
F	MN	***	**	**	**	NS	NS	NS	NS
M	NS	NS	*	*	MN	NS	NS	NS	**
Ni	*	NS	NS	NS	NS	NS	NS	NS	NS
F X M	NS	NS	NS	NS	NS	NS	NS	NS	NS
F X Ni	NS	***	NS	NS	NS	NS	NS	NS	NS
M X Ni	*	NS	MN	NS	*	NS	NS	NS	NS
F X M X Ni	NS	NS	NS	NS	NS	NS	NS	NS	NS
Root									
F	*	**	MN	NS	NS	MN	***	NS	NS
M	NS	MN	NS	NS	NS	**	NS	NS	NS
Ni	NS	NS	*	NS	NS	*	*	NS	NS
F X M	NS	*	NS	NS	NS	**	**	NS	NS
F X Ni	NS	NS	NS	NS	NS	NS	NS	NS	NS
M X Ni	NS	**	NS	NS	NS	NS	MN	NS	NS
F X M X Ni	NS	NS	NS	NS	NS	MN	NS	NS	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Table 2.24 Three-way ANOVA on leaf and root P concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	1.76	1.76	3.82	1	3.01	3.01	7.12
M	1	0.11	0.11	0.25	1	1.00	1.00	2.37
Ni	1	3.69	3.69	8.00	1	0.35	0.35	0.83
F X M	1	0.54	0.54	1.16	1	1.45	1.45	3.43
F X Ni	1	0.66	0.66	1.43	1	0.05	0.05	0.12
M X Ni	1	3.84	3.84	8.33	1	0.51	0.51	1.21
F X M X Ni	1	0.87	0.87	1.88	1	0.07	0.07	0.17

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.25 Three-way ANOVA on leaf (ranked data) and root K concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	494.08	494.08	42.22	1	0.19	0.19	13.36
M	1	0.083	0.083	0.007	1	0.05	0.05	3.43
Ni	1	4.90	4.90	0.419	1	0.01	0.01	0.59
F X M	1	20.45	20.45	1.75	1	0.11	0.11	7.59
F X Ni	1	222.45	222.45	19.01	1	0.01	0.01	0.84
M X Ni	1	4.90	4.90	0.42	1	0.13	0.13	8.91
F X M X Ni	1	0.75	0.75	0.06	1	0.026	0.026	1.77

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.26 Three-way ANOVA on leaf and root (ranked data) Ca concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	415.23	415.23	11.36	1	134.21	134.21	4.18
M	1	300.24	300.24	8.22	1	0.01	0.01	0.00
Ni	1	7.80	7.80	0.21	1	266.91	266.91	8.31
F X M	1	68.19	68.19	1.87	1	29.66	29.66	0.923
F X Ni	1	45.07	45.07	1.23	1	25.50	25.50	0.79
M X Ni	1	128.76	128.76	3.52	1	16.48	16.48	0.51
F X M X Ni	1	0.72	0.72	0.02	1	64.32	64.32	2.00

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.27 Three-way ANOVA on leaf and root Mg concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	73.96	73.96	10.32	1	0.69	0.69	2.09
M	1	25.77	25.77	3.60	1	0.36	0.36	1.10
Ni	1	0.61	0.61	0.08	1	0.23	0.23	0.69
F X M	1	1.28	1.28	0.18	1	0.38	0.38	1.16
F X Ni	1	10.49	10.49	1.46	1	7 0.01	0.01	0.04
M X Ni	1	11.94	11.94	1.67	1	0.01	0.01	0.02
F X M X Ni	1	2.54	2.54	0.35	1	0.54	0.54	1.63

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.28 Three-way ANOVA on ranked data of leaf and root Mn concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	280.01	280.01	10.57	1	40.04	40.04	0.81
M	1	108.09	108.09	4.079	1	66.67	66.67	1.35
Ni	1	7.15	7.15	0.27	1	45.37	45.37	0.92
F X M	1	19.85	19.85	0.75	1	26.04	26.04	0.53
F X Ni	1	13.42	13.42	0.51	1	1.50	1.50	0.03
M X Ni	1	189.42	189.42	7.15	1	35.04	35.04	0.71
F X M X Ni	1	10.68	10.68	0.40	1	130.67	130.67	2.65

SS = sum of squares, df = degrees of freedom, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.29 Three-way ANOVA on root[1]Cu concentration.

Parameters	Root			
	df	SS	MS	F-value
F	1	73.50	73.50	4.09
M	1	234.37	234.37	13.06
Ni	1	135.37	135.37	7.54
F X M	1	198.37	198.37	11.05
F X Ni	1	15.04	15.04	0.84
M X Ni	1	37.50	37.50	2.09
F X M X Ni	1	66.67	66.67	3.71

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

[1]Leaf ANOVA data are not shown since the levels were below the detectable limit.

Table 2.30 Three-way ANOVA on ranked data of leaf and root Zn concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	21.20	21.20	0.50	1	352.67	352.67	19.39
M	1	97.06	97.06	2.30	1	40.04	40.04	2.20
Ni	1	57.37	57.37	1.357	1	130.67	130.67	7.17
F X M	1	13.79	13.79	0.33	1	187.04	187.04	10.27
F X Ni	1	21.20	21.20	0.50	1	20.17	20.17	1.11
M X Ni	1	23.06	23.06	0.55	1	70.04	70.04	3.85
F X M X Ni	1	128.53	128.53	3.04	1	12.04	12.04	0.66

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.31 Three-way ANOVA on leaf and root Al concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.37	0.37	0.01	1	7.04	7.04	0.13
M	1	84.37	84.37	0.14	1	35.04	35.04	0.65
Ni	1	0.37	0.37	0.01	1	28.17	28.17	0.52
F X M	1	1.50	1.50	0.04	1	96.00	96.00	1.78
F X Ni	1	73.5	73.5	2.08	1	1.04	1.04	0.02
M X Ni	1	73.5	73.5	2.08	1	92.04	92.04	1.71
F X M X Ni	1	0.37	0.37	0.01	1	13.50	13.50	0.25

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.32 Three-way ANOVA on ranked data of leaf and root Fe concentration.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.48	0.48	0.02	1	0.16	0.16	0.00
M	1	404.01	404.0	13.31	1	79.41	79.41	1.67
Ni	1	38.91	38.91	1.28	1	32.98	32.98	0.69
F X M	1	25.50	25.50	0.84	1	45.33	45.33	0.95
F X Ni	1	38.91	38.91	1.28	1	1.41	1.41	0.03
M X Ni	1	5.19	5.19	0.17	1	118.63	118.63	2.49
F X M X Ni	1	25.50	25.50	0.84	1	1.41	1.41	0.03

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.33 Carbon (C) and nitrogen (N) content (%) means (n = 3) and (SEs) in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Treatment	N-type	%C		%N	
		Leaf	Root	Leaf	Root
M-Ni-	NO_3^-	37.4[a] (2.14)	40.2[a] (2.13)	2.90[a] (0.04)	1.04[a] (0.07)
	NH_4^+	39.1[a] (0.42)	39.3[a] (0.25)	2.78[a] (0.14)	1.04[a] (0.05)
M+Ni-	NO_3^-	39.6[a] (1.80)	40.1[a] (3.34)	3.19[a] (0.55)	1.08[a] (0.09)
	NH_4^+	39.0[a] (1.69)	38.4[a] (4.25)	3.08[a] (0.24)	1.42[a] (0.23)
M-Ni+	NO_3^-	39.6[a] (0.33)	40.1[a] (1.91)	2.59[a] (0.07)	1.11[a] (0.10)
	NH_4^+	39.8[a] (0.85)	32.2[a] (5.10)	2.61[a] (0.07)	0.85[a] (0.10)
M+Ni+	NO_3^-	35.4[a] (0.41)	38.5[a] (2.76)	2.74[a] (0.26)	1.08[a] (0.14)
	NH_4^+	40.0[a] (0.23)	41.1[a] (1.16)	3.25[a] (0.52)	1.24[a] (0.06)
ANOVA: Mycorrhiza (M), Fertilizer (F), Nickel (Ni)					
F		NS	NS	NS	NS
M		NS	NS	NS	*
Ni		NS	NS	NS	NS
F X M		NS	NS	NS	*
F X Ni		NS	NS	NS	NS
M X Ni		MN	NS	NS	NS
F X M X Ni		NS	NS	NS	NS

* $P < 0.05$; MN, $0.05 < P < 0.1$; NS, not significant

Table 2.34 Three-way ANOVA on leaf (ranked data) and root C content.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	73.50	73.50	1.63	1	23.52	23.52	0.87
M	1	6.00	6.00	0.13	1	15.30	15.30	0.56
Ni	1	2.67	2.67	0.059	1	13.89	13.89	0.51
F X M	1	28.17	28.17	0.63	1	36.16	36.16	1.33
F X Ni	1	48.17	48.17	1.07	1	2.80	2.80	0.10
M X Ni	1	150.00	150.00	3.33	1	25.13	25.13	0.92
F X M X Ni	1	121.50	121.50	2.70	1	48.11	48.11	1.77

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.35 Three-way ANOVA on leaf (ranked data) and root N content.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	4.17	4.17	0.07	1	0.02	0.02	0.53
M	1	60.17	60.17	1.07	1	0.23	0.23	5.38
Ni	1	96.00	96.00	1.71	1	0.03	0.03	0.68
F X M	1	42.67	42.67	0.76	1	0.21	0.21	5.02
F X Ni	1	8.17	8.17	0.14	1	0.08	0.08	1.81
M X Ni	1	37.50	37.50	0.67	1	0.00	0.00	0.04
F X M X Ni	1	2.67	2.67	0.05	1	0.00	0.00	0.06

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

2.4.6 Enzyme activity

Nitrate reductase (NR)

The leaf NR activity was affected significantly by the fertilizer ($P < 0.01$) and AM ($P < 0.001$) treatments, while the soil [Ni] treatment was marginally non-significantly ($P = 0.058$) (Tables 2.36 and 2.37). The interaction of fertilizer with AM ($P < 0.01$) was significant on leaf NR activity. According to the Tukey's test, the leaf NR activities were significantly higher in plants supplied with NH_4^+ than NO_3^-, except in M+Ni+. The leaf NR activity was also significantly higher in AM (M+Ni-, M+Ni+) than non-AM (M-Ni-, M-Ni+) plants supplied with NO_3^-.

The fertilizer ($P < 0.001$), AM ($P < 0.01$) and soil [Ni] ($P < 0.01$) treatments significantly affected the root NR activity (Tables 2.36 and 2.37). The interactions of fertilizer with AM ($P < 0.001$) and with soil [Ni] ($P < 0.001$) were significant on root NR activity, while the interaction of AM with soil [Ni] ($P = 0.075$) was marginally non-significant. According to the Tukey's test, the root NR activity was significantly higher in both AM (M+Ni+) and non-AM (M-Ni+) plants supplied with NH_4^+ than NO_3^-. The root NR activity was significantly lower in AM (M+Ni-, M+Ni+) than non-AM (M-Ni-, M-Ni+) plants supplied with NH_4^+.

Glutamine synthetase (GS)

The AM treatment had a significant ($P < 0.01$) effect on leaf GS activity (Tables 2.36 and 2.37). The interactions of fertilizer with soil [Ni] ($P < 0.01$), and the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.01$) were also significant on GS activity. According to the Tukey's test, the leaf GS activity was significantly higher in M-Ni+ with NH_4^+ than NO_3^-. The leaf GS activity was also significantly higher with (M+Ni+) than without (M-Ni+) AM colonization and with NO_3^-, and was significantly higher with

(M+Ni-) than without (M-Ni-) AM colonization with NH_4^+.

The fertilizer ($P < 0.01$), AM ($P < 0.05$) and soil [Ni] ($P < 0.05$) treatments had significant effects on the root GS activity (Tables 2.36 and 2.37). The interactions of fertilizer with soil [Ni] ($P < 0.01$) and AM with soil [Ni] ($P < 0.05$), and the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.01$) were also significant. According to the Tukey's test, it was significantly lower with (M+Ni+) than without (M-Ni-) AM when supplied with NO_3^-. The root GS activity was also significantly higher with (M-Ni+, M+Ni+) than without (M+Ni-, M-Ni-) Ni when supplied with NH_4^+.

Glutamate dehydrogenase (GDH)

The fertilizer ($P < 0.001$), AM ($P < 0.05$) and soil [Ni] ($P < 0.001$) treatments significantly affected the leaf GDH activity (Tables 2.36 and 2.37). The interactions of fertilizer with AM ($P < 0.05$) and with soil [Ni] ($P < 0.01$), and of AM with soil [Ni] ($P < 0.05$) were also significant. According to the Tukey's test, it was significantly higher in plants supplied with NO_3^- than NH_4^+, but significantly lower in M+Ni-. The Tukey's test also indicated that the leaf GDH activity was significantly lower in the AM than non-AM plants supplied with NO_3^-, but significantly higher when supplied with NH_4^+. The leaf GDH specific activity was significantly higher with (M-Ni+, M+Ni+) than without (M-Ni-, M+Ni-) Ni when supplied with NH_4^+, but significantly lower with (M+Ni+) than without (M+Ni-) Ni when supplied with NO_3^-.

The AM ($P < 0.001$) and soil [Ni] ($P < 0.05$) treatments resulted in significant effects on the root GDH activity (Tables 2.36 and 2.37). The interaction of fertilizer with soil [Ni] ($P < 0.01$), and the tripartite interaction of fertilizer, AM and soil [Ni] ($P = 0.092$) were significant and marginally non-significant, respectively. According to the Tukey's test, it was significantly higher with (M+Ni-) than without (M-Ni-) AM when supplied

with NH_4^+. The root GDH activity also tended to be higher when supplied with NH_4^+ than NO_3^-.

Soluble protein concentrations and specific activities

The total soluble protein concentrations (Appendices: Table A.3 and Figure A.5) and the specific activities for NR, GS, and GDH (Appendices: Tables A.5 and A.6) were also determined.

Table 2.36 Nitrate reductase (NR, μmol NO_2^- produced g^{-1} DM h^{-1}), glutamine synthetase (GS, μmol γ-GH produced g^{-1} DM h^{-1}), and glutamate dehydrogenase (GDH, μmol g^{-1} DM h^{-1}) activity means (n = 3 to 6) and (SEs) in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	N-type	NR activity		GS activity		GDH activity	
		Leaf	Root	Leaf	Root	Leaf	Root
M-Ni-	NO_3^-	0.42^{b}	0.027^{bc}	53.2^{ab}	26.1^{a}	5.56^{b}	4.73^{ab}
		(0.04)	(0.007)	(4.55)	(3.04)	(1.21)	(0.39)
	NH_4^+	0.94^{a}	0.061^{b}	35.3^{b}	13.1^{b}	3.07^{c}	2.11^{c}
		(0.13)	(0.010)	(7.87)	(3.67)	(0.59)	(0.38)
M+Ni-	NO_3^-	0.77^{ab}	0.032^{bc}	60.3^{a}	9.67^{b}	2.95^{c}	5.57^{ab}
		(0.06)	(0.007)	(5.23)	(0.69)	(0.70)	(0.41)
	NH_4^+	0.93^{a}	0.034^{bc}	55.8^{a}	12.7^{b}	6.19^{b}	4.68^{ab}
		(0.09)	(0.003)	(3.50)	(1.76)	(1.08)	(0.68)
M-Ni+	NO_3^-	0.55^{b}	0.019^{c}	33.7^{b}	10.3^{b}	16.0^{a}	3.25^{bc}
		(0.04)	(0.007)	(0.45)	(0.87)	(2.15)	(0.29)
	NH_4^+	0.88^{a}	0.136^{a}	55.3^{a}	34.7^{a}	4.84^{bc}	4.71^{ab}
		(0.09)	(0.012)	(3.20)	(4.96)	(0.75)	(0.99)
M+Ni+	NO_3^-	1.14^{a}	0.019^{c}	55.8^{a}	13.0^{b}	9.45^{b}	6.27^{ab}
		(0.13)	(0.003)	(5.30)	(2.84)	(1.78)	(0.92)
	NH_4^+	1.06^{a}	0.067^{b}	50.4^{ab}	28.1^{a}	2.23^{c}	7.20^{a}
		(0.17)	(0.02)	(3.91)	(5.04)	(1.43)	(0.77)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 2.37 ANOVA results for NR, GS, and GDH activities in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in two soil [Ni] treatments.

Parameters	NR activity		GS activity		GDH activity	
	Leaf	Root	Leaf	Root	Leaf	Root
F	**	***	NS	**	***	NS
M	***	**	**	*	*	***
Ni	MN	**	NS	*	***	*
F X M	**	***	NS	NS	*	NS
F X Ni	NS	***	**	**	**	**
M X Ni	NS	MN	NS	*	*	NS
F X M X Ni	NS	NS	**	**	NS	MN

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Table 2.38 Three-way ANOVA on leaf and root NR activity.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	0.03	0.03	12.33	1	0.01	0.01	66.14
M	1	0.04	0.04	17.21	1	0.00	0.00	13.52
Ni	1	0.01	0.01	3.90	1	0.00	0.00	11.99
F X M	1	0.02	0.02	9.21	1	0.00	0.00	16.86
F X Ni	1	0.01	0.01	2.64	1	0.01	0.01	26.83
M X Ni	1	0.00	0.00	1.47	1	0.00	0.00	3.63
F X M X Ni	1	0.00	0.00	0.01	1	0.00	0.00	2.18

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.39 Three-way ANOVA on leaf and root GS activity.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	24.51	24.51	0.24	1	0.18	0.18	13.36
M	1	1249.85	1249.85	12.18	1	0.10	0.10	7.04
Ni	1	54.47	54.47	0.53	1	0.10	0.10	7.31
F X M	1	114.62	114.62	1.12	1	0.03	0.03	1.89
F X Ni	1	923.65	923.65	9.00	1	0.46	0.46	33.13
M X Ni	1	67.32	67.32	0.66	1	0.07	0.07	5.11
F X M X Ni	1	1002.23	1002.23	9.76	1	0.14	0.14	9.86

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table 2.40 Three-way ANOVA on leaf and root GDH activity.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	124.10	124.10	24.23	1	0.03	0.03	1.86
M	1	29.75	29.75	5.81	1	0.42	0.42	23.94
Ni	1	86.72	86.72	16.93	1	0.07	0.07	4.22
F X M	1	37.16	37.16	7.26	1	0.02	0.02	1.40
F X Ni	1	145.71	145.71	28.45	1	0.20	0.20	11.49
M X Ni	1	37.34	37.34	7.29	1	0.00	0.00	0.17
F X M X Ni	1	1.32	1.32	0.26	1	0.05	0.05	3.06

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

2.5 Discussion

2.5.1 Nickel concentration and content

To our knowledge, this is the first study conducted on the combined effect of AM colonization and soil Ni input in sunflower tolerance. In this study, a maximum of 100 mg Ni kg^{-1} DS was added after 8 weeks of growth for both the NO_3^- and NH_4^+ fertilizer treatments. The AM sunflower plants supplied with NO_3^- had significantly higher Ni content than non-AM plants. Furthermore, the Ni content was predominantly distributed in roots (by 80%) than in shoots of NO_3^--fed plants. These results coincide with others that suggest that AM fungi contribute to an enhanced plant tolerance through reduced or selective transfer of HM(s) from roots to shoots (Audet and Charest 2006, Baker 1987, Chaney *et al.* 1997, Joner *et al.* 2000, Smith and Read 1997). Enhanced root sequestration of Ni has been observed in ectomycorrhizal symbiosis of birch (Jones and Hutchinson 1988a and 1988b), and of Cu, Ni, Pb and Zn in perennial bunchgrass colonized by *Glomus fasciculatum* (Killham and Firestone 1983). Joner *et al.* (2000) observed that the AM mycelium had a high binding affinity for Cd and Zn.

Interestingly, about 60% of the Ni content was detected in the shoots of AM plants compared to 20% in non-AM plants when supplied with NH_4^+. By contrast, Zornoza *et al.* (1999) suggested that the Ni transport from roots to shoots in sunflower was more limited under NH_4^+ than NO_3^- nutrition. Similarly, McGrath and Rorison (1982) observed in grasses, *Holcus lanatus* and *Bromus erectus*, that NH_4^+ inhibited Mn uptake, whereas NO_3^- enhanced it but resulted in a reduced Mn tolerance. However, Rorison (1985) observed that *Deschampsia flexuosa* treated with Al showed less toxic effects when supplied with NH_4^+ than NO_3^-, and suggested that pH differences, due to the two different inorganic N ions, affected plant tolerance to Al toxicity. Moreover, tolerance to Zn was shown to be enhanced in *Deschampsia caespitosa* when supplied with NH_4^+ than NO_3^- (Smirnoff and Stewart 1987b). This enhanced tolerance in NH_4^+-fed plants was

suggested to result from a higher content in asparagine which forms complexes with Zn (Smirnoff and Stewart 1987b). Therefore, N nutrition may indirectly play a significant role in plant HM tolerance, including Ni.

Our results on the Ni content suggest that Ni was transferred from roots to shoots. Cataldo *et al.* (1978b) reported that $\sim$ 85% of Ni in soybean was distributed in roots 24 h after Ni feeding, but after 21 days, it decreased to $\sim$ 50%. As Ni is considered an essential microelement (Brown *et al.* 1987, Marschner 1995), the transport of Ni to sunflower shoots may have been facilitated through chelation with organic acids such as malate or citrate (Lee *et al.* 1977), or amino acids such as histidine (Krämer *et al.* 1996). Saber *et al.* (1999) reported significant increases of malic and citric acids in the shoots and roots of sunflower seedlings treated with Al, Cd and Zn. Cataldo *et al.* (1978b) concluded that the chemical fate of Ni may be dependent of plant species. In the present study, the AM colonization significantly increased the Ni extracted % by approximately 1.3 times in plants supplied with NO_3^-. The total Ni extracted %, ranging from 2.63 to 3.51%, is substantial considering that most plant species usually have less than 0.01% Ni, under "normal" conditions (Baker and Brooks 1989, Marschner 1995, Prasad 2003). Similar observations were reported by Turnau and Mesjasz-Przybylowicz (2003) who showed that AM plants of *Berkheya coddii* had higher Ni content in shoots than roots. Davies *et al.* (2001) also reported higher Cr levels in sunflower shoots than roots.

2.5.2 Physiological parameters

Mycorrhizae are known to be highly adaptable even under adverse environmental conditions. In the present study, *Glomus intraradices* was shown to be well tolerant to soil Ni input, as the root colonization level reached 40%. Del Val *et al.* (1999) observed the presence of six *Glomus* species in soils with sewage-amended sludge containing

Ni, Zn, Cd, Pb and Cu. Davies *et al.* (2001) reported 100% root colonization for another sunflower cultivar colonized with *G. intraradices* grown in Cr contaminated soil. Mehrotra and Baijal (1994) obtained colonization levels of 60 to 90% in sunflower with a mixture of *G. intraradices* and *G. mosseae* grown under low, medium and high N and P levels. Thus, the AM fungal species, and sunflower cultivar may contribute to varying colonization levels.

In the present study, the AM association significantly contributed to higher root and total plant dry masses under NO_3^- fertilization without soil Ni input. An enhanced growth by AM colonization had also been reported for sunflower treated with Cr (Davies *et al.* 2002) and red clover treated with Zn (Chen *et al.* 2003). By contrast, Audet and Charest (2006) did not observe any biomass differences between AM and non-AM plants of the wild tobacco species grown in Zn contaminated soils. Guo *et al.* (1996) reported no differences in biomass of AM or non-AM bean or maize plants grown with Cd or Ni, while Takács and Vörös (2003) reported lower biomass in AM than non-AM ryegrass exposed to Cd, Zn and Ni. Mycorrhizal contribution to plant growth is highly variable and can be attributed, directly or indirectly, to improved mineral nutrition (Clark and Zeto 2000, Gupta *et al.* 2000, Smith and Read 1997), and/or increased water uptake (Subramanian and Charest 1998, 1999) under drought conditions. Therefore, growth is dependent upon the specific combination of fungal and plant species, as well as the experimental conditions.

The NO_3^- over NH_4^+ fertilization significantly increased the plant biomass, shoot height and R/S ratios. Thus, the ammonium fertilization may have contributed to reduced growth of sunflower plants as NH_4^+ accumulation leads to lower cytosolic pH and photosynthetic activity (Lasa *et al.* 2001, Marschner 1995). These results coincide with other studies which showed the preference of sunflower plants for NO_3^- than NH_4^+ (Lasa *et al.* 2000 and 2001, Zornoza *et al.* 1999).

Surprisingly, the addition of Ni did not significantly affect the sunflower biomass nor shoot height. Ouzounidou and Ilias (2005) suggested that sunflower shoot height is less affected than root length to excess Cu because of lower Cu content in shoots. By contrast, Zornoza *et al.* (1999) observed that Ni not only caused stunted root and shoot in sunflower supplied with NO_3^-, but also interveinal chlorosis and necrosis in younger leaves. Our soil Ni concentration supplied to sunflower plants may be below their toxicity threshold level, as their high biomass may have resulted in a growth dilution effect. However, their chlorophyll concentrations were significantly decreased by the soil Ni input. Other studies reported lower photosynthetic rates and pigments, as chlorophylls, in sunflower plants under Cr (Davies *et al.* 2002), Cu (Ouzounidou and Ilias 2005), and Cd (Panković *et al.* 2000), and in birch seedlings under Ni stress (Jones and Hutchinson 1988b). By contrast, Zornorza *et al.* (1999) reported that excess Ni caused an increase in chlorophyll concentrations in sunflower. Other studies with sunflower showed that Ni toxicity causes a decrease in lipid content and a degradation of the chloroplast membrane system, the Golgi complex and the endoplasmic reticulum (Slivinskaya 1991), and a decrease in peroxidase activity (Pillay *et al.* 1996). These plant disruptions could contribute to growth stunting and chlorosis, which are considered the two major symptoms of Ni toxicity (Jones and Hutchinson 1988a,b).

2.5.3 Mineral concentrations

The AM treatment led to increased concentrations of Ca, Mg, Mn and Fe in the leaves, and of K and Cu in the roots. In addition, the interaction of AM with soil Ni input tended to enhance the concentrations of P, Ca and Mn in the leaves of AM plants, while the interaction of AM with the fertilizer tended to increase the concentrations of K, Cu and Zn in the roots of AM plants supplied with NH_4^+. Killham and Firestone (1983) also reported enhanced uptake of Cu, Ni, Pb and Zn in AM bunchgrass. Using a compartmented glass bead system, Chen *et al.* (2001) observed that P, Cu and Zn

concentrations were higher in the fungal biomass than in roots or shoots of maize. Davies *et al.* (2002) reported higher K but lower levels of Fe and Al in AM than non-AM Cr-treated sunflower plants. Azcón *et al.* (2003) observed a decrease or no effect on Mg, Cu, Fe, and S in shoots, and on P, K, Ca, Cu, Fe and S in roots of AM lettuce plants under high N and P levels. These discrepancies in AM contribution to nutrient acquisition may be attributed to differences in the experimental design, the fungal inoculum, the host plant species, as well as, a growth dilution effect (Chen *et al.* 2003, Liu *et al.* 2000).

It is known that Ni excess counteracts the uptake of other essential elements, inducing nutrient deficiencies. In particular, Ni competes with divalent cations such as Ca, Mg, Fe, Mn, Cu and Zn (Cataldo *et al.* 1978a, Marschner 1995, Zornoza *et al.* 1999). This was observed in the present study, as the addition of Ni tended to lower the concentrations of Ca, Cu and Zn in AM and non-AM roots, and of P in leaves of non-AM plants. Zornoza *et al.* (1999) also observed decreased concentrations of Ca and Cu, but not of Zn in roots of Ni treated sunflower plants supplied with NO_3^-. Jones and Hutchinson (1988b) reported reduced concentrations of P, Mg, Ca and Fe in shoots of mycorrhizal birch seedlings. By contrast, Madejón *et al.* (2003) reported that Cu and Zn concentrations increased, especially in the roots, in a sludge polluted as compared to a non-polluted site. Surprisingly, the soil Ni input did not result in any significant differences in Fe and Mg concentrations, as Ni usually induces their reduction leading to a decrease in chlorophyll concentrations (Jones and Hutchinson 1988b, Zornoza *et al.* 1999).

In the present study, plants supplied with NO_3^- tended to have higher concentrations of P, Ca, Mg and Mn in leaves and of Ca in roots. Gerendás *et al.* (1997) argued that since a considerable proportion of Ca is localised in the vacuole to balance mineral anions such as nitrate, the Ca requirement of urea-grown plants may be lower than for NO_3^--grown plants. This observation is in agreement with our findings, as plants supplied with NH_4^+ had lower Ca concentrations in leaves and roots as compared to plants supplied

with NO_3^-. Conversely, we found that concentrations of P, Cu and Zn in roots tended to be higher in plants fertilized with NH_4^+. Lasa *et al.* (2000) also reported higher P content in plants grown with NH_4^+ than NO_3^-. The enhanced concentration of P may have resulted from its increased mobility in soils brought about by a decrease in rhizosphere pH from the NH_4^+ fertilizer (Marschner 1995). Surprisingly, we also observed that the K concentrations in leaves and roots tended to be higher in plants supplied with NH_4^+ than NO_3^-, as the uptake of K is usually inhibited by NH_4^+ in order to regulate the charge balance (Marschner 1995, Smith and Read 1997). However, the AM associations greatly affect the uptake of minerals, and in many cases, enhance their acquisition particularly in mineral or water deficient soils (Clark and Zeto 2000, Marschner 1995, Smith and Read 1997). In this study, the significant increase of K concentrations in roots due to the interaction of AM with fertilizer may account for the higher levels we observed in NH_4^+-fed plants.

2.5.4 Nitrogen assimilating enzymes

The AM colonization tended to increase the activities of NR, GS and GDH and the specific activities of NR and GS in leaves. In addition, the GDH activity and the specific activities of NR and GDH in roots also tended to be increased as a result of the AM association. Subramanian and Charest (1998) reported enhanced NR, GS and GOGAT activities in AM maize plants. Cliquet and Stewart (1993) also reported increased NR and GS activities in roots and shoots of maize plants when colonized with *Glomus fasciculatum*. Smith *et al.* (1985) observed higher GS than GDH activity in roots of onion plants colonized with *Glomus mosseae*. By contrast, Toussaint *et al.* (2004) reported higher GDH activity in AM than non-AM transformed carrot roots, suggesting that this enhanced GDH activity is increased by preferential uptake of NH_4^+ by *G. intraradices*. The possible involvement of NADP-GDH in NH_4^+ assimiliation has also been reported in ectomycorrhizal associations (Martin *et al.* 1988, Pierleoni *et al.* 2001). Further-

more, Martin *et al.* (1988) concluded that NH_4^+ assimiliation in the ectomycorrhizal *Cenococcum geophilum* occurs by the concurrent activity of the GDH and GS pathways. This was also observed in ectomycorrhizal *Laccaria bicolor* (Ahmad *et al.* 1990), and in AM transformed carrot roots (Toussaint *et al.* 2004). In the present study, we also observed enhanced root GDH activity and specific activity in AM plants supplied with NO_3^-. Moreover, the activities of GDH in leaves and of GS in roots were enhanced in AM plants under NH_4^+ fertilization. Our results do suggest that AM colonization contribute to an enhanced N assimilation in sunflower via concurrent activities of GS/GOGAT and GDH pathways. Labelled N studies in compartmented systems may provide further insight in AM contribution to NH_4^+ assimilation as reported by Toussaint *et al.* (2004) and Subramanian and Charest (1999).

In this study, we observed that soil Ni input is closely linked to different N fertilization. The combined effect of soil Ni input and NH_4^+ fertilization resulted in higher activities of leaf GS and GDH, and of root NR, GS and GDH than treatments without soil Ni, whereas, the combined effect of soil Ni input and NO_3^- significantly increased the leaf GS and GDH activities in treatments without soil Ni. Furthermore, the activities of NR and GS were significantly higher in plants supplied with NH_4^+ than NO_3^-. Similarly, Gouia *et al.* (2003) observed that Cd induced high levels of ammonium and increased the GDH activity in bean seedlings. According to Chugh *et al.* (1992), the role of GDH in NH_4^+ assimilation may be enhanced under HM stresses. Furthermore, they proposed that GDH and GS/GOGAT activities may also be influenced by Ni contamination. In addition, plants occurring on acidic (NH_4^+-N) and calcareous (NO_3^--N) soils differ in their response to the N source (Baker and Brooks 1989, Marschner 1995, Rorison 1985). Ammonium assimilation via GDH activity in roots of grapevines treated with Cu (Llorens *et al.* 2000) and of beans treated with Cd (Gouia *et al.* 2003) were also proposed as an alternative system to the GS/GOGAT pathway. In our study, the increased activities of GS and GDH in plants supplied with NH_4^+ suggest that, under soil Ni input, N assimilation is

enhanced via the concurrent activities of GS/GOGAT and GDH pathways. Therefore, our results, in agreement with others, suggest that the role of GDH may be important under HM stresses.

2.6 Conclusion

In this study, we have investigated the combined effects of AM colonization with N fertilization and soil Ni treatments on N metabolism in sunflower, as well as on the uptake and tolerance of Ni. To our knowledge, this is the first study to examine this representative plant-fungal relationship in Ni contaminated soils.

The AM colonized sunflower plants supplied with NO_3^- had significantly higher Ni content than non-AM plants, and significantly higher Ni content than plants supplied with NH_4^+. This Ni content, predominantly distributed in roots (80%) of AM sunflower plants, suggests that some of the Ni was also sequestered in the fungal hyphae. This enhanced plant tolerance due to AM symbiosis is in agreement with others who also reported reduced or selectively transfer of HM(s) from roots to shoots (Audet and Charest 2006, Guo *et al.* 1996, Joner *et al.* 2000).

We showed that N nutrition indirectly plays a significant role in Ni uptake, as 60% of the Ni content was distributed in the shoots of AM colonized plants when supplied with NH_4^+. Several studies also reported similar enhancement of Al (Rorison 1985), and Zn (Smirnoff and Stewart 1987b) in plants under NH_4^+ fertilization. We also observed that plants supplied with NH_4^+ had increased activities of N-assimilating enzymes when treated with Ni. Our results, in accordance with others, suggest that N metabolism is enhanced under NH_4^+ nutrition with soil Ni input (Chugh *et al.* 1992, Llorens *et al.* 2000, Srivastava and Singh 1987).

The AM symbiosis contributed to enhanced biomass and mineral concentrations when plants were supplied with NO_3^-. These results are in accordance with other studies (Azcón *et al.* 2003, Chen *et al.* 2003, Liu *et al.* 2000, Phelps 2004). Furthermore, we observed enhanced GS and GDH activities in AM sunflower plants, suggesting that the AM colonization contributes to an improved N metabolism via concurrent activities of GS/GOGAT and GDH pathways. Others also reported similar observations in AM transformed carrot roots (Toussaint *et al.* 2004), in maize under drought conditions (Subramanian and Charest 1998, 1999) and in ectomycorrhizal *Laccaria bicolor* (Ahmad *et al.* 1990).

Our results reveal that sunflower is a promising "candidate" plant species for phytoremediation and Ni phytoextraction. Furthermore, colonization with AM fungi may aid in enhancing the uptake and translocation of Ni from roots to shoots. As very little symptoms of Ni toxicity were observed in sunflower plants at 100 mg Ni kg^{-1}, the Ni tolerance threshold in sunflower has likely not been reached. Increased input of soil Ni concentration may shed more light on the extent of AM colonization on plant tolerance to Ni toxicity.

Chapter 3

Phytoremediation of Ni contaminated soils using sunflower colonized by AM fungi

3.1 Introduction

The sources of heavy metals (HMs) in soils include the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments, dyes and batteries (Leyval *et al.* 1997). The toxicity of a metal depends on its bioavailability, that is, its ability to be transferred from soil to organisms (Leyval *et al.* 1997). The bioavailability depends on the relative lipophilicity of the compound, the soil components (pH, organic matter and clay content or type), and the contaminant duration (Cunningham *et al.* 1995, Leyval *et al.* 1997). Since HMs cannot be degraded, current remediation strategies include either *in situ* (e.g., solidification, burial, soil washing, acid leaching) or *ex situ* (e.g., excavation, disposal to landfill sites) methods (Cunningham *et al.* 1995). These physico-chemical methods are expensive and environmentally degradative, as they remove any fauna, useful microbes, nitrogen-fixing bacteria and mycorrhizal fungi from the soil (Khan *et al.* 2000).

Phytoremediation has been proposed as an alternative to these physico-chemical methods (Chaney *et al.* 1997). Early studies on plants growing on metal-contaminated soils posed intriguing questions about the mechanisms of adaptation involved (Baker 1987). Baker (1981) proposed three types of plant-soil relationships: *accumulators*, where metals are concentrated and detoxified in aboveground organs; *indicators*, where metal uptake and transport to the shoot are regulated so that internal concentrations reflect

external levels; and *excluders*, whereby metal uptake and/or root to shoot transport are restricted.

Plants capable of accumulating HMs require a highly specialized physiology, as metal concentrations in plant tissues are extreme and considered as toxic (McGrath *et al* 2001). Plants exhibiting these traits are termed hyperaccumlators; defined as plant species whose shoots contain more than 100 mg Cd kg^{-1}, 1000 mg Ni, Pb or Cu kg^{-1}, or 10,000 mg Zn or Mn kg^{-1} on a dry mass basis when grown in metal-rich soils (Baker 1977, Dahmani-Muller *et al.* 2000). These metal concentrations in hyperaccumulators are one to three order(s) magnitude higher than in non-hyperaccumulators (Cunningham 1995, Dahmani-Muller *et al.* 2000).

Approximately two-thirds of the known hyperaccumulator plant species accumulate nickel. Geobotanical studies of ultramafic floras have provided insight on their ecophysiology (Baker and Brooks 1989, McGrath *et al.* 2001). Only recently has nickel been considered an essential micronutrient in plant growth and production (Baker and Brooks 1989, Brown *et al.* 1987, Marschner 1995). In plants, Ni has been shown to be the metallic component of urease in jack bean (Dixon *et al.* 1975). Nickel concentrations ranged between 0.5-1.0 $\mu g\ g^{-1}$ for most plants found on non-nickeliferous soils, in contrast to 5-100 $\mu g\ g^{-1}$ for plants growing on Ni-rich substrates, and can be as high as 30,000 $\mu g\ g^{-1}$ in hyperaccumulators (Reeves *et al.* 1983). Excess of Ni can cause inhibition of root elongation and interveinal chlorosis (Krämer *et al.* 1997), as well as biochemical changes in cell membranes, such as decreases in lipid and phospholipid content, formation of Golgi bodies, endoplasmic reticulum swelling, and starch accumulation in chloroplasts (Slivinskaya 1991).

Phytoremediation seeks to exploit the metabolic capabilities and growth habits of hyperaccumulator plant species (Cunningham 1995, Pankovic *et al.* 2000). However,

the potential of these hyperaccumulators for use as phytoremediation candidate species are limited by a number of factors, of which their slow growth and small biomass are their main drawbacks (Cunningham 1995, Raskin and Ensley 2000). As such, current strategies of phytoremediation focus on increasing HM plant uptake and tolerance. The use of mycorrhizal fungi has been proposed since they not only improve the vitality of host plant (e.g. nutrition, resistance against pathogens, drought), but also contribute to alleviate HM plant stresses (Gildon and Tinker 1983, Joner *et al.* 2000, Jones and Hutchinson 1988, Schwitzguébel 2001). Mycorrhizal fungi provide a direct link between the soil and roots, allowing for an enhanced HM availability and tolerance to plants (Cunningham *et al.* 1995).

3.2 Hypothesis and Objectives

This study focuses on the effect of AM colonization on phytoremediation potential of sunflower under soil Ni treatments. In addition, it aimed to determine the threshold or toxic Ni concentration in sunflower. It was hypothesized that AM colonization increases tolerance and uptake of Ni in sunflower plants at different developmental stages, under increasing soil Ni input. To test this hypothesis, the specific objectives were to determine if the AM symbiosis in sunflower plants increases: (1) the content of Ni in plant organs; (2) the threshold or toxic Ni level in plants exposed at the vegetative or reproductive stage of development; (3) the physiological parameters such as biomass, shoot height, Root/Shoot ratio, chlorophyll and mineral concentrations; and (4) the soluble protein concentration and the activity of glutamine synthetase (GS, EC 6.3.1.2).

3.3 Materials and Methods

The detailed methods of growth conditions, root colonization, analyses of chlorophylls, minerals, GS activity, and statistical analyses are presented in Chapter 2 (2.3.1 to 2.3.10). Only the changes that were brought here are indicated below.

3.3.1 Growth conditions and treatments

Two growth experiments were performed to test our hypothesis. In Experiment #1, plants of sunflower were grown with or without the AM fungal inoculum, *Glomus intraradices* Schenck & Smith, in soil treated with 0, 100, 200 or 400 mg Ni kg^{-1} dry soil (DS), applied as $NiCl_2 \cdot 6H_2O$, after 8 weeks of growth (56 days after sowing, DAS). Plants not receiving Ni were given dH_2O. Eight pots per treatment with 3 seeds per pot were sown (64 pots total). In Experiment #2, sunflower seeds were directly placed in soil (0 DAS) containing 0, 200 and 400 mg Ni kg^{-1} DS, with or without the AM inoculum. Four pots per 0 soil [Ni], and 6 pots per 200 or 400 mg Ni kg^{-1} Ni treatment, with 3 seeds per pot were sown, for a total of 32. Plants were fertilized with a NH_4NO_3-type LANS (Hewitt and Smith 1975) once a week (250 mL) for 2 weeks, and bi-weekly (500 mL) thereafter for 6 weeks. The LANS fertilizer composition is described in the Appendix 2. Plants were harvested at week 10 for both experiments.

3.4 Results

3.4.1 Mycorrhizal colonization

Sunflower roots were highly colonized even with the increasing soil Ni input (Figure 3.1). In Experiment #1, the AM root colonization levels were 78, 76, 64 and 64% at 0, 100, 200 and 400 mg Ni kg^{-1} soil Ni, respectively. In Experiment #2, at 400 mg Ni kg^{-1} soil Ni input, germination had occurred ($\sim$ 80%), yet growth subsided after 3 weeks, and

by the 4th week all plants died. However, plants (5 of 6 replicates) exposed to 200 mg Ni kg^{-1} Ni survived until the end of the experiment with a 42% root colonization.

3.4.2 Physiological data

Experiment #1

The soil [Ni] significantly ($P < 0.05$) affected the leaf dry mass (DM) (Table 3.1). According to the Tukey's test, the leaf DM of 0M+ treatment was significantly higher (by 1.4 times) than 200M+. No significant effects were observed for flower DMs.

The soil [Ni] ($P < 0.01$), and its interaction with AM ($P < 0.01$) were significant on stem DM (Table 3.1). According to the Tukey's test, with increasing soil [Ni], the stem DMs significantly decreased in AM plants, but was unchanged in non-AM plants. The 0M+ treatment was also significantly higher than all other treatments. Furthermore, stem DM was significantly higher at 100M(±) than the 400M+ treatment.

The AM ($P < 0.001$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.001$), had significant effects on root DM (Table 3.1). According to the Tukey's test, at 0 and 100 soil [Ni], the root DMs were significantly higher in AM than non-AM plants, and 3 to 4 times higher than all other treatments.

The AM ($P < 0.05$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.01$), had significant effects on total plant DM (Table 3.1). According to the Tukey's test, the total DM of AM plants were significantly decreased with increasing soil [Ni], but were unchanged in non-AM plants. The total DM for 0M+ and 100M+ treatments were significantly higher (by 1.3 to 1.8 times) than at 200M(±) and 400M(±). In addition, the total plant DM at 0M+ was significantly higher than at 0M- and 100M- (by 1.5 and 1.4 times, respectively).

The Root/Shoot (R/S) ratio was significantly ($P < 0.001$) decreased with increasing soil [Ni] for AM and non-AM plants (Table 3.4). In addition, the interaction of soil [Ni] with AM was also significant ($P < 0.05$). According to the Tukey's test, the R/S ratios at 0M(±) and at 100M+ were significantly higher (by 1.5 to 2.5 times) than all other treatments. The soil [Ni] was marginally non-significant on the leaf concentrations of Chl*a* ($P = 0.057$), Chl*b* ($P = 0.084$) and Chl*tot* ($P = 0.057$). According to the Tukey's test, Chl*a* and Chl*tot* concentrations were significantly lower at 400M+ than all other treatments. No significant differences were observed for shoot height in any of the treatments (Table 3.4).

Experiment #2

The DM of AM plants was significantly higher in stem ($P < 0.01$), but significantly lower in leaf ($P < 0.01$), and marginally non-significantly lower in flower ($P = 0.088$), than non-AM plants. No significant differences were observed for root or total plant DMs (Table 3.7).

The shoot height was significantly ($P < 0.01$) higher, and the R/S ratio was marginally non-significantly ($P = 0.098$) lower in AM than non-AM plants (Table 3.9). No significant differences were observed for chlorophyll concentrations (Table 3.9).

Figure 3.1 AM structures of *Glomus intraradices* Schenck & Smith in sunflower roots grown in Ni at 400 mg kg^{-1} DS (**A**), 200 mg kg^{-1} DS (**B**) and 0 mg kg^{-1} DS (**C**).
A. Intraradical hyphae (HY) and vesicles (VE) .
B. Arbuscule (AR) and hyphae.
C. Magnified view of vesicles.

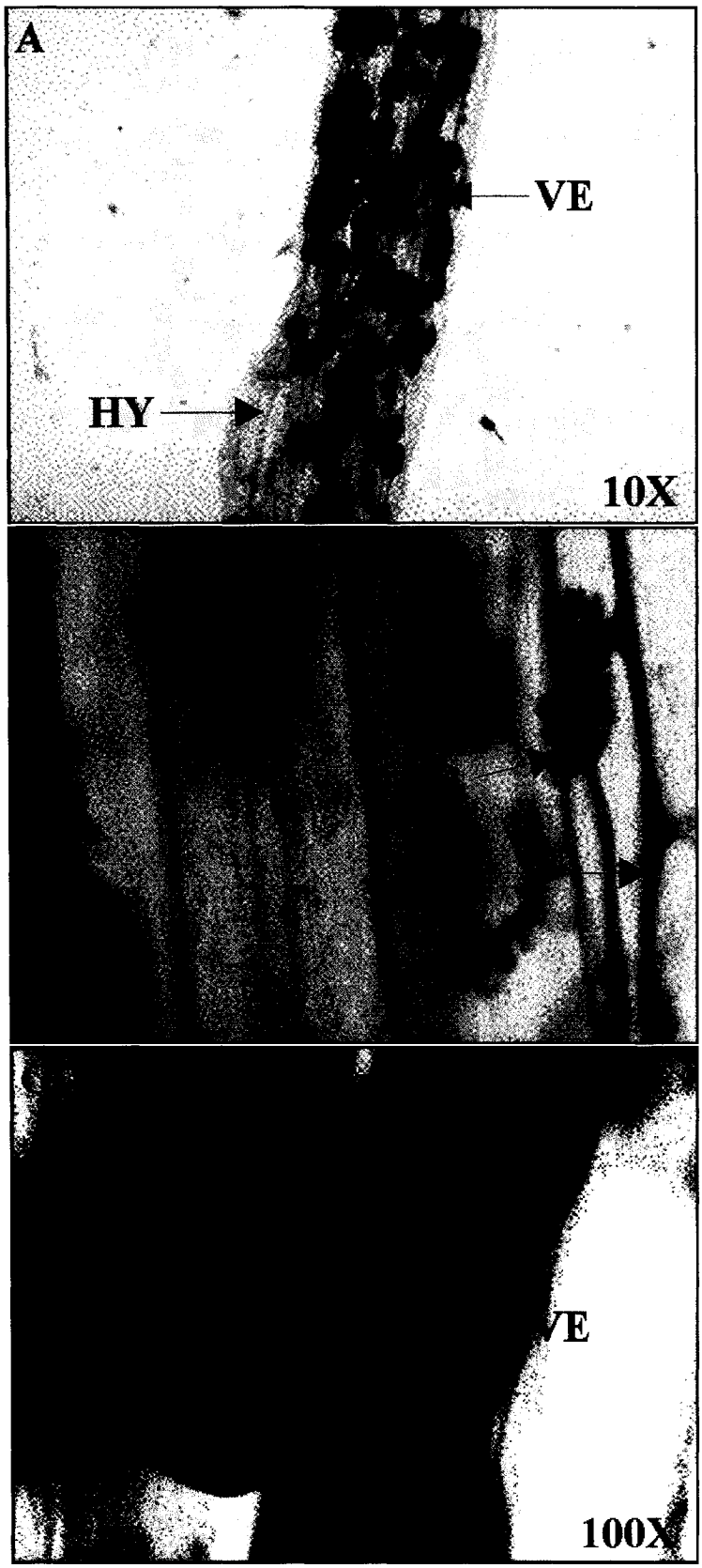

A
VE
HY
10X
VE
100X

Table 3.1 Dry mass (DM) means and (SEs) (n = 3 to 6) for each organ and total plant DM of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	DM (g)				
		Flower	Leaf	Stem	Root	Total
0	M -	2.70^{a} (0.58)	21.2^{ab} (0.99)	30.7^{b} (2.24)	9.94^{b} (1.07)	64.5^{c} (1.12)
	M+	3.51^{a} (1.27)	24.1^{a} (1.24)	45.6^{a} (3.63)	21.2^{a} (1.94)	94.4^{a} (5.23)
100	M -	5.01^{a} (1.25)	21.9^{ab} (1.64)	32.0^{b} (3.31)	9.52^{b} (1.36)	68.4^{bc} (6.48)
	M+	4.44^{a} (0.83)	22.0^{ab} (1.43)	29.8^{b} (4.46)	20.0^{a} (0.40)	81.5^{ab} (9.91)
200	M -	3.76^{a} (1.11)	20.4^{ab} (1.69)	26.8^{bc} (2.45)	8.78^{b} (0.85)	59.7^{c} (7.19)
	M+	3.76^{a} (0.96)	17.2^{b} (1.45)	26.6^{bc} (1.50)	7.08^{b} (1.01)	54.6^{c} (4.38)
400	M -	2.97^{a} (0.91)	21.7^{ab} (1.82)	27.6^{bc} (2.71)	8.80^{b} (0.22)	61.1^{c} (3.23)
	M+	3.85^{a} (0.66)	19.3^{ab} (0.60)	21.4^{c} (1.76)	6.35^{b} (0.85)	50.9^{c} (3.99)
ANOVA: AM fungi (M), Nickel (Ni)						
M		NS	NS	NS	***	*
Ni		NS	*	**	***	***
M X Ni		NS	NS	**	***	**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.2 Two-way ANOVA on ranked data of shoot dry masses.

Source of variation	Flower			Leaf			Stem		
	M	Ni	M X Ni	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3	1	3	3
SS	1.00	20.23	4.65	30.41	2554.19	1539.91	3.96	2422.71	2315.14
MS	1.00	6.74	1.55	30.41	851.40	513.30	3.96	807.57	771.71
F-value	0.14	1.98	0.22	0.13	3.52	2.12	0.02	4.59	4.39

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.3 Two-way ANOVA on root (ranked data) and total dry masses.

Source of variation	Root			Total		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	63.04	744.92	789.55	535.46	3305.31	2552.21
MS	63.04	248.30	263.18	535.46	1101.77	850.74
F-value	2.25	8.86	9.39	4.74	9.76	7.54

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.4 Height (n = 8), Root/Shoot ratio (R/S, n = 5) and leaf chlorophyll concentration (mg g^{-1} FM) (n = 3) means and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg Ni kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	Height (cm)	R/S	Chl concentration		
				Chl*a*	Chl*b*	Chl*tot*
0	M -	143^{a} (8.01)	0.33^{a} (0.09)	1.42^{a} (0.05)	0.55^{a} (0.00)	1.97^{a} (0.05)
	M+	136^{a} (14.9)	0.31^{a} (0.02)	1.37^{a} (0.03)	0.55^{a} (0.01)	1.92^{ab} (0.05)
100	M -	148^{a} (6.63)	0.14^{b} (0.02)	1.33^{a} (0.11)	0.56^{a} (0.06)	1.89^{ab} (0.17)
	M+	159^{a} (10.5)	0.26^{a} (0.03)	1.43^{a} (0.02)	0.57^{a} (0.02)	2.00^{a} (0.12)
200	M -	151^{a} (9.35)	0.15^{b} (0.02)	1.40^{a} (0.14)	0.56^{a} (0.05)	1.96^{a} (0.19)
	M+	147^{a} (15.2)	0.13^{b} (0.01)	1.36^{a} (0.02)	0.57^{a} (0.02)	1.93^{a} (0.04)
400	M -	136^{a} (9.47)	0.17^{b} (0.02)	1.13^{ab} (0.06)	0.49^{a} (0.02)	1.62^{ab} (0.08)
	M+	142^{a} (6.88)	0.14^{b} (0.05)	0.76^{b} (0.20)	0.34^{a} (0.09)	1.10^{b} (0.29)
ANOVA: AM fungi (M), Nickel (Ni)						
M		NS	NS	NS	NS	NS
Ni		NS	***	MN	MN	MN
M X Ni		NS	*	NS	NS	NS

** $P < 0.01$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.5 Two-way ANOVA on ranked data of height and R/S ratio.

Source of variation	Height			R/S ratio		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	58.14	630.81	152.42	122.50	2442.10	796.20
MS	58.14	210.27	50.81	122.50	814.03	265.40
F-value	0.15	0.56	0.13	2.02	13.43	4.38

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.6 Two-way ANOVA on ranked data of chlorophyll concentrations.

Source of variation	Chl*a*			Ch*b*			Chl*tot*		
	M	Ni	M X Ni	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3	1	3	3
SS	0.87	259.87	32.31	1.14	237.22	33.10	2.29	258.31	35.07
MS	0.87	86.62	10.77	1.14	79.07	11.03	2.29	86.10	11.69
F-value	0.03	3.39	0.42	0.04	2.88	0.40	0.09	3.39	0.46

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.7 Dry mass (g, DM) (n = 3 to 4) means and (SEs) for each organ and total plant of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS (0 DAS). ANOVA results are also presented.

AM treatment	Flower	Leaf	Stem	Root	Total
M -	2.66^a (0.44)	16.4^a (0.41)	25.6^b (1.76)	12.4^a (1.76)	57.1^a (2.47)
M+	1.43^a (0.32)	13.5^b (0.64)	38.7^a (2.87)	10.1^a (2.38)	63.7^a (2.22)
M	MN	**	**	NS	NS

** $P < 0.01$; MN, $0.05 < P < 0.1$; NS, not significant

Table 3.8 One-way ANOVA on dry masses.

Source of variation	Flower	Leaf	Stem	Root	Total
df	1	1	1	1	1
SS	2.60	16.96	290.83	7.64	8.00
MS	2.60	16.96	290.83	7.64	8.00
F-value	5.39	14.76	16.82	0.58	1.41

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

Table 3.9 Height (n = 8), R/S ratio (n = 5) and leaf chlorophyll concentration (mg g^{-1} FM) (n = 3) means and (SEs) for each physiological parameter of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

AM treatment	Height (cm)	R/S ratio	Chl*a*	Chl*b*	Chl*tot*
M -	90.3[b]	0.29[a]	0.86[a]	0.30[a]	1.16[a]
	(7.5)	(0.04)	(0.19)	(0.07)	(0.26)
M+	129[a]	0.18[a]	1.08[a]	0.38[a]	1.46[a]
	(13.0)	(0.10)	(0.11)	(0.03)	(0.14)
M	**	MN	NS	NS	NS

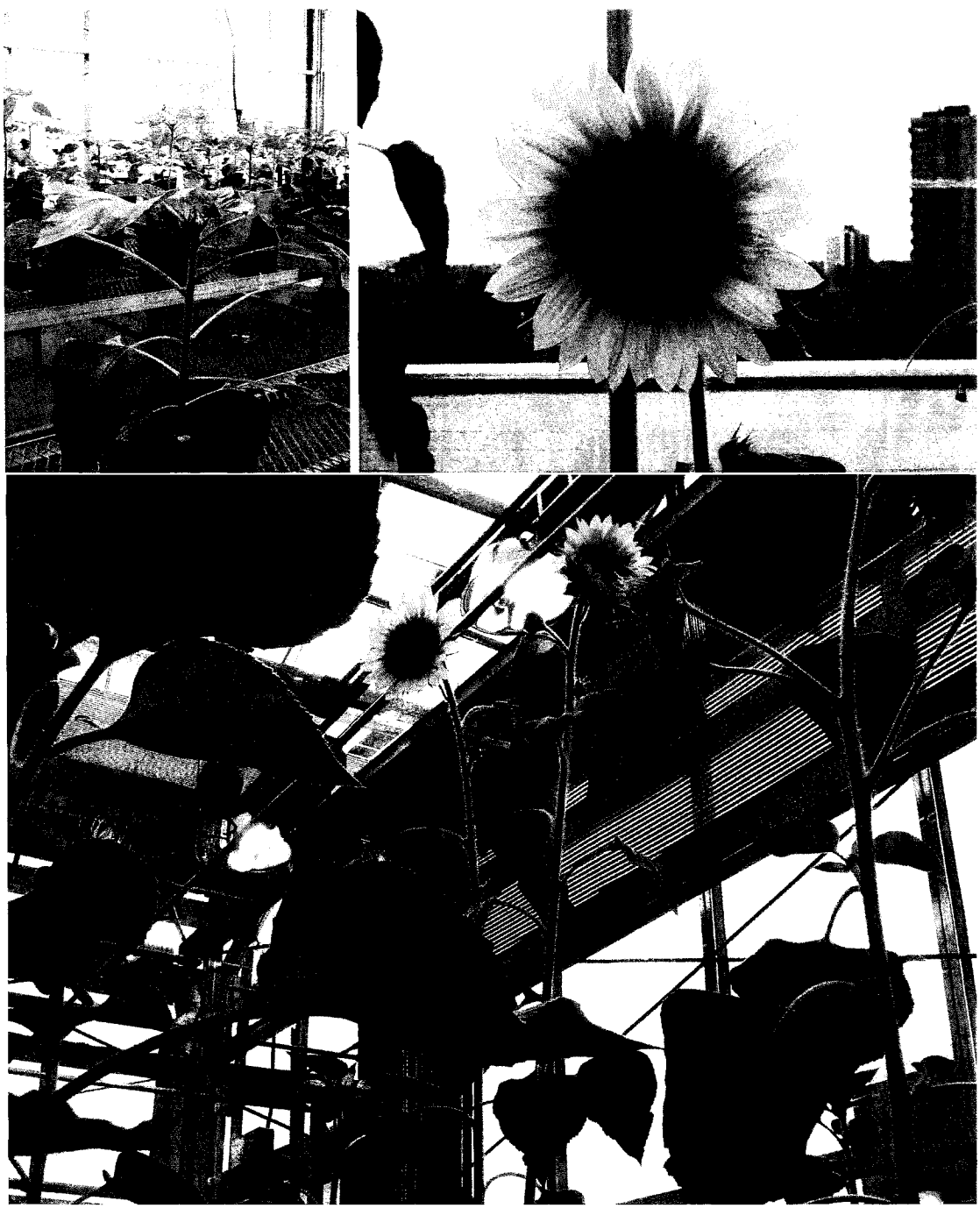
** $P < 0.01$; MN, $0.05 < P < 0.1$; NS, not significant

Table 3.10 One-way ANOVA on height, R/S ratio, and chlorophyll concentrations.

Source of variation	Height	R/S ratio	Chl*a*	Chl*b*	Chl*tot*
df	1	1	1	1	1
SS	0.06	0.02	0.13	0.02	0.25
MS	0.06	0.02	0.13	0.02	0.25
F-value	46.01	3.82	0.89	1.14	0.93

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

Figure 3.2 Growth stages of sunflower treated with 0 mg Ni kg^{-1} DS. **Top left:** Sunflower at 4 weeks of growth. Note the inflorescence, marked by the presence of a bud, has begun to occur. **Top right:** Sunflower head at maturity. Photo taken at 10 weeks of growth. **Bottom:** Sunflower at 10 weeks of growth.



3.4.3 Nickel concentration and content

Experiment #1

The flower [Ni] was significantly ($P < 0.001$) increased with increasing soil [Ni] (Table 3.11). According to the Tukey's test, the flower [Ni] significantly follows this order: 400 > 200 > 100 > 0. The flower Ni content was also significantly affected by the soil [Ni] ($P < 0.001$), and by its interaction with AM ($P < 0.05$) (Table 3.12). According to the Tukey's test, the Ni content at 400M(±) and 200M- were significantly the highest, followed by 200M+ and 100M(±), then 0M(±). The flower Ni content was also significantly lower (by 1.9 times) at 200M+ than at 200M-.

The leaf [Ni] was significantly ($P < 0.001$) increased with increasing soil [Ni] (Table 3.11). According to the Tukey's test, the leaf [Ni] significantly follows this order: 400 > 200 > 100 > 0. The leaf Ni content was also significantly affected by the soil [Ni] ($P < 0.001$), and was marginally non-significant for the AM ($P = 0.065$) treatment (Table 3.12). According to the Tukey's test, the leaf Ni content significantly follows this order: 400 > 200 > 100 > 0. In addition, the leaf Ni content was significantly higher (by 1.4 times) at 400M+ than at 400M-.

The stem [Ni] ($P < 0.001$) and Ni content ($P < 0.001$) were significantly increased with increasing soil [Ni] (Tables 3.11 and 3.12). According to the Tukey's test, the [Ni] and Ni content significantly follows this order: 400 > 200 > 100 ≅ 0.

The root [Ni] was significantly ($P < 0.001$) increased with increasing soil [Ni] (Table 3.11). According to the Tukey's test, the root [Ni] significantly follows this order: 400 > 200 ≅ 100 > 0. The AM ($P < 0.05$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.05$), significantly affected root Ni content (Table 3.12). According to the Tukey's test, the root Ni content significantly follows this order: 400 > 100M+ > 200 ≅ 100M- > 0. In addition, the root Ni content was significantly higher (by 3 times) at 100M+ than at

100M-.

The total [Ni] was significantly ($P < 0.001$) increased with increasing soil [Ni] (Table 3.11). According to the Tukey's test, the [Ni] significantly follows this order: 400 > 200 $\cong$ 100 > 0. The AM ($P < 0.001$) and soil Ni ($P < 0.001$), and their interaction ($P < 0.05$), significantly affected total Ni content (Table 3.12). According to the Tukey's test, the Ni content significantly follows this order: 400 > 200 $\cong$ 100M+ > 100M- > 0. In addition, the total Ni content was significantly higher (by 1.6 times) at 100M+ than at 100M-.

The shoot Ni extracted % was significantly affected by the AM ($P < 0.05$) and soil [Ni] ($P < 0.001$), with their interaction being marginally non-significant ($P = 0.057$) (Table 3.19). According to the Tukey's test, the shoot Ni extracted % significantly follows this order: 400M+ > 400M- $\cong$ 200 > 100. In addition, at 400M+ it was significantly higher than in all other treatments (by 1.4 to 2.2 times).

The AM ($P < 0.01$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.01$), significantly affected the root Ni extracted % (Table 3.19). The Tukey's test indicated that the Ni extracted % was significantly higher at 100M+ than at treatments 400M+, 100M-, 200M+ and 200M- by 2 to 4.5 times. In addition, at 400M-, it was significantly 3-fold higher than at 200M-.

The AM ($P < 0.001$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.05$), significantly affected total Ni extracted % (Table 3.19). According to the Tukey's test, it significantly follows this order: 400 $\cong$ 100M+ > 200 $\cong$ 100M-. In addition, the Ni extracted % was significantly higher (by 1.6 times) at 100M+ than at 100M-.

The soil [Ni] ($P < 0.001$) significantly affected the Ni distribution % at all soil [Ni], while its interaction with AM ($P < 0.01$) was significant at 100 and 400 soil [Ni] (Table

3.20). According to the Tukey's test, at 0 soil [Ni], the Ni content significantly follows this order: stem > leaf ≅ root > flower of AM and non-AM plants. At 100 soil [Ni], the Ni content was significantly higher in AM than non-AM roots, but lower in AM than non-AM leaves. The Tukey's test also indicated that the Ni content was significantly higher in roots than leaves of AM plants (by 2.5 times), but lower in roots than leaves of non-AM plants (by 1.4 times). At 200 and 400 soil [Ni], the Ni content significantly follows this order: leaf > root > stem ≅ flower of AM and non-AM plants. The Tukey's test indicated that the Ni content in roots was significantly lower at 400M+ than at 400M- (by 1.4 times). Although not significant, the leaf Ni content tended to be higher at 400M+ than at 400M-.

Experiment #2

The soil [Ni] significantly ($P < 0.001$) affected the Ni distribution % (Table 3.20). According to the Tukey's test, the Ni content significantly follows this order: root > leaf > stem > flower, in both AM and non-AM plants. Although not significant, the Ni content in stems was 2.3-fold higher in AM than non-AM plants. The root [Ni] was marginally non-significantly ($P = 0.059$) lower in AM than non-AM plants (Table 3.24). There were no significant effects on the Ni extracted % (Table 3.27).

Table 3.11 Ni concentration (mg Ni g^{-1} (x 10^3) DM) means (n = 3 to 5) and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	Ni concentration			
		Flower	Leaf	Stem	Root
0	M -	5.80^{d} (0.80)	5.00^{d} (0.00)	6.33^{c} (1.33)	5.65^{c} (0.38)
	M+	5.90^{d} (0.90)	5.00^{d} (0.00)	5.00^{c} (0.00)	5.50^{c} (0.24)
100	M -	37.3^{c} (1.38)	59.8^{c} (4.78)	6.33^{c} (0.67)	111^{b} (21.1)
	M+	48.2^{c} (6.87)	51.8^{c} (8.39)	8.21^{c} (1.26)	128^{b} (14.3)
200	M -	78.3^{b} (2.60)	155^{b} (23.3)	18.4^{b} (1.08)	167^{b} (26.7)
	M+	76.7^{b} (4.26)	159^{b} (19.3)	20.0^{b} (10.7)	192^{b} (45.8)
400	M -	116^{a} (3.28)	388^{a} (24.6)	57.4^{a} (7.67)	662^{a} (96.4)
	M+	125^{a} (8.69)	440^{a} (18.5)	73.5^{a} (9.01)	728^{a} (83.4)
ANOVA: AM fungi (M), Nickel (Ni)					
M		NS	NS	NS	NS
Ni		***	***	***	***
M X Ni		NS	NS	NS	NS

*** $P < 0.001$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.12 Ni content (mg Ni organ^{-1} or mg Ni plant^{-1} DM) means (n = 3 to 4) and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	Ni content				
		Flower	Leaf	Stem	Root	Total
0	M -	0.02^{c} (0.00)	0.10^{e} (0.01)	0.18^{c} (0.03)	0.11^{d} (0.03)	0.41^{d} (0.05)
	M+	0.02^{c} (0.01)	0.11^{e} (0.01)	0.23^{c} (0.02)	0.12^{d} (0.01)	0.48^{d} (0.03)
100	M -	0.21^{b} (0.03)	1.22^{d} (0.12)	0.20^{c} (0.02)	0.86^{c} (0.24)	2.49^{c} (0.15)
	M+	0.21^{b} (0.02)	1.07^{d} (0.15)	0.30^{c} (0.05)	2.48^{b} (0.20)	4.06^{b} (0.09)
200	M -	0.41^{a} (0.08)	2.81^{c} (0.37)	0.60^{b} (0.15)	1.15^{c} (0.09)	4.96^{b} (0.34)
	M+	0.22^{b} (0.05)	2.90^{c} (0.10)	0.51^{b} (0.23)	1.57^{c} (0.53)	4.90^{b} (0.53)
400	M -	0.54^{a} (0.10)	7.97^{b} (0.35)	1.85^{a} (0.37)	6.58^{a} (0.94)	16.8^{a} (1.06)
	M+	0.62^{a} (0.14)	10.9^{a} (1.02)	2.25^{a} (0.32)	5.23^{a} (0.53)	19.0^{a} (1.08)
ANOVA: AM fungi (M), Nickel (Ni)						
M		NS	MN	NS	*	***
Ni		***	***	***	***	***
M X Ni		*	NS	NS	*	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.13 Two-way ANOVA on flower Ni concentration and content.

Source of variation	Ni concentration			Ni content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	9.01	1417.02	56.54	12.90	1391.34	84.55
MS	9.01	472.34	18.85	12.90	463.78	28.18
F-value	1.14	59.83	2.39	1.60	57.69	3.51

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.14 Two-way ANOVA on ranked data of leaf Ni concentration and content.

Source of variation	Ni concentration			Ni content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	0.61	0.61	0.13	20.23	2557.84	23.62
MS	2324.70	774.90	161.83	20.23	852.61	7.87
F-value	8.56	2.85	0.60	3.75	157.93	1.46

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.15 Two-way ANOVA on ranked data for stem Ni concentration and content.

Source of variation	Ni concentration			Ni content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	0.04	960.58	40.54	13.50	878.33	47.50
MS	0.04	320.19	13.51	13.50	292.78	15.83
F-value	0.00	39.16	1.65	1.02	22.24	1.20

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.16 Two-way ANOVA on ranked data of root Ni concentration and content.

Source of variation	Ni concentration			Ni content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	12.51	1784.50	51.81	0.06	1.58	0.09
MS	12.51	594.83	17.27	0.02	0.53	0.05
F-value	1.34	63.96	1.86	1.50	41.06	3.63

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.17 Two-way ANOVA on total Ni content and total Ni extracted % (ranked data).

Source of variation	Ni content			Ni extracted %		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	2	2
SS	6.00	1057.33	2.67	24.50	277.08	11.08
MS	6.00	352.44	0.89	24.50	138.54	5.54
F-value	1.15	67.53	0.17	1.71	9.70	0.39

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.18 Two-way ANOVA on shoot and root (ranked data) Ni extracted %.

Source of variation	Shoot Ni extracted %			Root Ni extracted %		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	2	2	1	2	2
SS	0.03	0.54	0.05	12.50	310.33	56.33
MS	0.03	0.27	0.03	12.50	155.17	28.17
F-value	4.85	37.97	3.68	1.44	17.85	3.24

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.19 Ni extracted percentage (%) means and (SEs) (n = 3) of sunflower grown with (M+) or without (M-) AM fungi in Ni (100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.[1]

Soil [Ni]	AM treatment	Ni extracted (%)[2]		
		Shoot[3]	Root	Total
100	M -	0.42^{c} (0.03)	0.23^{bc} (0.06)	0.65^{b} (0.04)
	M+	0.41^{c} (0.05)	0.66^{a} (0.05)	1.07^{a} (0.02)
200	M -	0.51^{bc} (0.05)	0.15^{c} (0.01)	0.66^{b} (0.05)
	M+	0.49^{bc} (0.02)	0.21^{bc} (0.07)	0.70^{b} (0.07)
400	M -	0.68^{b} (0.01)	0.44^{ab} (0.06)	1.12^{a} (0.07)
	M+	0.92^{a} (0.06)	0.35^{bc} (0.04)	1.27^{a} (0.07)
ANOVA: AM fungi (M), Nickel (Ni)				
M		*	**	***
Ni		***	***	***
M X Ni		MN	**	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN $0.05 < P < 0.1$

[1]Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[2]Data for treatments without Ni are not shown since the Ni extracted % were below 0.001 %.

[3]Shoot Ni extracted % was calculated by combining the Ni content for flower, leaf and stem.

Table 3.20 Ni distribution percentage (%) means (n = 3) and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Plant organ	AM treatment	Experiment #1				Experiment #2
		0	100	200	400	200
Flower	M -	4.0^{c} (1.0)	10^{d} (1.0)	8.0^{c} (1.0)	3.0^{d} (1.0)	5.0^{d} (1.0)
	M+	5.0^{c} (1.0)	6.0^{d} (1.0)	5.0^{c} (1.0)	3.0^{d} (1.0)	2.0^{d} (1.0)
Leaf	M -	27^{b} (5.0)	47^{ab} (5.0)	56^{a} (3.0)	47^{ab} (3.0)	36^{b} (8.0)
	M+	24^{b} (1.0)	25^{c} (4.0)	58^{a} (7.0)	57^{a} (3.0)	32^{b} (8.0)
Stem	M -	48^{a} (4.0)	8.0^{d} (1.0)	12^{c} (2.0)	11^{d} (2.0)	12^{c} (2.0)
	M+	48^{a} (1.0)	7.0^{d} (1.0)	9.0^{c} (3.0)	12^{d} (2.0)	27^{c} (11)
Root	M -	21^{b} (6.0)	34^{b} (6.0)	23^{b} (2.0)	39^{b} (3.0)	47^{a} (9.0)
	M+	23^{b} (1.0)	62^{a} (4.0)	28^{b} (6.0)	28^{c} (2.0)	45^{a} (3.0)
ANOVA: AM fungi (M), Organ (O)						
M		NS	NS	NS	NS	NS
O		***	***	***	***	***
M X O		NS	***	NS	**	NS

** $P < 0.01$; *** $P < 0.001$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.21 Two-way ANOVA on Ni distribution % for Experiment #1.

Source of variation	0 mg Ni kg^{-1}			100 mg Ni kg^{-1}		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	3.38	986.08	2.21	0.02	0.79	0.12
MS	3.38	328.69	0.74	0.02	0.26	0.04
F-value	0.34	33.53	0.97	0.48	63.50	9.95

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.22 Two-way ANOVA on Ni distribution % for Experiment #1.

Source of variation	200 mg Ni kg^{-1}			400 mg Ni kg^{-1}		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	16.67	960.25	18.92	0.00	0.86	0.036
MS	16.67	320.08	6.31	0.00	0.29	0.01
F-value	1.77	33.99	0.67	0.00	130.36	5.38

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.23 Two-way ANOVA on ranked data of Ni distribution % for Experiment #2

Source of variation	200 mg Ni kg^{-1}		
	M	Ni	M X Ni
df	1	3	3
SS	0.45	632.55	40.23
MS	0.45	210.85	13.41
F-value	0.03	14.31	0.91

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.24 Ni concentration (mg Ni g^{-1} (x 10^3) DM) and content (mg Ni $organ^{-1}$ or mg Ni $plant^{-1}$ DM) means (n = 3) and (SEs) for each organ and total plant of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

Parameter	AM treatment	Flower	Leaf	Stem	Root	Total
Concentration	M -	72.4 (11.7)	156 (29.8)	36.0 (6.85)	305 (58.9)	–
	M+	77.9 (14.7)	136 (28.4)	36.1 (9.20)	152 (23.7)	–
M		NS	NS	NS	MN	–
Content	M -	0.40 (0.04)	2.89 (0.75)	0.96 (0.15)	3.66 (0.57)	7.91 (0.32)
	M+	0.67 (0.55)	1.76 (0.30)	1.44 (0.42)	3.18 (0.66)	7.05 (1.17)
M		NS	NS	NS	NS	NS

MN, $0.05 < P < 0.1$; NS, not significant

Table 3.25 One-way ANOVA on Ni concentration for Experiment #2.

Source of variation	Flower	Leaf	Stem	Root
df	1	1	1	1
SS	45.26	564.73	28134.88	59968.00
MS	45.26	564.73	28134.88	59968.00
F-value	0.08	0.22	3.85	6.83

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

Table 3.26 One-way ANOVA on Ni content for Experiment #2.

Source of variation	Flower	Leaf	Stem	Root	Total
df	1	1	1	1	1
SS	0.11	1.90	0.34	3.45	5.36
MS	0.11	1.90	0.34	3.45	5.36
F-value	0.25	1.94	1.15	1.49	1.17

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

Table 3.27 Ni extracted percentage (%) means and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

Parameter	Shoot[1]	Root	Total
M-	0.57 (0.11)	0.39 (0.06)	0.96 (0.15)
M+	0.52 (0.15)	0.42 (0.15)	0.94 (0.11)
M	NS	NS	NS

NS, not significant

[1]Shoot Ni extracted % was calculated by combining the Ni content for flower, leaf and stem.

Table 3.28 One-way ANOVA on Ni extracted % for Experiment #2.

Source of variation	Shoot	Root	Total
df	1	1	1
SS	0.00	0.04	0.06
MS	0.00	0.04	0.06
F-value	0.07	1.43	1.20

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

3.4.4 Mineral concentrations and total C and N contents (%)

Experiment #1

The AM treatment led to significant effects on the leaf concentrations of Ca, Mg, and Mn ($P < 0.05$), and Cu ($P < 0.001$), and a marginally non-significant effect on Zn ($P = 0.079$) (Tables 3.29 and 3.31). The soil [Ni] also had significant effects on the leaf concentrations of Ca ($P < 0.05$) and Cu ($P < 0.001$), and marginally non-significant effects on Mg ($P = 0.099$), Mn ($P = 0.051$) and Fe ($P = 0.062$). According to the Tukey's test, the concentration of Ca at 100M+ was significantly higher (by 1.7 times) than at 400M-. Although not significant, the concentrations tended to be higher in the leaves of AM than non-AM plants for Ca at 100, 200 and 400 soil [Ni], and Mg, Mn and Fe at all the soil [Ni].

The AM treatment had significant effects on the root concentrations of P, K, Cu, and Fe ($P < 0.05$), Mn ($P < 0.01$), and Ca, Zn, and Al ($P < 0.001$) (Tables 3.30 and 3.31). The soil Ni input also had significant effects on the concentration of Ca ($P < 0.01$), and marginally non-significant effects on Zn ($P = 0.087$), and Al ($P = 0.098$). There were also significant or marginally non-significant interactions on the concentrations of Al ($P < 0.05$) and Ca ($P = 0.095$). According to the Tukey's test, the Ca concentration was significantly higher at 100M+ (by 1.2 to 1.7 times) than all other treatments, and at 0M+ significantly higher (by 1.4 times) than at 400M-. The root Zn concentration was significantly higher (by 1.6 to 2.8 times) in AM than non-AM plants. The Tukey's test also indicated that the root Cu concentration at 0M+ was significantly higher than at 0M- and 400M- (by 2.2 times). The root Al concentration at 100M+ was significantly higher than at 0M-, 100M-, 200M- and 400M(±) (by 2.1 to 3.4 times), while 0M+ and 200M+ were significantly higher than 100M- (by 2.8 times). Although not significant, the concentrations tended to be higher in roots of AM than non-AM plants for P, Mn, and Fe at all soil [Ni], but lower for K at 0 and 100 soil [Ni].

The AM ($P < 0.05$) and soil [Ni] ($P < 0.05$) significantly affected the leaf C content (Table 3.41). According to the Tukey's test, the leaf C content was significantly higher at 400M- than 100M+ (by 1.2 times). Although not significant, the leaf C content tended to be lower in AM than non-AM plants. The AM treatment was marginally non-significant ($P = 0.052$) on root N content ($P = 0.052$) (Table 3.41). Although not significant, the root N content tended to be higher in AM than non-AM plants.

Experiment #2

The AM ($P < 0.05$) treatment significantly decreased (by 2.4 times) the concentrations of Mn and Fe in leaves, and significantly increased (by 1.2 times) the P concentration in roots (Table 3.44). No other significant differences were detected for the other mineral concentrations, nor the C and N content (Table 3.46).

Table 3.29 Mineral concentration (mg g^{-1} DM) means (n = 3) and (SEs) in leaves of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment.[1,2]

Soil [Ni]	AM treatment	P	K	Ca	Mg	Mn	Zn	Cu	Fe
0	M -	3.41^{a} (0.38)	29.3^{a} (3.38)	39.3^{ab} (5.02)	10.7^{a} (1.90)	0.74^{a} (0.19)	0.06^{a} (0.01)	0.02^{c} (0.00)	0.33^{a} (0.23)
	M+	3.26^{a} (0.22)	28.3^{a} (2.91)	39.9^{ab} (5.63)	12.1^{a} (2.92)	1.35^{a} (0.26)	0.09^{a} (0.04)	0.06^{b} (0.00)	0.70^{a} (0.31)
100	M -	3.88^{a} (0.37)	29.0^{a} (3.79)	46.7^{ab} (3.74)	14.6^{a} (4.21)	1.99^{a} (0.58)	0.08^{a} (0.02)	0.03^{c} (0.00)	2.33^{a} (1.13)
	M+	3.56^{a} (0.17)	30.3^{a} (2.19)	57.7^{a} (5.63)	20.5^{a} (3.13)	2.42^{a} (0.36)	0.13^{a} (0.00)	0.07^{b} (0.00)	2.47^{a} (1.02)
200	M -	3.89^{a} (0.60)	37.0^{a} (1.15)	42.9^{ab} (5.00)	13.9^{a} (3.37)	2.00^{a} (0.74)	0.11^{a} (0.03)	0.03^{c} (0.01)	2.10^{a} (1.16)
	M+	3.86^{a} (0.14)	30.3^{a} (3.67)	53.2^{ab} (4.27)	19.2^{a} (1.43)	2.97^{a} (0.69)	0.11^{a} (0.03)	0.08^{ab} (0.01)	2.47^{a} (0.32)
400	M -	3.17^{a} (0.06)	32.7^{a} (1.86)	33.2^{b} (3.44)	9.80^{a} (1.63)	1.05^{a} (0.29)	0.07^{a} (0.02)	0.04^{c} (0.00)	0.63^{a} (0.43)
	M+	3.84^{a} (0.23)	29.7^{a} (1.20)	45.2^{ab} (3.43)	14.7^{a} (2.23)	2.26^{a} (0.58)	0.10^{a} (0.02)	0.10^{a} (0.01)	1.63^{a} (0.61)

[1]Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

[2]Aluminum concentration data are not shown as the levels were below the detectable limit.

Table 3.30 Mineral concentration (mg g^{-1} DM) means (n = 3) and (SEs) in roots of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment.[1]

Soil [Ni]	AM treatment	P	K	Ca	Mg	Mn	Zn	Cu	Fe	Al
0	M -	1.80^{a}	31.0^{a}	6.70^{bc}	5.02^{a}	0.07^{a}	0.04^{c}	0.05^{b}	0.87^{a}	0.53^{bc}
		(0.17)	(6.56)	(0.45)	(1.29)	(0.01)	(0.00)	(0.01)	(0.12)	(0.07)
	M+	1.82^{a}	18.7^{a}	8.66^{b}	4.42^{a}	0.13^{a}	0.07^{ab}	0.11^{a}	1.40^{a}	0.93^{ab}
		(0.20)	(3.38)	(0.26)	(0.48)	(0.01)	(0.00)	(0.01)	(0.12)	(0.03)
100	M -	1.85^{a}	32.0^{a}	7.79^{bc}	3.73^{a}	0.09^{a}	0.05^{bc}	0.07^{ab}	0.97^{a}	0.33^{c}
		(0.19)	(3.46)	(0.45)	(0.37)	(0.01)	(0.00)	(0.01)	(0.23)	(0.09)
	M+	2.34^{a}	23.3^{a}	10.7^{a}	3.96^{a}	0.14^{a}	0.09^{a}	0.07^{ab}	1.73^{a}	1.13^{a}
		(0.23)	(2.19)	(0.53)	(0.13)	(0.02)	(0.01)	(0.01)	(0.22)	(0.24)
200	M -	1.99^{a}	26.7^{a}	7.39^{bc}	4.53^{a}	0.12^{a}	0.05^{bc}	0.06^{ab}	1.07^{a}	0.53^{bc}
		(0.08)	(3.28)	(0.44)	(0.71)	(0.01)	(0.01)	(0.01)	(0.09)	(0.09)
	M+	3.05^{a}	26.3^{a}	8.51^{b}	4.11^{a}	0.17^{a}	0.08^{a}	0.07^{ab}	1.40^{a}	0.93^{ab}
		(0.59)	(5.24)	(0.37)	(0.26)	(0.05)	(0.01)	(0.00)	(0.25)	(0.09)
400	M -	2.05^{a}	31.7^{a}	6.28^{c}	4.24^{a}	0.10^{a}	0.04^{bc}	0.05^{b}	1.17^{a}	0.47^{bc}
		(0.42)	(2.03)	(0.20)	(0.76)	(0.01)	(0.01)	(0.01)	(0.37)	(0.12)
	M+	2.43^{a}	30.7^{a}	7.24^{bc}	3.16^{a}	0.13^{a}	0.08^{a}	0.06^{ab}	1.13^{a}	0.50^{bc}
		(0.06)	(4.06)	(0.43)	(0.25)	(0.02)	(0.00)	(0.01)	(0.19)	(0.10)

[1]Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.31 ANOVA results for leaf and root mineral concentrations of sunflower grown with or without AM fungi (M) in soil Ni treatment.

Parameters	P	K	Ca	Mg	Mn	Zn	Cu	Fe	Al
Leaf									
M	NS	NS	*	*	*	MN	***	NS	NS
Ni	NS	NS	*	MN	MN	NS	***	MN	NS
M X Ni	NS	NS	NS	NS	NS	NS	NS	NS	NS
Root									
M	*	*	***	NS	**	***	*	*	***
Ni	NS	NS	**	NS	NS	MN	NS	NS	MN
M X Ni	NS	NS	MN	NS	NS	NS	NS	NS	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05\ P < 0.1$; NS, not significant

Table 3.32 Two-way ANOVA on ranked data of leaf and root P concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	24.00	169.00	160.33	253.50	209.33	608.00
MS	24.00	56.33	53.44	253.50	69.78	26.39
F-value	0.48	1.13	1.08	6.67	1.84	0.69

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.33 Two-way ANOVA on leaf and root K concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	1	1	1	1	1
SS	1.60	23.33	10.08	288.01	107.86	113.67
MS	1.60	23.33	10.08	288.01	107.86	113.67
F-value	0.07	1.03	0.45	6.55	2.45	2.58

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.34 Two-way ANOVA on leaf and root Ca concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	426.73	741.27	127.01	18.27	19.08	3.70
MS	426.73	247.09	42.34	18.27	6.36	1.23
F-value	6.72	3.89	0.67	37.29	12.98	2.52

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.35 Two-way ANOVA on leaf and root (ranked data) Mg concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	113.93	168.84	17.96	32.67	150.58	107.58
MS	113.93	56.28	5.99	32.67	50.19	35.86
F-value	5.00	2.47	0.26	0.61	0.93	0.67

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.36 Two-way ANOVA on leaf and root (ranked data) Mn concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	0.11	0.17	0.01	486.00	83.25	79.25
MS	0.11	0.06	0.00	486.00	27.75	26.42
F-value	6.16	3.22	0.22	15.89	0.91	0.86

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.37 Two-way ANOVA on ranked data of leaf and root Zn concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	170.67	125.67	51.33	770.67	108.50	14.17
MS	170.67	41.89	17.11	770.67	36.17	4.72
F-value	3.51	0.86	0.35	55.75	2.61	0.34

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.38 Two-way ANOVA on leaf and root (ranked data) Cu concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	0.06	0.02	0.00	192.67	127.75	167.08
MS	0.06	0.01	0.00	192.67	42.58	55.69
F-value	148.86	13.53	0.55	5.09	1.12	1.47

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.39 Two-way ANOVA on leaf and root Fe concentration.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	1.31	14.98	0.62	0.96	0.18	0.52
MS	1.31	4.99	0.21	0.96	0.06	0.17
F-value	0.78	2.99	0.12	0.02	0.74	0.33

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.40 Two-way ANOVA on root Al concentration.

Source of variation	Root[1]		
	M	Ni	M X Ni
df	1	3	3
SS	0.06	0.02	0.03
MS	0.06	0.01	0.01
F-value	26.46	2.48	3.72

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

[1]ANOVA for leaf data are not shown as the levels were below the detectable limit.

Table 3.41 Carbon (C) and nitrogen (N) content (%) means (n = 3) and (SEs) in leaves and roots of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM	%C		%N	
	treatment	Leaf	Root	Leaf	Root
0	M -	38.3^{ab}	38.8^{a}	2.61^{a}	1.00^{a}
		(0.98)	(0.79)	(0.47)	(0.14)
	M+	39.0^{ab}	36.1^{a}	2.68^{a}	1.02^{a}
		(1.40)	(5.17)	(0.09)	(0.13)
100	M -	38.2^{ab}	41.2^{a}	2.83^{a}	0.98^{a}
		(1.15)	(0.25)	(0.29)	(0.02)
	M+	34.2^{b}	35.0^{a}	2.27^{a}	1.33^{a}
		(1.25)	(6.01)	(0.22)	(0.28)
200	M -	37.4^{ab}	40.8^{a}	2.67^{a}	1.24^{a}
		(1.64)	(0.80)	(0.08)	(0.11)
	M+	35.3^{ab}	39.3^{a}	2.70^{a}	1.45^{a}
		(0.59)	(2.27)	(0.17)	(0.09)
400	M -	40.6^{a}	40.3^{a}	2.60^{a}	1.12^{a}
		(0.67)	(0.71)	(0.01)	(0.21)
	M+	37.8^{ab}	41.0^{a}	2.25^{a}	1.49^{a}
		(0.84)	(0.51)	(0.04)	(0.16)
ANOVA: AM fungi (M), Nickel (Ni)					
M		*	NS	NS	MN
Ni		*	NS	NS	NS
M X Ni		NS	NS	NS	NS

* $P < 0.05$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.42 Two-way ANOVA on leaf and root C content.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	–	–	–	1	3	3
SS	24.62	44.33	18.36	0.06	0.02	0.03
MS	24.62	14.78	6.12	0.06	0.01	0.01
F-value	6.57	3.94	1.63	26.46	2.48	3.72

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.43 Two-way ANOVA on leaf (ranked data) and root N content.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	32.67	110.83	252.17	0.34	0.41	0.12
MS	32.67	36.94	84.06	0.34	0.14	0.04
F-value	0.69	0.78	1.79	4.39	1.78	0.51

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.44 Mineral concentration (mg g^{-1} DM) means (n = 3) and (SEs) in leaves and roots of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

AM treatment	P	K	Ca	Mg	Mn	Zn	Cu	Fe	Al
Leaf									
M -	3.28[a] (0.12)	31.4[a] (1.91)	23.0[a] (4.74)	6.77[a] (0.69)	0.66[a] (0.24)	0.04[a] 0.01	*nd*[1]	0.65[a] (0.25)	0.15[a] (0.01)
M+	2.97[a] (0.34)	28.7[a] (1.08)	21.6[a] (1.05)	4.97[a] (0.72)	0.28[b] (0.12)	0.03[a] (0.01)	*nd*	0.29[b] (0.04)	0.15[a] (0.01)
M	NS	NS	NS	NS	*	NS	–	*	NS
Root									
M -	1.55[b] (0.15)	19.8[a] (0.64)	6.61[a] (0.56)	5.56[a] (0.56)	0.11[a] 0.04)	0.04[a] (0.01)	0.02[a] (0.00)	0.76[a] (0.12)	0.67[a] (0.06)
M+	1.87[a] (0.42)	23.8[a] (6.01)	7.92[a] (0.54)	5.75[a] (1.11)	0.07[a] (0.01)	0.04[a] (0.00)	0.03[a] (0.01)	0.87[a] (0.25)	0.84[a] (0.22)
M	*	NS	NS	NS	NS	NS	NS	NS	NS

* $P < 0.05$; NS, not significant

[1] *nd*, not detectable

Table 3.45 One-way ANOVA on leaf minerals for Experiment #2.

Source of variation	P	K	Ca	Mg	Mn	Zn	Cu[1]	Fe	Al
Leaf									
df	1	1	1	1	1	1	–	1	1
SS	0.15	10.96	3.05	4.84	0.45	0.00	–	0.55	0.00
MS	0.15	10.96	3.05	4.84	0.45	0.00	–	0.55	0.00
F-value	0.78	1.52	0.09	3.24	14.79	1.60	–	11.04	0.00
Root									
df	1	1	1	1	1	1	1	1	1
SS	0.16	0.00	2.56	0.06	4.17	0.00	0.00	0.02	0.04
MS	0.16	0.00	2.56	0.06	4.17	0.00	0.00	0.02	0.04
F-value	0.54	0.15	2.83	0.03	1.35	0.00	0.00	0.17	0.54

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

[1]ANOVA results are not shown as the levels were below the detectable limit.

Table 3.46 Carbon (C) and nitrogen (N) content (%) means (n = 5 to 6) and (SEs) in leaves and roots of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

AM	%C		%N	
treatment	Leaf	Root	Leaf	Root
M -	41.0[a]	36.1[a]	3.29[a]	1.11[a]
	(0.82)	(2.05)	(0.35)	(0.18)
M+	41.2[a]	35.6[a]	3.06[a]	0.91[a]
	(0.46)	(3.97)	(0.05)	(0.15)
ANOVA	NS	NS	NS	NS

NS, not significant

Table 3.47 One-way ANOVA for leaf and root C and N content.

AM	%C		%N	
treatment	Leaf	Root	Leaf	Root
df	1	1	1	1
SS	0.05	0.98	1.50	0.06
MS	0.05	0.98	1.50	0.06
F-value	0.03	0.03	0.37	0.71

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

3.4.5 Glutamine synthetase

Experiment #1

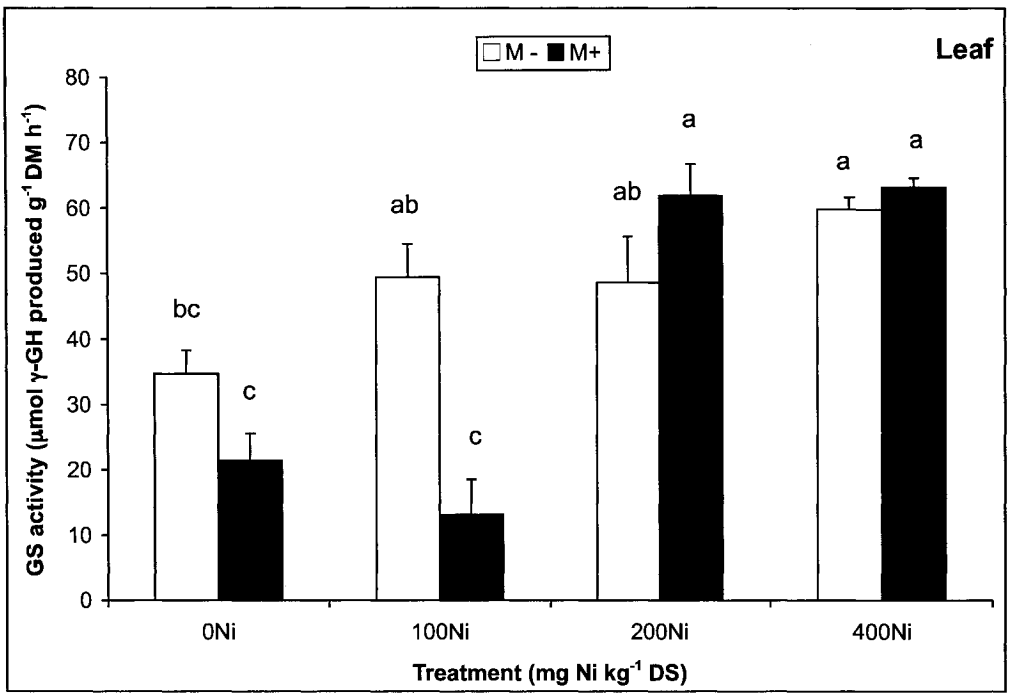
The AM ($P < 0.05$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.01$), significantly affected the leaf GS activity (Figure 3.3 and Table 3.48). According to the Tukey's test, with increasing soil [Ni], the leaf GS activity significantly increased in both AM and non-AM plants. The Tukey's test indicated that the GS activities were significantly lower in the leaves of AM than non-AM plants at 0 and 100 soil [Ni] (by 1.6 and 3.8 times, respectively). Although not significant, the leaf GS activity tended to be higher at 200M+ than at 200M- .

The AM ($P < 0.001$) treatment and its interaction with soil [Ni] ($P < 0.01$) significantly affected the root GS activity (Figure 3.3 and Table 3.48). According to the Tukey's test, with increasing soil [Ni], the root GS activity tended to be decreased in AM plants, but increased in non-AM plants. The GS activities were significantly higher in the roots of AM than non-AM plants at 0 and 100 soil [Ni] (by 4 and 1.8 times, respectively). Although not significant, the root GS activity tended to be higher at 200M+ than at 200M-.

Experiment #2

The GS activities in leaves ($P < 0.05$) and roots ($P < 0.05$) were significantly higher (by 1.8 and 3.6 times, respectively) in AM than non-AM plants (Figure 3.4 and Table 3.48).

Figure 3.3 Glutamine synthetase (GS) activity (μmol γ-GH produced g^{-1} DM h^{-1}) in sunflower leaves (top) and roots (bottom) grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for leaves and roots. Means (n = 3 to 5) and SEs are shown and different letters refer to significant differences according to the Tukey's test at $P < 0.05$.


Leaf
□M - ■M+
GS activity (µmol γ-GH produced g-1 DM h-1)
80
70
60
50
40
30
20
10
0
bc
c
ab
c
ab
a
a
a
0Ni
100Ni
200Ni
400Ni
Treatment (mg Ni kg-1 DS)

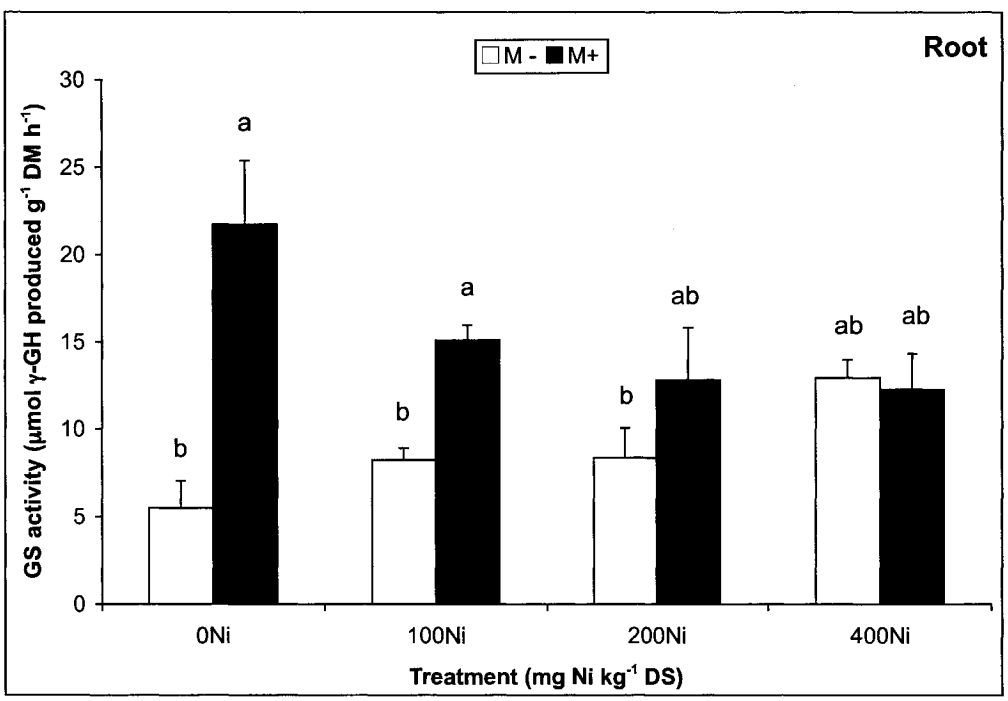

Root
□M - ■M+
GS activity (µmol γ-GH produced g-1 DM h-1)
30
25
20
15
10
5
0
b
a
b
a
b
ab
ab
ab
0Ni
100Ni
200Ni
400Ni
Treatment (mg Ni kg-1 DS)

Figure 3.4 Glutamine synthetase (GS) activity (μmol γ-GH produced g^{-1} DM h^{-1}) means (n = 3 to 5) and SEs in leaves and roots of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. Statistical analyses were done separately for leaves and roots. Different letters refer to significant differences according to the Tukey's test at $P < 0.05$.

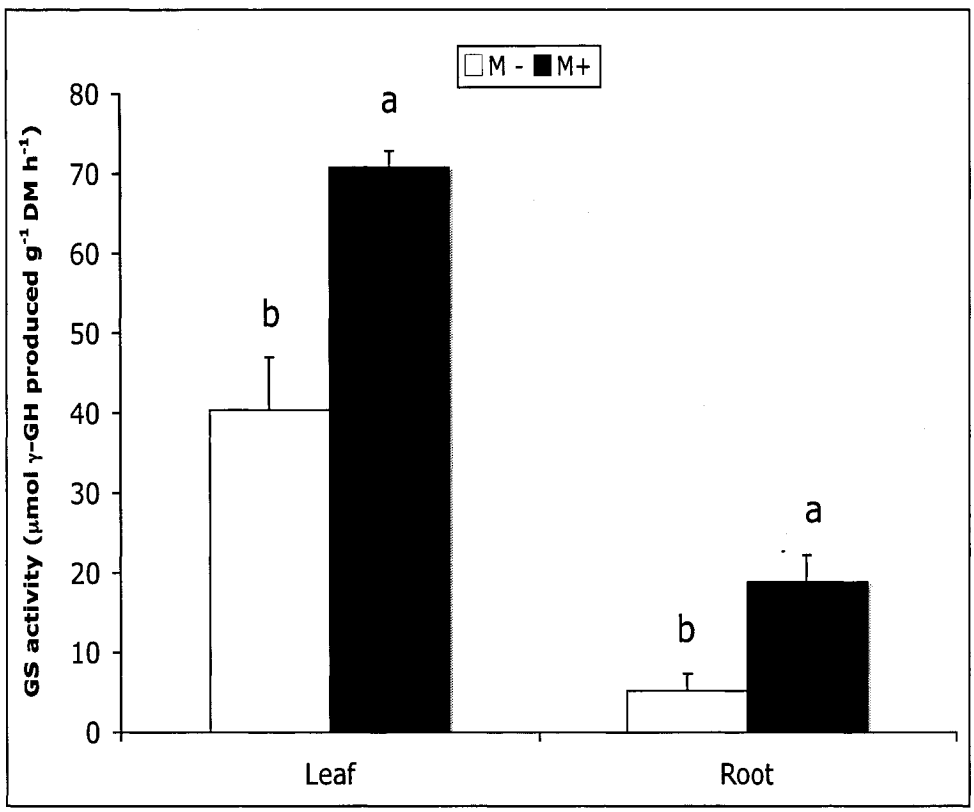

M - M+
GS activity (µmol γ-GH produced g-1 DM h-1)
80
70
60
50
40
30
20
10
0
a
b
a
b
Leaf
Root

Table 3.48 ANOVA results for GS activity in leaves and roots of sunflower grown with or without AM fungi (M) in Ni soil treatment.

Parameters	Leaf	Root
Experiment #1		
M	*	***
Ni	***	NS
M X Ni	**	**
Experiment #2		
M	*	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant

Table 3.49 Two-way ANOVA on leaf and root GS activity for Experiment #1.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	433.45	5585.36	2133.12	727.77	62.63	416.02
MS	433.45	1861.79	711.04	727.77	20.88	138.67
F-value	7.20	30.94	11.82	26.06	0.75	4.97

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.50 Two-way ANOVA on leaf and root GS activity and its specific activity for Experiment #2.

Source of variation	GS activity	
	Leaf	Root
df	1	1
SS	1587.75	276.76
MS	1587.75	276.76
F-value	14.27	11.37

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

3.4.6 GS specific activity and protein concentrations

Experiment #1

The soil [Ni] ($P < 0.01$) and its interaction with AM ($P = 0.098$) were significant and marginally non-significant on the GS specific activity in leaves (Table 3.51). According to the Tukey's test, at 200M+ it was significantly higher than at 0M+ and 100M+ (by 2.2 and 2.8 times, respectively). Although not significant, it tended to be lower in the leaves at 0 and 100 soil [Ni], but higher at 400 soil [Ni] in AM than non-AM plants.

The AM ($P < 0.01$) and soil [Ni] ($P < 0.001$), and their interaction ($P < 0.001$), significantly affected the GS specific activity in roots (Table 3.51). According to the Tukey's test, with increasing soil [Ni], it tended to decrease in the roots of both AM and non-AM plants. In addition, the GS specific activities were significantly higher in the roots of AM than non-AM plants at 0 and 100 soil [Ni] (by 4 and 2 times, respectively).

The leaf protein concentrations were significantly affected by the AM treatment ($P < 0.05$), and was marginally non-significant for the soil [Ni] ($P = 0.052$) (Table 3.51). According to the Tukey's test, the leaf protein concentration was significantly lower in

AM than non-AM plants at 100 soil [Ni] (by 1.9 times). Although not significant, it also tended to be lower in AM than non-AM plants at 200 soil [Ni].

The soil [Ni] ($P < 0.001$) significantly affected the protein concentration in roots (Table 3.51). According to the Tukey's test, with increasing soil [Ni], it was significantly increased in the roots of both AM and non-AM plants. Furthermore, at 400M+, it was significantly higher than at 200, 100 and 0 soil [Ni]; while at 200M+ it was significantly higher than at 0 soil [Ni]. Although not significant, the root protein concentrations tended to be higher in AM than non-AM plants at 200 and 400 soil [Ni].

Experiment #2

The leaf GS specific activity was significantly ($P < 0.05$) 2-folds higher in the leaves of AM than non-AM plants, while the root protein concentration was also marginally non-significantly ($P = 0.098$) increased in AM than non-AM plants (Table 3.54). Although not significant, the GS specific activity tended to be higher in the roots of AM than non-AM plants.

Table 3.51 GS specific activity (μmol mg^{-1} proteins h^{-1}) and protein concentration (mg g^{-1} DM) means (n = 3 to 5) and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatments. ANOVA results are also presented.

Soil [Ni]	AM treatment	GS specific activity		Protein concentration	
		Leaf	Root	Leaf	Root
0	M -	1.07^{ab} (0.18)	4.71^{bc} (0.71)	32.4^{ab} (3.27)	1.16^{c} (0.34)
	M+	0.65^{b} (0.46)	19.2^{a} (5.47)	32.7^{ab} (6.83)	1.13^{c} (0.17)
100	M -	1.05^{ab} (0.09)	3.08^{bc} (0.43)	47.2^{a} (4.71)	2.67^{bc} (0.26)
	M+	0.52^{b} (0.11)	6.23^{b} (2.10)	25.2^{b} (4.62)	2.42^{bc} (0.63)
200	M -	0.96^{ab} (0.24)	2.65^{bc} (0.62)	50.6^{a} (3.93)	3.15^{bc} (0.27)
	M+	1.45^{a} (0.09)	3.54^{bc} (0.74)	42.7^{ab} (1.81)	3.61^{b} (0.87)
400	M -	1.70^{a} (0.18)	3.08^{bc} (0.47)	35.2^{ab} (2.99)	4.20^{ab} (0.40)
	M+	1.75^{a} (0.40)	1.94^{c} (0.33)	36.1^{ab} (7.99)	6.33^{a} (0.51)
ANOVA: AM fungi (M), Nickel (Ni)					
M		NS	**	*	NS
Ni		**	***	MN	***
M X Ni		MN	***	NS	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 3.52 Two-way ANOVA on leaf (ranked data) and root GS specific activity for Experiment #1.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	9.33	743.07	300.26	0.32	0.83	0.94
MS	9.33	247.69	100.08	0.32	0.28	0.31
F-value	0.22	5.95	2.40	9.97	8.72	9.86

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.53 Two-way ANOVA on leaf and root (ranked data) protein concentration for Experiment #1.

Source of variation	Leaf			Root		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	2.47	5.07	3.87	14.99	1669.50	72.61
MS	2.47	1.69	1.29	14.99	556.50	24.20
F-value	4.55	3.12	2.38	0.51	18.82	0.82

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 3.54 GS specific activity (μmol mg^{-1} proteins $h\text{-}^{-1}$) and protein concentration (mg g^{-1} DM) means (n = 3 to 4) and (SEs) of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

AM treatment	GS specific activity		Protein concentration	
	Leaf	Root	Leaf	Root
M -	0.81^{b} (0.13)	1.43^{a} (0.80)	48.4^{a} (1.08)	3.63^{a} (0.54)
M+	1.56^{a} (0.25)	2.84^{a} (0.36)	45.8^{a} (3.16)	6.89^{a} (2.07)
M	*	NS	NS	MN

* $P < 0.05$; MN, $0.05 < P < 0.1$; NS, not significant

Table 3.55 Two-way ANOVA on leaf and root GS specific activity and protein concentrations for Experiment #2.

Source of variation	GS specific activity		Protein concentration	
	Leaf	Root	Leaf	Root
df	1	1	1	1
SS	1.11	3.44	9.78	16.13
MS	1.11	3.44	9.78	16.13
F-value	6.90	3.18	0.58	3.82

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

3.5 Discussion

3.5.1 Nickel content and distribution

The Ni concentration was greater in roots than shoots at low soil Ni input of 100 mg Ni kg^{-1}, of which 62% of Ni was found in roots of AM plants. Furthermore, the total Ni extracted % was almost 2-fold higher in AM than non-AM plants at 100 mg Ni kg^{-1}. These results coincide with other studies which showed that AM fungi contribute to HM tolerance by sequestering HMs in their tissues (Audet and Charest 2006, Bi *et al.* 2003, Jones and Hutchinson 1988a and 1988b, Leyval *et al.* 1997, Takács and Vörös 2003). Root accumulation of Cu, Ni, Pb and Zn in AM perennial grass (Killham and Firestone 1983), and of Zn in AM wild tobacco roots (Audet and Charest 2006) have been reported.

However, in the present study, at the higher soil Ni input of 200 and 400 mg Ni kg^{-1}, the Ni distribution did not follow this trend as the Ni content was 2 to 3 times higher in shoots than roots of both AM and non-AM plants. This accounts for a Ni extracted % of 0.7 to 1.3%. Furthermore, at these soil [Ni], the plant Ni content was distributed in this order: leaf > root > stem > flower. This suggests that a high Ni proportion was transferred to the aboveground organs, a trait especially important in phytoextraction (Chaney *et al.* 1997, Li *et al.* 2003). This Ni extracted % is substantial as most plants have less than 0.01% Ni under non-contaminated soils (Baker and Brooks 1989, Cunningham 1995, Marschner 1995). For comparison, a hyperaccumulator species such as *Berkheya coddii* can accumulate up to 4% Ni in leaves under non-contaminated soils (Li *et al.* 2003, Turnau and Mesjasz-Przybylowicz 2003), and *Sebertia acuminata* up to 26% in dry latex (Sagner *et al.* 1998). Sunflower is not considered as a hyperaccumulator of any HM. However, Davies *et al.* (2001) reported significant amounts of Cr accumulated in sunflower shoots, especially in AM plants. Turnau and Mesjasz-Przybylowicz (2003) also reported higher Ni content in *Berkheya coddii* in AM (1.3%) than non-AM plants (0.5%).

Li *et al.* (1991) suggested that Cu is selectively transported with P by the fungal hyphae from roots to shoots in AM white clover. Our results suggest that AM colonization of sunflower enhanced the Ni uptake. Furthermore, the large biomass of sunflower, in particular the leaves and stem, may have contributed to greater Ni accumulation into shoots. In addition, an enhanced plant tolerance through a growth dilution effect may have been afforded by this large biomass.

In our study, we observed that a soil [Ni] of 400 mg Ni kg^{-1} was toxic for sunflower directly sown in Ni treated soils, as all plants died within four weeks after seeding. Sunflower plants directly seeded in 200 mg Ni kg^{-1} soil Ni grew to maturity, although these plants had lower chlorophyll concentrations than when exposed at a later stage of development. This is consistent with other studies that indicated severe chlorosis due to Ni toxicity, with indirect effects of Fe-deficiency (Davies *et al.* 2001 and 2002, Jones and Hutchinson 1988b, Zornoza *et al.* 1999). We did not observe any effect of AM association on Ni uptake in sunflower when directly sown in Ni treated soil. However, at 200 mg Ni kg^{-1}, the Ni extracted % ($\sim$ 0.95%) was higher than plants exposed at the later stage ($\sim$ 0.70 %). Thus, direct seeding of sunflower in Ni contaminated soils not only resulted in an increase of Ni extraction, it is also a straightforward and economical planting method that may help to reduce the costs associated with phytoremedation practices (Li *et al.* 2003).

It has been proposed that Ni is transported through plant tissues by complexing with organic compounds such as malate or citrate (Baker 1987, Bhatia *et al.* 2005, Lee *et al.* 1978), or histidine (Krämer *et al.* 1996, 2000). In many hyperaccumulator plant species, Ni has been found chelated and/or stored in the vacuoles, indicating an HM transport through the cytoplasm (Bhatia *et al.* 2005, Leopold *et al.* 1999). This plant ability to bind nickel affords protection to the cytosol and organelles as it results in decreased free ion activity (Baker 1981, Bhatia *et al.* 2005, Leopold *et al.* 1999). Krämer *et al.*

(1996) reported that exposing the hyperaccumulator *Alyssum lesbiacum* to Ni elicited a proportional increase of histidine levels. In *Stackhousia tryonii* and *Sebertia acuminata*, Bhatia *et al.* (2005) and Sagner *et al.* (1998) determined that Ni was complexed to malate and citrate or to nitrate, respectively. Saber *et al.* (1999) reported significant increases in malate and citrate contents in shoots and roots of sunflower seedlings exposed to Al, Cd and Zn, suggesting that these compounds play a prominent role in the HM plant tolerance. In light of the "substantial" Ni proportion that we observed in sunflower shoots, the chelation of Ni whether by organic or amino acids poses interesting questions into the mechanism of plant Ni tolerance, and thus further investigation may shed more light on their tolerance mechanisms.

3.5.2 Mycorrhizal root colonization

The AM colonization levels in this study are comparable to other studies using either sunflower as a test species, or Ni as a soil contaminant. In this study, colonization levels ranged from 78% to 64% for sunflower grown in 0 to 400 mg Ni kg^{-1}. In the hyperaccumulator *Berheya coddii* colonized with *G. intraradices* and grown in ultramafic soils (1100 mg Ni kg^{-1}), Turnau and Mesjasz-Przybylowicz (2003) reported 85% AM colonization levels. Takács and Vörös (2003) reported lower colonization levels (33%) for ryegrass grown in 270 mg Ni kg^{-1}. Davies *et al.* (2001, 2002) showed that AM colonization was as high as 100% in sunflower grown in Cr treated soils. In our study, the AM colonization was substantial even at 400 mg Ni kg^{-1}, which is considered a toxic soil level (McGrath *et al.* 2001).

We also examined the effect of Ni toxicity on AM colonization levels of early versus late exposed sunflower. For sunflower seeds directly sown in soil Ni of 200 mg Ni kg^{-1}, the root colonization level of 42% represented a 22% reduction as compared to late exposed plants. This colonization level of 42% is still quite substantial and demonstrates the

hardiness of sunflower plant and AM fungus under Ni stress (Pawlowska and Charvat 2004, Smith and Read 1997).

3.5.3 Physiological parameters

Decreased root growth has been found to be one of the first signs of HM toxicity (Ahonen-Jonnarth and Finlay 2001). This observation was also apparent in the present study, as the dry masses (DM) of all the organs were the most affected by the soil Ni input, resulting in decreased total plant DM and R/S ratio for late exposed sunflower plants. Early exposed plants had similar DMs, however plants generally had lower shoot height but higher R/S ratios than late exposed plants. Zornoza *et al.* (1999) reported a decrease in sunflower stem and root DM as a result of Ni treatment. Turgut *et al.* (2004) also reported a decrease in plant DM of sunflower grown in a combination of Cd, Cr and Ni, with EDTA or citric acid as chelators. However, neither of these two studies incorporated AM fungi. Studies investigating Ni toxicity in mycorrhizal interactions reported either a slight reduction or no effect of mycorrhizal fungi on biomass of birch seedlings (Jones and Hutchinson 1988a, b), or maize and bean plants (Guo *et al.* 1996). In the present study, the AM colonization significantly increased total sunflower plant DM at 0 and 100 mg Ni kg^{-1}. In addition, early exposed plants had significantly higher stem DM and shoot height in AM than non-AM plants at 200 mg Ni kg^{-1}. Yet, at 400 mg Ni kg^{-1}, early exposed plants died, while a 47% reduction in biomass was observed for late exposed plants. Thus, at the low and moderate soil Ni input of 100 and 200 mg Ni kg^{-1}, the AM contribution to plant growth and tolerance was more prominent.

3.5.4 Mineral concentrations

Mycorrhizal colonization often results in enhanced plant uptake of relatively immobile micronutrients such as Mn, Cu, Zn and Fe (Marschner 1995, Liu *et al.* 2000), as a result

of an increased mycorrhizosphere. In this study, late exposed AM plants had higher concentrations of Ca, Mg, Mn and Cu in leaves, and of P, Ca, Mn, Zn, Cu, Fe and Al in roots as compared to non-AM plants. This would suggest that AM fungi contributed to an enhanced plant tolerance under soil Ni contamination by increasing the minerals available for plant growth. These results are in accordance with other studies which reported higher concentrations of Cu, Zn and Fe (Liu *et al.* 2000), of P, Cu and Zn (Chen *et al.* 2001), and of P, K, Al, Fe (Davies *et al.* 2002) in AM maize, red clover and sunflower, respectively. However, in early exposed sunflower, concentrations of Mn and Fe were significantly lower in leaves, while P was significantly higher in roots of AM than non-AM plants. The enhancement of nutrient acquisition by AM colonization can be directly attributed to hyphal uptake or indirectly to changes in the rhizosphere (Azćon *et al.* 2003, Clark and Zeto 2000, Kothari *et al.* 1991, Li *et al.* 1991). According to Clark and Zeto (2000), AM fungal acquisition of Ca, K and Mg was evident in acidic soils that are deficient in these elements. Other factors affecting trace element uptake by AM hyphae include plant species or cultivar, AM fungal species or isolate, and the P levels (Douds *et al.* 2000).

In our study, the AM association also resulted in marginally significant increases in root N content. It has been reported that enhanced N in AM plants may be mediated by high N demand as a result of enhanced P (Clark and Zeto 2000, Smith and Read 1997). This may be the case in our study since P concentrations were higher in AM than non-AM plants. Moreover, Jones and Hutchinson (1988b) concluded that the high root Ni content of mycorrhizal birch seedlings correlated with an increased P content suggesting a detoxifying mechanism through polyphosphate binding to Ni. In our study, the leaf C content was significantly lower in AM than non-AM plants, and tended to decrease with increasing soil Ni input. As mycorrhizal fungi are dependent on the host plant for photosynthates, it is likely that the decrease in leaf C content results from a C drain to the fungal tissues (Smith and Read 1997).

Ni is an essential micronutrient (Brown *et al.* 1987, Dixon 1975, Eskew *et al.* 1984, Marschner 1995), but in excess, it can inhibit the uptake of essential elements. In particular, Ni competes with other divalent cations such as Ca, Mg, Fe, Mn, Cu and Zn (Cataldo *et al.* 1978a, Marschner 1995, Zornoza *et al.* 1999). Therefore, one would expect lower concentrations of these elements when plant [Ni] is high. In our study, the mineral concentrations did not follow this trend. In leaves of AM plants, the Cu concentration increased with increasing soil [Ni], especially at 400 mg Ni kg^{-1}. Similarly, the concentrations of Ca, Mg, Mn, and Fe followed the same trend. The root concentrations of Ca, Zn and Al were all significantly higher in AM than non-AM plants at soil Ni of 100 mg Ni kg^{-1}. Similar results were reported by Yang *et al.* (1985) in which Ni in leaves correlated positively with Co, Cr, Mn, Na and Zn in hyperaccumulators of the genus *Flacourtiaceae* from New Caledonia. Cataldo *et al.* (1978b) observed that Mg and Mn did not inhibit Ni uptake in soybean seedlings; however Co, Cu, Fe and Zn were shown to inhibit its absorption. It is likely that the AM association contributed to an enhanced mineral acquisition in order to alleviate high [Ni] toxicity. Similar observations of enhanced P, K, Mg, Ca and S levels were reported in mycorrhizal *Pinus* seedlings supplied with Ni (Ahonen-Jonnarth and Finlay 2001). Davies *et al.* (2002) also reported increased Al, Zn and Fe concentrations in AM sunflower plants under Cr contaminated soils.

3.5.5 Glutamine synthetase

The GS activity and specific activity were significantly increased with the increasing soil [Ni] in leaves of both AM and non-AM plants. As GS catalyzes the formation of glutamine via the combined enzymatic activity of GOGAT, it is possible that the increase in GS activity may be required in order to provide the glutamine necessary for Ni complexation. Bhatia *et al.* (2005) also reported that glutamine was the dominant amino acid in the xylem sap of the Ni hyperaccumulator, *Stacckhousai tryonii*, suggesting

an enhanced N assimilation via the induction of GS/GOGAT activity. Krämer *et al.* (2000) proposed that Ni chelation to histidine is part of a highly specialized detoxification response in hyperaccumulators as this chelate could be safely transported through the cytoplasm and stored in the vacuole. However, non-hyperaccumulators bind to other amino acids such as glutamine which are incapable of storage in the vacuoles and remain in the cytoplasm, thereby resulting in damage to cellular processes (Krämer *et al.* 2000). These results suggest that non-hyperaccumulators, such as sunflower, may not have, or do have but to a lesser extent, the capability of Ni detoxification via complexation with citrate or histidine. Thus, glutamine may aid in Ni transport through the cytoplasm, but this may ultimately result in cellular damage. These observations are intriguing but require further examination. Analyses on amino acid production such as glutamine, glutamate and histidine, as well as, determination of citrate or malate levels may aid in clarifying these results.

Most studies of the HM effects on plants reported general declines in the activities of N-assimilating enzymes. The GS activity was lower in sugar beet when exposed to Ni or Cd, but higher in the presence of Mo (Kevrešan *et al.* 1998). Gouia *et al.* (2003) observed similar declines in GS and GOGAT activities in Cd-treated bean plants. In grapevines exposed to Cu, Llorens *et al.* (2000) observed decreases in the activities of NR, NiR, GS, GOGAT and GDH. However, none of the above studies incorporated the AM symbiosis. In our study, the AM symbiosis aided N metabolism in sunflower plants as the GS activity was significantly higher in AM than non-AM roots at 0 and 100 mg Ni kg^{-1}. Similarly, early exposed plants had significantly higher leaf and root GS activity in AM than non-AM plants. Smith *et al.* (1985) argued that an increased NH_4^+ assimilation via GS activity in AM roots may be a result of the fungal contribution. Subramanian and Charest (1999) and Toussaint *et al.* (2004) also showed that AM fungi possess their own N-assimilating enzymatic systems.

3.6 Conclusion

This study showed that AM colonization significantly contributes to enhancing Ni uptake in AM plants, especially at moderately contaminated soil Ni input of 100 mg Ni kg^{-1}. At 100 mg Ni kg^{-1}, the Ni content was 2-fold higher in AM than non-AM plants. Furthermore, at soil Ni input of 200 and 400 mg Ni kg^{-1}, an approximately 45% reduction in plant DM was observed in AM plants as compared to only a 14% reduction for plants grown at 100 mg Ni kg^{-1}. We observed that soil [Ni] input of 400 mg kg^{-1} was toxic for early exposed sunflower, as all seedlings died within 4 weeks after sowing. Thus, the AM contribution to Ni uptake was optimal at 100 mg Ni kg^{-1} as 62% of the Ni was distributed in the roots, suggesting an enhanced sequestration by the fungal tissues. These results coincide with others which showed that AM fungi contribute to HM tolerance by sequestering HMs in their fungal tissue within plant roots (Audet and Charest 2006, Jones and Hutchinson 1988a and 1988b, Leyval *et al.* 1997, Takács and Vörös 2003). However, the total Ni extracted % (ranging from $\sim$ 0.70 to 1.3%) may indicate that a proportion of Ni was transferred to shoots, a trait especially important in phytoextraction (Chaney *et al.* 1997, Davies *et al.* 2001, Li *et al.*. 2003).

The AM symbiosis also contributed to sunflower plant tolerance under Ni input by increasing uptake of minerals in both leaves and roots. Acquisition of these nutrients may have been facilitated by the fungal hyphae which are known to increase the uptake of immobile soil elements such as Zn, Cu and Fe (Azćon *et al.* 2003, Clark and Zeto 2000, Liu *et al.* 2000). The AM plants also had higher GS activity in roots and leaves. This may suggest a possible requirement of sunflower plant tolerance to Ni toxicity, as GS catalyzes the formation of glutamine, a N-based amino acid that has been reported to bind with Ni (Bhatia *et al.* 2005, Krämer *et al.* 1996, 2000). Further investigation into Ni chelation with amino and organic acids may provide more insight into sunflower tolerance mechanisms.

Chapter 4

Effect of AM colonization and Ni input on sesquiterpene lactone synthesis in sunflower

4.1 Introduction

Hyperaccumulation of nickel is a relatively rare response, occurring in only 1-2% of known plant species in ultramafic soils (Baker 1987, Boyd and Martens 1998). Ultramafic or serpentine soils are typically infertile (low N and P levels), with usually unfavourable Mg/Ca ratios, and enriched with Ni, Cr or Co (Baker 1987). These harsh conditions often result in floras that are specifically adapted to ultramafic soils (Davis *et al.* 2001). The questions of how and why these unique species arose and the mechanisms by which large amounts of HMs can be acquired and tolerated by these plant species still remain unanswered. Although several hypotheses were proposed to explain metal hyperaccumulation by plants (Boyd and Martens 1998), to date, only the defense hypothesis has been extensively tested. Metal hyperaccumulation has been suggested to represent an "elemental defense" that differs from other secondary compound defenses (Boyd and Martens 1998). Firstly, elemental defense involves a toxic element taken up externally as compared to an internal organic compound produced in response to attack; secondly, elemental defenses cannot be degraded as it can occur with secondary compounds (Boyd and Martens 1998).

Boyd and Martens (1998) suggested that one benefit from the metal-based defense is the decreased resource (i.e. C and N) allocation into organic defenses. The metabolic cost for metal-based defenses is presumed to be low compared with the costs of producing and

maintaining secondary metabolites as the metals are speculated to be complexed with common metabolic products such as citrate (Lee *et al.* 1977, 1978), malate (Bhatia *et al.* 2005), and amino acids (Krämer *et al.* 1996). Thus, the translocation and compartmentalization of these metal-complexes may represent the only direct metabolic costs for metal-based defenses (Davies *et al.* 2001, Martens and Boyd 1998). Yet, the energy expenditure involved in mechanisms of tolerance may be great as manifested by slow growth rates and low biomass production of several hyperaccumulating plant species (Baker 1987). In contrast, since many organic defenses are inducible, the energy expenditure to produce secondary metabolites would be required only in response to attack. This would greatly decrease the energy cost for organic defenses as compared to metal-based defenses.

The colonization of plant roots by AM fungi has been reported to produce plant defense-like responses (Harrison and Dixon 1993 and 1994, Maier *et al.* 1997, Piepp *et al.* 1997). Maier *et al.* (1997) reported that colonization with *Glomus. intraradices* induced an accumulation of sesquiterpenoid cyclohexenone glycosides in roots of barley, with blumenin being found to accumulate in AM roots of oat, rye and wheat. These authors suggested that AM colonization might initiate a cascade of metabolic events, among which the accumulation of the cyclohexenone derivatives could be part of some stress-induced reactions. Piepp *et al.* (1997) also observed an increase of phenolics in AM barley roots, thus corroborating the assumption that accumulation of secondary compounds reflects a transient defense response of the plant upon penetration of the AM fungus. In tobacco roots colonized with *G. intraradices*, Maier *et al.* (1999) proposed that the sesquiterpenoid cyclohexenone derivatives found in roots may be involved in their AM control process.

Within the Asteraceae family (i.e. sunflower), sesquiterpene lactones (STLs) constitute the largest group of terpenoid secondary metabolites (Picman 1986, Singer *et al.*

2003). STLs have a diverse and large range of biological activities including antifeedant, antibiotics, insect juvenile hormone mimics, phytoalexins (antifungal), plant growth regulators and antioxidants (Picman 1986, Singer *et al.* 2003). Secondary plant metabolites such as flavonoids and terpenoids were also shown to stimulate microorganisms for xenobiotic biodegradation (Singer *et al.* 2003). In addition, there are concerns as to whether implementation of phytoremediation schemes may have ecotoxicological consequences, towards native fauna or mobilization of metals into food chains (Pollard 2000).

4.2 Hypotheses and Objectives

This study aimed to examine the relationship between production of sesquiterpene lactones (STLs) and the elemental defense hypothesis for Ni accumulation in sunflower. In addition, the effect of AM colonization in STL induction was also investigated. The first hypothesis of the present study is that Ni accumulation in sunflower results in a decrease in the production of STLs. Secondly, it is hypothesized that AM colonization induces an accumulation of STLs in sunflower. To test these hypotheses, the specific objectives were: 1) to investigate the elemental defense hypothesis by comparing the levels of STL production to C/N ratios; and 2) to compare the effect of AM colonization under *a priori* and *a posteriori* soil Ni input. It is predicted that the STL concentrations and content in sunflower will be increased when colonized by AM fungi, but will decrease under increasing soil Ni input.

4.3 Materials and Methods

Extracts from the leaves of sunflower plants from the greenhouse experiments #1 (Ni treatments of 0, 100, 200 and 400 mg kg^{-1} DS at 56 DAS) and #2 (Ni treatment of 200 mg kg^{-1} DS at 0 DAS) described in Chapter 3 were prepared as indicated below.

4.3.1 HPLC analyses of sesquiterpene lactones

Sample preparation

Sesquiterpene lactones were determined using a modified method from the Laboratory of Dr. Arnason, University of Ottawa, and Awang *et al.* (1991). For each sample, 0.5 g of freeze-dried leaf material, reduced to powder, was extracted in 10 mL of 95% ethyl acetate (EtAC) for 10 min in an ultrasonic water bath. After a first centrifugation at 12 100g for 3 min, the pellet was washed with 5 mL of 95% EtAC. After a 2^{nd} centrifugation, the supernatant was adjusted to 15 mL. The STL extract was separated (2X) from the water portion using a separatory funnel and subsequently rotovapped to dryness. The concentrated SQL extract was recollected with two acetonitrile washings (1 mL each). The extract (1 mL) was filtered through a 0.22 μm PTFE (polytetrafluoroethylene) membrane, prior to injection of 1 μL into the HPLC system.

Instrumentation

The STL extracts were analyzed using Liquid Chromatography/Mass Spectrometry (LC/MS). The LC/MS instrument consisted of 1100 Series LC/MSD (VL) system (Agilent Technologies, Mississauga, ON, Canada) with a mass range of 50-1500 DA, consisting of G1313A Autosampler (Module 502), with 5 μL loop, G1311A Quaternary pump, G1315A Photodiode array detector (Module 168), G1322A In-line solvent degasser, Solvent delivery system (Module 126), and System Gold software (version 8.10).

Separations were achieved on a YMC ODS-AM Spherical 3 μm 120A, 2.0 mm x 50 mm cartridge (Waters, Mississauga, ON, Canada) at 50^0C. A gradient system based on solvent A: 80% water and B: 20% acetonitrile, at a flow rate of 0.5 mL min^{-1}, allowed separation and quantification of the STLs.

HPLC Analyses

Comparison with commercially available known STL standards, Helanalin and Isohelanalin (Sigma-Aldrich, Canada; Picman 1986) did not reveal that these compounds were found in the sunflower leaf extracts. Therefore, parthenolide was used as a basis for comparison. A STL standard curve was generated from injection of pure parthenolide (Sigma-Aldrich, Canada). Quantifications were done at 210-220 nm. Elution times were 8.66, 9.88, 19.27 and 24.55 min for the putative terpenes A, B, C and D, respectively (Appendix: Figure A.6). Peak identities in samples were chosen based on the UV absorbance profile at 220 nm similar to the known STL parthenolide (Appendix: Figure A.7). MS profiles were also performed and compared to the parthenolide standard (Appendix: Figure A.8), but could not conclusively determine the molecular masses of the putative STLs.

4.4 Results

4.4.1 Experiment #1

Putative STL A concentration and content

The AM ($P < 0.05$) and soil Ni ($P < 0.001$) treatments and their interaction ($P < 0.001$) were significant on the putative STL [A] (Table 4.1). According to the Tukey's test, with increasing soil [Ni], the [A] tended to be increased in non-AM but decreased in AM plants. At 0 soil [Ni], the [A] was significantly higher (by 3.2 times), but at 200

soil [Ni] significantly lower (by 1.4 times) in AM than non-AM plants. Overall, the [A] tended to be higher at 0 and 100, but lower at 200 and 400 soil [Ni], in AM than non-AM plants.

The AM treatment ($P < 0.001$) and its interaction with soil Ni ($P < 0.001$) were significant on the A content (Table 4.2). According to the Tukey's test, with the increasing soil [Ni], the A content tended to be increased in non-AM, but decreased in AM plants. The A content was significantly higher (by 2.6, 1.3, and 1.4 times) in AM than non-AM plants at 0, 100 and 200 soil [Ni], respectively.

Putative STL B concentration and content

The AM ($P < 0.001$) and soil Ni ($P < 0.001$) treatments, and their interaction ($P < 0.01$), significantly affected the [B] (Table 4.1). According to the Tukey's test, with increasing soil [Ni] (except at 100M-), the [B] tended to be increased in non-AM plants. The [B] in AM plants was significantly lower (by 8.7 times) at 100 mg Ni kg^{-1}, but at 400 mg Ni kg^{-1} the [B] was significantly higher (by 1.7 times) than at 0 mg Ni kg^{-1}. At 0 and 400 soil [Ni], the [B] were significantly higher (by 3.3 and 2.4 times, respectively) in AM than non-AM plants.

The AM ($P < 0.001$) and soil Ni ($P < 0.01$) treatments had significant effects on the B content (Table 4.2). According to the Tukey's test, these contents were significantly higher in AM than non-AM plants at soil Ni input of 0, 100 and 400 mg Ni kg^{-1} (by 2.2, 5.1, and 2.2 times, respectively). In AM plants, the B contents were significantly lower at 100 and 200 mg Ni kg^{-1} but similar between 400 and 0 mg Ni kg^{-1}. In non-AM plants, the B content was significantly lower at 100 soil [Ni] and constant at 200 and 400 soil [Ni] as compared to 0 soil [Ni].

Putative STL C concentration and content

The AM ($P < 0.05$) and soil Ni ($P < 0.001$) treatments, and their interaction ($P < 0.001$), had significant effects on the [C] (Table 4.1). According to the Tukey's test, with increasing soil [Ni], the [C] tended to increase in non-AM and decrease in AM plants. The [C] was significantly higher (by 5.5 times) at 0M+ than at 0M-.

The AM ($P < 0.001$) and soil Ni ($P < 0.001$) treatments, and their interaction ($P < 0.001$), had significant effects on the C content (Table 4.2). According to the Tukey's test, with increasing soil [Ni], the C content significantly decreased in AM plants, while remaining constant in non-AM plants. The Tukey's test indicated that the C content was significantly higher in 0M+ and 400M+ treatments (by 2.0 to 5.1 times, respectively) than in all other treatments, with 0M+ having the highest overall C content.

Putative STL D concentration and content

The AM ($P < 0.01$) and soil Ni ($P < 0.001$) treatments, and their interaction ($P < 0.05$), had significant effects on the [D] (Table 4.1). According to the Tukey's test, the [D] was the highest in 100M- treatment, while in AM plants, the [D] tended to decrease at 200 and 400 mg Ni kg^{-1}. The [D] was significantly higher at 0M+ and 200M+ than at 0M- and 200M- (by 1.8 and 1.1 times), respectively.

The AM treatment had a marginally significant ($P = 0.075$) effect on the D content (Table 4.2). Although not significant, the D content tended to be higher in AM than non-AM plants. According to the ANOVA, the soil [Ni] had a significant effect ($P < 0.05$) on the D content. The Tukey's test indicated that the D content at 100M+ was significantly higher than at 200M+ treatment (by 1.8 times).

Total putative STL content

The AM ($P < 0.001$) and its interaction with soil [Ni] ($P < 0.001$), were significant on the total putative STL content (Table 4.2). According to the Tukey's test, the total content was significantly higher in AM than non-AM plants at soil [Ni] of 0, 100 and 200 (by 3.0, 1.7, and 1.8 times, respectively), with the 0M+ treatment having the highest total putative STL content. With increasing soil [Ni], this content in AM plants significantly decreased, while it remained unchanged in non-AM plants, except at 400M- where it was significantly increased. Overall, the putative STL A had the highest content or concentration, followed by D and B (approximately equal), then C.

4.4.2 Experiment #2

The AM treatment significantly increased the putative terpene concentrations of A ($P < 0.01$), B ($P < 0.05$), C ($P < 0.01$) and D ($P < 0.001$) (by 3.0, 3.4, 3.6, and 6.0 times, respectively), as well as the content for terpene A ($P = 0.097$), B ($P < 0.01$), C ($P < 0.01$) and total ($P < 0.01$) (by 1.4, 7.0, 3.4 and 2.0 times, respectively) of AM sunflower directly seeded in 200 soil [Ni] (Table 4.8).

4.4.3 C/N Ratio

The AM treatment significantly ($P < 0.01$) lowered root C/N ratios, without any differences observed according to the Tukey's test in Experiment #1 (Tables 4.10). No significant effects of AM treatment were observed in leaf, root or total C/N ratios in Experiment #2. There were no significant effects of soil [Ni] input on leaf, root or total C/N ratio from Experiments #1 and #2.

Table 4.1 Putative STL concentration (mg g^{-1} DM) means and (SEs) (n = 3) in leaves of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	Putative STLs			
		A	B	C	D
0	M -	5.89^{b}	1.66^{c}	0.72^{c}	2.26^{b}
		(0.63)	(0.12)	(0.17)	(0.17)
	M+	18.6^{a}	5.54^{b}	3.98^{a}	4.11^{a}
		(1.68)	(0.65)	(0.88)	(0.63)
100	M -	5.88^{b}	1.34^{c}	1.17^{bc}	4.17^{a}
		(0.80)	(0.11)	(0.16)	(0.32)
	M+	10.1^{ab}	0.64^{c}	1.08^{bc}	5.65^{a}
		(0.42)	(0.03)	(0.08)	(0.41)
200	M -	7.06^{b}	2.15^{b}	1.01^{bc}	3.63^{b}
		(1.25)	(0.84)	(0.03)	(0.31)
	M+	5.22^{c}	3.84^{b}	0.85^{c}	4.16^{a}
		(0.59)	(1.04)	(0.05)	(0.34)
400	M -	13.0^{a}	3.90^{b}	2.16^{ab}	2.68^{b}
		(0.69)	(0.38)	(0.10)	(0.34)
	M+	10.4^{ab}	9.27^{a}	2.43^{a}	2.45^{b}
		(2.67)	(1.77)	(0.21)	(0.33)
ANOVA: AM fungi (M), Nickel (Ni)					
M		*	***	*	**
Ni		***	***	***	***
M X Ni		***	**	***	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 4.2 Putative STL content (mg leaf^{-1} DM) means and (SEs) (n = 3) in leaves of sunflower grown with (M+) or without (M-) AM fungi in Ni (0, 100, 200 and 400 mg kg^{-1} DS) soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	Putative STLs				
		A	B	C	D	Total
0	M -	113^{b}	66.7^{b}	22.7^{c}	94.4^{ab}	209^{c}
		(14.4)	(2.96)	(2.08)	(8.19)	(26.3)
	M+	294^{a}	150^{a}	85.5^{a}	115^{ab}	624^{a}
		(96.5)	(26.1)	(4.73)	(28.4)	(99.6)
100	M -	150^{b}	20.4^{c}	16.6^{c}	88.9^{ab}	247^{bc}
		(6.10)	(3.06)	(1.74)	(2.17)	(21.7)
	M+	196^{a}	105^{b}	22.4^{c}	136^{a}	429^{a}
		(12.7)	(1.10)	(1.40)	(24.8)	(19.5)
200	M -	143^{b}	68.8^{b}	19.3^{c}	81.5^{ab}	205^{c}
		(4.67)	(27.6)	(2.89)	(7.64)	(32.5)
	M+	194^{a}	90.2^{b}	18.3^{c}	77.2^{b}	362^{b}
		(30.8)	(21.9)	(5.26)	(0.74)	(16.3)
400	M -	193^{a}	82.2^{b}	30.7^{bc}	89.6^{ab}	363^{ab}
		(3.93)	(7.15)	(4.57)	(9.54)	(22.3)
	M+	199^{ab}	180^{a}	44.7^{b}	112^{ab}	289^{bc}
		(48.8)	(27.2)	(2.68)	(3.60)	(28.6)
ANOVA: AM fungi (M), Nickel (Ni)						
M		***	***	***	MN	***
Ni		NS	**	***	*	NS
M X Ni		***	NS	***	NS	***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 4.3 Two-way ANOVA on putative STL A concentration (ranked data) and content.

Source of variation	Concentration			Content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	96.00	409.67	437.67	384.00	51.67	446.33
MS	96.00	136.56	145.89	384.00	17.22	148.78
F-value	7.43	10.57	11.29	22.93	1.03	8.88

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.4 Two-way ANOVA on ranked data of putative STL B concentration and content.

Source of variation	Concentration			Content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	106.01	595.47	145.49	468.08	189.20	21.52
MS	106.01	198.49	48.50	468.08	63.07	7.17
F-value	19.88	37.22	9.09	65.78	8.86	1.01

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.5 Two-way ANOVA on putative STL C concentration (ranked data) and content.

Source of variation	Concentration			Content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	80.67	430.33	484.33	2215.26	4074.01	2787.88
MS	80.67	143.44	161.44	2215.26	1358.00	929.29
F-value	8.45	15.03	16.92	66.24	40.61	27.79

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.6 Two-way ANOVA on ranked data of putative STL D concentration and content.

Source of variation	Concentration			Content		
	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3
SS	4.63	17.97	3.68	94.45	320.20	138.60
MS	4.63	5.99	1.22	94.45	106.73	46.20
F-value	13.31	17.22	3.52	3.69	4.17	1.80

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.7 Two-way ANOVA on ranked data of putative STL total content.

Source of variation	Content		
	M	Ni	M X Ni
df	1	3	3
SS	416.67	55.67	379.00
MS	416.67	18.56	126.33
F-value	22.36	1.00	0.00

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.8 Putative STL concentration (mg g^{-1} DM) and content (mg $leaf^{-1}$ DM) means and (SEs) (n = 3) in leaves of sunflower grown with (M+) or without (M-) AM fungi in 200 mg Ni kg^{-1} DS. ANOVA results are also presented.

AM treatment	Putative STLs				
	A	B	C	D	Total
Concentration					
–	4.57^{b}	3.31^{b}	1.31^{b}	1.76^{b}	–
	(0.98)	(0.04)	(0.28)	(0.77)	
+	13.6^{a}	11.2^{a}	4.66^{a}	10.5^{a}	–
	(1.02)	(1.36)	(0.37)	(0.43)	
M	**	*	**	***	
Content					
–	127^{a}	22.3^{b}	23.7^{b}	116^{a}	289^{b}
	(18.9)	(1.26)	(6.93)	(33.1)	(34.8)
+	179^{a}	157^{a}	80.7^{a}	147^{a}	565^{a}
	(14.9)	(26.7)	(7.77)	(11.5)	(39.3)
M	MN	**	**	NS	**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 4.9 One-way ANOVA on putative STL concentrations and content.

Source of variation	Concentration				Content				
	A	B	C	D	A	B	C	D	Total
df	1	1	1	1	1	1	1	1	1
SS	122.22	13.50	16.87	114.93	4057.56	27336.15	4876.92	1482.40	113822.80
MS	122.22	13.50	16.87	114.93	4057.56	27336.15	4876.92	1482.40	113822.80
F-value	40.93	13.5	16.87	98.59	4.68	25.42	28.21	0.81	27.53

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

Table 4.10 C/N ratio means and (SEs) (n = 3) of sunflower grown with (M+) or without (M-) AM fungi in Ni soil treatment. ANOVA results are also presented.

Soil [Ni]	AM treatment	C/N ratio		
		Leaf	Root	Total
Experiment #1				
0	M -	15.4[a]	40.1[a]	21.9[a]
		(2.02)	(4.82)	(2.23)
	M+	14.6[a]	35.2[a]	20.3[a]
		(1.00)	(1.90)	(0.35)
100	M -	13.7[a]	42.1[a]	21.0[a]
		(1.15)	(1.00)	(1.28)
	M+	15.2[a]	27.0[a]	19.3[a]
		(0.88)	(1.50)	(0.60)
200	M -	14.0[a]	33.6[a]	20.1[a]
		(0.31)	(3.97)	(0.92)
	M+	13.2[a]	27.1[a]	18.0[a]
		(0.80)	(0.25)	(0.77)
400	M -	15.6[a]	38.3[a]	21.9[a]
		(0.17)	(5.92)	(1.10)
	M+	16.8[a]	28.1[a]	21.2[a]
		(0.62)	(2.77)	(0.80)
ANOVA: AM fungi (M), Nickel (Ni)				
M		NS	**	NS
Ni		NS	NS	NS
M X Ni		NS	NS	NS
Experiment #2				
200	M -	12.8[a]	34.0[a]	17.9[a]
		(1.31)	(4.62)	(1.69)
	M+	13.5[a]	39.3[a]	19.2[a]
		(0.35)	(2.57)	(0.80)
M		NS	NS	NS

** $P < 0.01$; NS, not significant

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table 4.11 Two-way ANOVA on leaf (ranked data), root and total C/N ratios for Experiment #1.

Source of variation	Leaf			Root			Total		
	M	Ni	M X Ni	M	Ni	M X Ni	M	Ni	M X Ni
df	1	3	3	1	3	3	1	3	3
SS	10.67	277.67	121.67	0.08	0.03	0.02	88.17	321.33	61.83
MS	10.67	92.56	40.56	0.08	0.01	0.01	88.17	107.11	20.61
F-value	0.23	2.00	0.88	15.61	1.81	1.09	2.08	2.52	0.49

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

M = AM fungi, Ni = nickel

Table 4.12 One-way ANOVA on leaf (ranked data), root and total C/N ratios for Experiment #2.

Source of variation	Leaf	Root	Total
df	1	1	1
SS	1.50	42. 35	2.56
MS	1.50	42.35	2.56
F-value	0.37	1.01	0.49

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

4.5 Discussion

4.5.1 Effects of soil Ni input on STL production

The significance of metal hyperaccumulation in plants is still largely unknown, but has been increasingly debated over the last decade (Boyd and Martens 1998, Davis *et al.* 2001, Peterson *et al.* 2003). One hypothesis proposed by Boyd and Martens (1998) states that hyperaccumulation represents an elemental defense against herbivory and pathogen attack such that at high internal HM concentrations, the secondary metabolite concentration should be low. In this study, the putative STLs were determined in relation to soil Ni input and AM colonization in sunflower plants. These STLs, characteristics of the Asteraceae family, have been reported to possess antifeedant and antifungal properties (Picman 1986). According to the elemental defense hypothesis, we propose that the plant metal concentrations are inversely proportional to the synthesis of organic defense compounds. The results of the present study do not coincide with this elemental defense hypothesis. Overall, with increasing soil [Ni], the concentrations of the STLs (putative STLs A, B, C and D) in non-AM plants were significantly increased, especially at soil Ni input of 200 and 400 mg Ni kg^{-1}.

Martens and Boyd (1998) suggested that metal-based defenses compensate for plant resource investment into organic defenses (i.e. C and N). Although the effect of herbivory was not investigated in the present study, the relationship between STL level and soil Ni input revealed that an increase in one component also results in an increase in the other, as observed at soil Ni input of 200 and 400 mg Ni kg^{-1} in non-AM treatments. It is likely that a synergistic effect is occurring, where the level of Ni input has resulted in a concomitant increase in STL levels. As our sunflower test species is not a hyperaccumulator of any metal, a strict elemental versus organic based defense (or vice versa) may result in a cost-benefit ratio that would be detrimental to overall plant growth and survival. Indeed, the total C/N ratio was not affected by Ni input in this study, suggesting that

even at high Ni levels, the C and N allocation was probably not directed towards defense but towards primary production. Sunflower has been shown to accumulate heavy metals exceeding what would normally be observed in non-hyperaccumulating plants (Chen *et al.* 2000, Davies *et al.* 2000 and 2001). Thus, it may be that non-hyperaccumulator species such as sunflower, utilize synergism among elemental and organic based defenses to increase their effectiveness for overall herbivory protection while maintaining growth and Ni tolerance during Ni stress (Davis *et al.* 2001).

4.5.2 Induction of STLs by AM colonization

We have also investigated the AM colonization effect in sunflower in order to determine its contribution in an induction of STL synthesis under soil Ni input. Interestingly, the AM plants showed an inverse relationship in STL levels, in that with increasing soil Ni input, the STL concentrations tended to decrease. This trend contrasts with the one previously described in non-AM plants, where an increase in soil Ni input resulted in an increase in STL concentrations. The AM association represents a large C drain, as 4 to 20% of the photosynthates are transferred to the fungus (Smith and Read 1997), resulting in a decline of STLs in leaves as the terpenoid biosynthesis requires an important C investment (Gange and West 1994, Gershenzon and Croteau 1991). In the present study, the leaf and total C/N ratios were not affected by AM association; however, a significant decrease in root C/N ratio in AM plants was observed. In terms of C allocation, this represents decreases of 7, 15 and 4% in the roots of AM as compared to non-AM plants for soil Ni input of 0, 100 and 200 mg Ni kg^{-1}, respectively. These reductions in C/N ratios may account for the decline in STL levels when mycorrhiza was a factor.

Yet, significant increases in the concentrations of all the parthenolide derivatives in the leaves of AM as compared to non-AM plants, particularly at 0 and 200 mg Ni kg^{-1}, do support the hypothesis that AM symbiosis induces an increase of STLs. This response

was even more obvious when looking at the STL contents, especially in the AM plants, which were significantly higher (by 2 to 3 times) than non-AM plants at soil Ni input of 0 to 200 mg Ni kg^{-1}. Furthermore, the STL concentrations in sunflower directly seeded in 200 mg Ni kg^{-1}, were more than 3 times higher for the putative STLs A, B and C, and 6 times higher for D in AM than non-AM plants. Similar results were reported by Maier *et al.* (1999) who found that the same AM fungal species, *G. intraradices*, induced an isoprenoid induction of cyclohexenone derivatives in tobacco roots. Akiyama *et al.* (2005) isolated a branching factor, the STL strigolactones from root exudates of *Lotus japonicus*, and suggested that this STL acts as a plant signal molecule to induce hyphal branching in the AM fungus *Gigaspora margarita*. The induction of STLs from the AM association in concert with the "defense" properties afforded by high Ni levels would maximize defense strategies with minimal carbon-based defenses required.

4.6 Conclusion

The objectives of this study were to determine whether the STLs in sunflower were induced with increasing soil Ni input and/or AM colonization. Specifically, the elemental based hypothesis (Boyd and Martens 1998) was investigated, and it was found that for sunflower, the increasing soil [Ni] resulted in a concomitant increase of the STLs in sunflower leaves. These findings contradict the hypothesis proposed by Boyd and Martens (1998). The elemental defense hypothesis is considered to be a highly specialized defense mechanism found only in hyperaccumulating plant species (Behmer *et al.* 2005, Boyd and Moar 1999). As sunflower is not a Ni hyperaccumulator, the validity of this hypothesis remains to be verified. However, it is probable that the high levels of Ni found in sunflower tissues may be deterrent to herbivores feeding on the leaves (Boyd and Wall 2001). Further studies incorporating feeding experiments of vertebrate and invertebrate species may aid to clarify this relationship.

The induction of STLs by AM symbiosis was also investigated. Significantly higher concentration and content of putative STLs were observed in AM as compared to non-AM plants, which suggests plant defense-like responses to AM colonization. However, with increasing soil Ni input, the STL levels in AM plants decreased. These results contrast with the increasing trend of STL levels in non-AM plants. As the AM root colonization could be as much as 20% C drain, a decline in STL levels could be expected. In addition, a synergistic relationship between AM colonization and soil Ni input may alleviate the cost associated with secondary metabolite production when Ni levels may contribute 'per se' to plant defenses.

General Conclusion

This research thesis investigated the contribution of AM colonization on Ni uptake and Ni tolerance, and its effect on the physiology and N metabolism in sunflower plants, supplied with either NH_4^+ or NO_3^- fertilization. Sunflower, one of the most worldwide important crops valued for the production of oil, was chosen as a test species for its high biomass and responsiveness to AM colonization. Whereas other studies had reported sunflower to be metal tolerant (Davies *et al.* 2001 and 2002, Mei *et al.* 2002, Turgut *et al.* 2004), to our knowledge, this is the first study that examined the effect of AM colonization on sunflower in Ni contaminated soils.

The first study (Chapter 2) was conducted in order to investigate the combined effects of AM colonization with two different N-fertilizers and soil Ni treatments on N assimilation and Ni uptake and Ni tolerance in sunflower. Our study revealed that the AM colonization significantly enhanced the shoot, root and total Ni content in sunflower, especially when supplied with NO_3^-. The total plant Ni extracted % was 1.3 times higher in NO_3^--fed AM than non-AM plants, indicating a significant AM contribution. These results are in accordance with other studies which showed that AM fungi contribute to an enhanced plant tolerance by sequestering HM(s) in their fungal tissue and limiting the translocation to the shoots (Audet and Charest 2006, Jones and Hutchinson 1988a and 1988b, Leyval *et al.* 1997). The AM symbiosis also significantly enhanced the plant biomass, mineral concentrations and activities of key N-assimilating enzymes (NR, GS and GDH) in leaves and roots of sunflower plants under NO_3^- fertilzation. These increased activities of GS and GDH suggest that the AM colonization contributes to an enhanced N assimilation via the concurrent GS/GOGAT and GDH pathways (Ahmad *et al.* 1990, Subramanian and Charest 1998, Toussaint *et al.* 2004).

Notably, about 60% of the Ni content was observed in the shoots of AM plants

when supplied with NH_4^+, while by only 20% in the non-AM plants. Furthermore, N-assimilation tended to be higher in plants supplied with NH_4^+, with their NR and GS activities being significantly higher than in plants supplied with NO_3^-, suggesting that the effects of Ni stress and N nutrition are closely linked. The combined effect of N nutrition and HM input on N metabolism has also been reported in other studies (Chugh *et al.* 1992, Llorens *et al.* 2000, Srivastava and Singh 1987). It is also concluded that AM colonization contributes to enhanced sunflower tolerance, through increases in plant biomass and mineral concentrations. It is likely that the critical level for Ni toxicity to become a factor in sunflower growth was not met at soil Ni input of 100 mg Ni kg^{-1}. In addition, the high biomass of sunflower may have contributed to an enhanced Ni tolerance through a growth dilution effect.

The investigation into AM fungal contribution to sunflower Ni tolerance was further studied since the threshold of Ni toxicity had not been reached, nor the AM contribution been fully determined (Chapter 3). The AM colonization significantly increased the Ni content in roots under 100 mg Ni kg^{-1} soil [Ni], resulting in significantly higher total plant Ni content. However, unlike in our first study, at this same soil Ni treatment, over 65% of the Ni content was distributed in the roots, not in shoots of AM plants. This could be a result of the combined NH_4NO_3 fertilizer supplied, whereas in the first study, plants were fertilized with either NH_4^+ or NO_3^-. Other studies have also reported that the type of N nutrition affected plant tolerance and uptake of HMs (McGrath and Rorison 1982, Rorison 1985, Smirnoff and Stewart). With a higher soil Ni input of 400 mg Ni kg^{-1}, the AM plants had a significantly higher shoot Ni extracted % than non-AM plants. Furthermore, at this highest soil Ni input, the AM plants had over 70% Ni content distributed in shoots, suggesting an important Ni translocation, a trait especially desirable in phytoextraction. The GS activities were also increased in leaves and roots of both Ni early and late exposed AM plants. This suggests a possible detoxification mechanism that explains a higher Ni content in the shoots of AM plants, as GS catalyzes

the formation of glutamine, that has been reported to bind with Ni (Bhatia *et al.* 2005, Kämer *et al.* 2000). The enhanced mineral concentrations in AM plants also suggest that their uptake has been facilitated by the intra and extraradical hyphae. These results show the importance of AM fungi in improving host plant N assimilation and nutritional status, especially under Ni stress.

A comparison of Ni exposure at an early (0 DAS or vegetative) or a late (56 DAS or reproductive) development stage showed that the a soil [Ni] of 400 mg Ni kg^{-1} was toxic for the early exposed plants, as all seedlings died within 4 weeks after sowing. In late exposed plants, a reduction of 45% in plant DM was determined at this soil [Ni] stress. Plants exposed to soil [Ni] of 200 mg Ni kg^{-1} at the vegetative stage had 45% higher Ni extracted % ($\sim$ 0.95%) than at the reproductive stage ($\sim$ 0.70 %). However, no significant AM contribution in Ni uptake was detected in early exposed plants. These results suggest that the AM contribution to Ni uptake was optimal at a soil Ni input of 100 mg Ni kg^{-1}, which is considered a moderate toxic concentration. This direct seeding of sunflower in Ni contaminated soils would allow for a straightforward and economical methodology for phytoremediation purposes.

As phytoremediation technology becomes more viable, one of the concerns associated with its implementation involves HM transfer from plants to invertebrate and vertebrate organisms. We therefore conducted an investigation to determine whether AM colonization and/or soil [Ni] would induce the production of STLs in sunflower (Chapter 4). Our results showed that both AM colonization and increasing soil [Ni] induce the increase of STL levels in sunflower leaves. The induction of secondary metabolites in AM plants had been reported under a number of stressful conditions (Akiyama *et al.* 2005, Dixon and Harrison 1994, Maier *et al.* 1999, Phelps 2004), and under HM conditions (Davis *et al.* 2001, Davis and Boyd 2000, Mithöfer *et al.* 2004). Notably, from our study, the combination of AM colonization and soil Ni input has resulted in a synergistic effect,

whereby the AM induction of STLs acted in concert with the "defense" properties of high Ni levels. Thus, the antifeedant properties of STLs could deter herbivores, thereby safeguarding against possible HM transfer into higher trophic levels. To validate these findings, it is therefore suggested that future studies incorporate feeding experiments to examine whether high Ni levels, and/or in concert with AM fungi, deters herbivory of selected invertebrate or vertebrate species. Furthermore, isolation and identification of the putative STLs through column chromatography (Melek *et al.* 1985) or through a microsampling technique of glandular trichomes, as described by Spring *et al.* 1989, followed by identification using NMR and MS spectra, would provide greater insight into the effect of AM fungi and/or high internal Ni concentrations on sunflower synthesis of defense compounds.

Overall, this study showed that AM fungi play an integral role in alleviating plant metal toxicity as well as augmenting plant Ni uptake. Thus, our stated hypotheses have been supported by our results. However, the AM effect was not clearly demonstrated when sunflower was directly seeded in Ni treated soils at 200 or 400 mg Ni kg^{-1}. It is possible that the AM effect would be more pronounced at a moderately contaminated soil [Ni] of 100 mg Ni kg^{-1}, as we propose that this AM-plant interaction is optimal at this concentration. It is therefore recommended that a future study implementing direct seeding be conducted at soil [Ni] of 100 mg Ni kg^{-1} to verify these assumptions. We also suggest that hyphal uptake and transfer of HMs to plant roots using compartmented pot systems and mechanisms of transport to aboveground organs through chelation of organic and amino acids, should be the focus of future research.

The integration of AM fungi into strategies of phytoremediation provides economical and environmentally safer alternative to current physico-chemical remediation techniques. This strategy shows promise to advancing the viability of phytoremediation in large-scale applications, as the AM fungi may also contribute to revegetation of de-

graded lands. Furthermore, use of chelators as amendments and/or addition of useful bacteria for the degradation of organic compounds (i.e. polycyclic aromatic hydrocarbons) may be combined with AM fungal strategies to further augment HM removal from soils. Moreover, phytomining of valuable metals (i.e. Ni, Cu, Au) can be exploited as an economical offshoot of phytoextraction. In light of this research, sunflower as a 'candidate' species and in association with AM fungi, is a heralding technology in the field of phytoremediation.

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Appendix A

Supplementary figures and tables

Figure A.1 Biosynthesis of the isoprenoid end product geranyl-pyrophosphate (GPP) from isopentenyl diphosphate (IPP) pathway **A** leads to the biogenetic transformation of farnesyl pyrophosphate (FPP) which provides the germacradiene skeleton of the sesquiterpene lactones. *Abbreviations:* AACT, acetyl CoA acetyltransferase; HMG, β-hydroxy-β-methyl-glutaryl; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonic acid kinase; PMK, phosphomevalonate kinase; MDC, diphospho-mevalonate decarboxylase; DXPS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; CMT, 2-*C*-methylerythritol-4-phosphate cytidyltransferase; CMK, (cytidine 5'-diphospho)-2-*C*-methylerythritol kinase; MECPS, 2-*C*-methylerythritol 2,4-cyclodiphosphate synthase; MTS, monoterpene synthase; SES, sesquiterpene synthase.

(Dey and Harborne 1991, Lange *et al.* 2000)

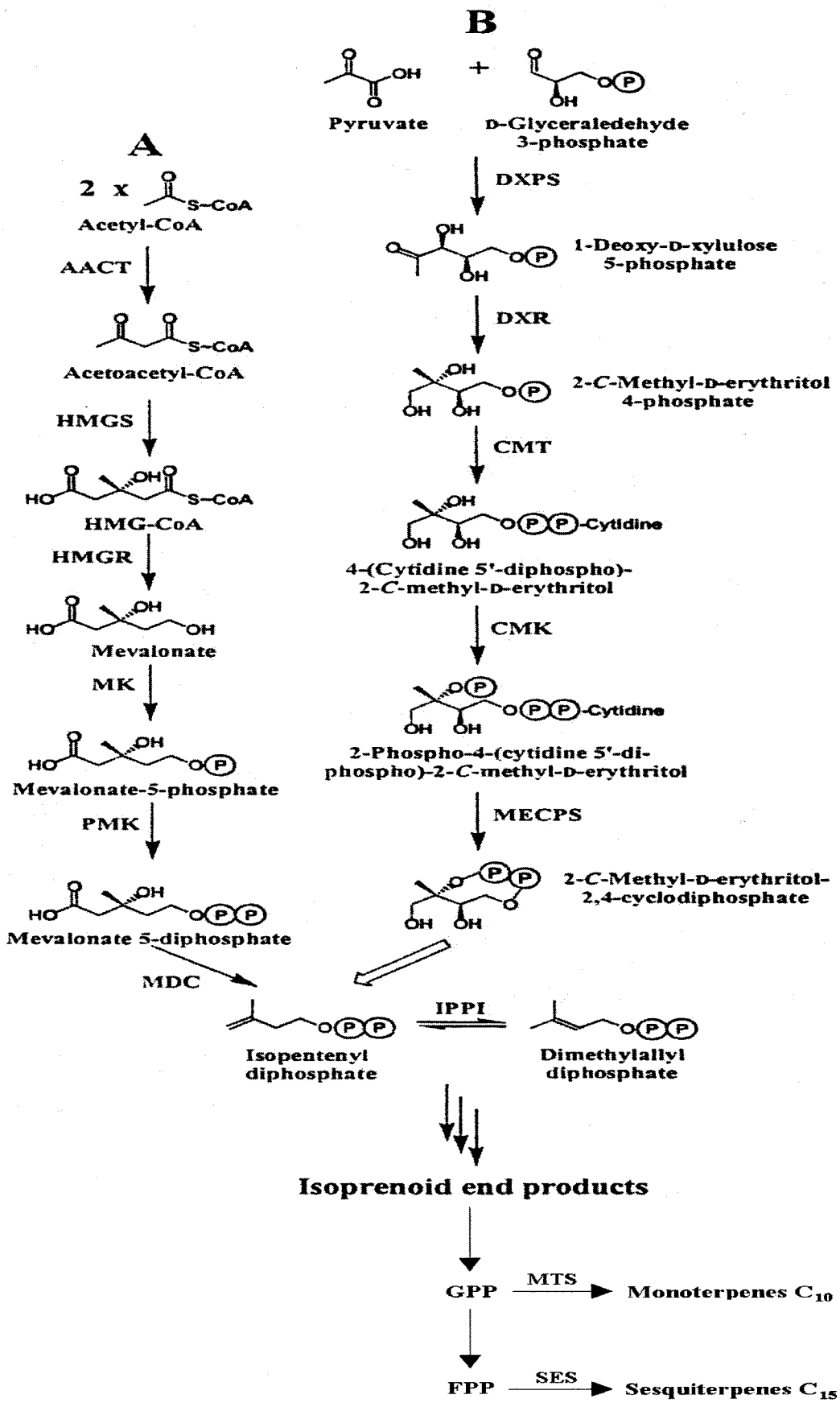

A
2 x
S-CoA
Acetyl-CoA
AACT
S-CoA
Acetoacetyl-CoA
HMGS
HO
OH
S-CoA
HMG-CoA
HMGR
HO
OH
OH
Mevalonate
MK
HO
OH
P
Mevalonate-5-phosphate
PMK
HO
OH
P P
Mevalonate 5-diphosphate
MDC
B
OH
O
Pyruvate
+
O
P
OH
D-Glyceraledehyde 3-phosphate
DXPS
OH
O
P
OH
1-Deoxy-D-xylulose 5-phosphate
DXR
OH
O
P
OH OH
2-C-Methyl-D-erythritol 4-phosphate
CMT
OH
O P P -Cytidine
OH OH
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol
CMK
O P
O P P -Cytidine
OH OH
2-Phospho-4-(cytidine 5'-di-phospho)-2-C-methyl-D-erythritol
MECPS
O P P
O
OH OH
2-C-Methyl-D-erythritol-2,4-cyclodiphosphate
IPPI
O P P
Isopentenyl diphosphate
O P P
Dimethylallyl diphosphate
Isoprenoid end products
GPP
MTS
Monoterpenes C10
FPP
SES
Sesquiterpenes C15

Figure A.2 Representative sesquiterpene lactone compounds isolated from sunflower, *Helianthus annuus* L. (Fischer 1991, Picman 1986).

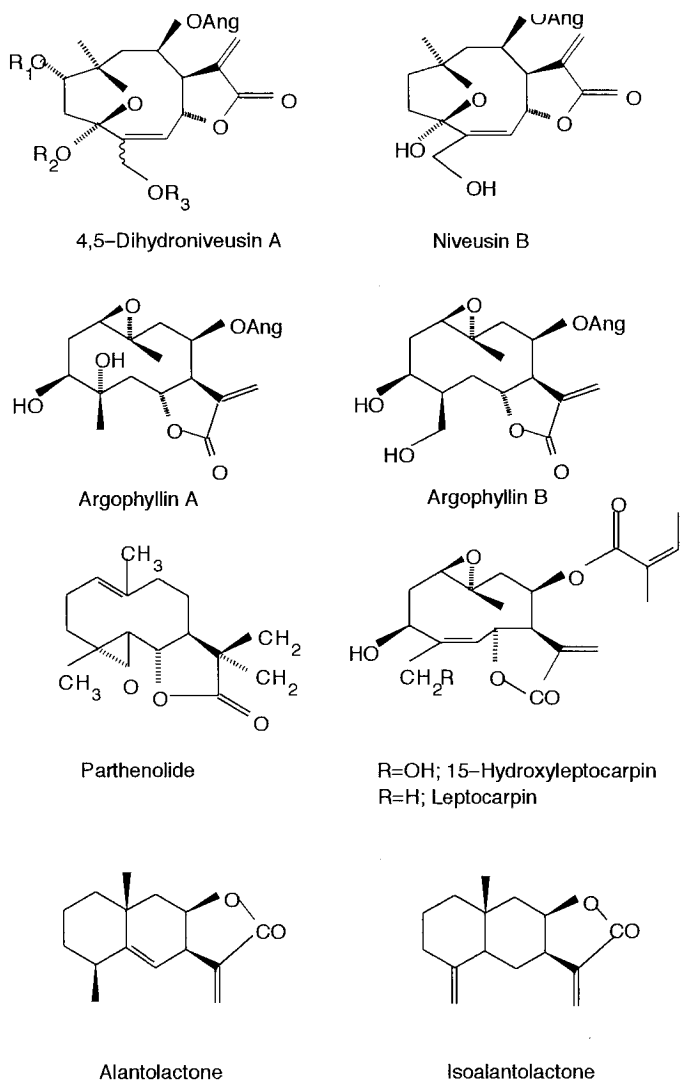


Table A.1 Soil physico-chemical properties[1](tested by LCOI, Québec, Canada).

Parameters	Values
Electrical conductivity (mS cm^{-1})	0.10
pH	5.0
Organic matter	6.12 %
Sand (> 0.050 mm)	95 %
Silt (> 0.002 - 0.050 mm)	< 1 %
Clay (< or = 0.002 mm)	4 %
N-NO_3 (Nitrate)	4 ppm
Total Kjeldahl Nitrogen	0.12 %
Total Organic Carbon	2.67 %
P ($NaHCO_3$ Extractable)	7 ppm
K (NH_4 Acetate Extractable)	35 ppm
Mg (NH_4 Acetate Extractable)	109 ppm
Ni	8 $\mu g\ g^{-1}$
Ca	4100 $\mu g\ g^{-1}$
Na	697 $\mu g\ g^{-1}$
Cu	10 $\mu g\ g^{-1}$
Fe	7160 $\mu g\ g^{-1}$
Mn	137 $\mu g\ g^{-1}$
Mo	< 1 $\mu g\ g^{-1}$
Zn	22 $\mu g\ g^{-1}$
B	1.1 ppm

[1]The soil was previously autoclaved for 20 min at 121^0C and 15PSI .

Table A.2 A nitrate-type, ammonium-type and ammonium-nitrate type Long Ashton Nutrient Solution (LANS) (Hewitt and Smith 1975).

Nitrogen type	Compound	Concentration ($g\ L^{-1}$)	Molarity (mM)
Macronutrient			
Nitrate	KO_3	0.404	4
	$Ca(NO_3)_2{\cdot}4H_2O$	0.950	4
	$MgSO_4{\cdot}7H_2O$	0.368	1.5
	$NaH_2PO_4{\cdot}H_2O$	0.208	1.5
Ammonium	K_2SO_4	0.348	2
	$CaCl_2$anhydrous	0.444	4
	$MgSO_4{\cdot}7H_2O$	0.368	1.5
	$NaH_2PO_4{\cdot}H_2O$	0.208	1.5
	$(NH_4)_2SO_4$	0.528	4
Ammonium-nitrate	K_2SO_4	0.348	2
	$CaCl_2$anhydrous	0.444	4
	$MgSO_4{\cdot}7H_2O$	0.368	1.5
	$NaH_2PO_4{\cdot}H_2O$	0.208	1.5
	NH_4NO_3	0.402	5
Micronutrient			
All types	$MnSO_4{\cdot}H_2O$	0.0017	0.01
	$CuSO_4{\cdot}5H_2O$	0.00025	0.001
	$ZnSO_4{\cdot}7H_2O$	0.0003	0.001
	H_3BO_3	0.003	0.048
	NaCl	0.005	0.085
	$Na_2MoO_4{\cdot}2H_2O$	0.00012	0.0005
	EDTA-Fe (13%)	N/A	5.7[1]

[1]in ppm

Figure A.3 A standard curve of γ-glutamylhydroxamate (γ-GH) concentration for the determination of glutamine synthetase (GS) activity.

γ-GH conc. (μmol)	OD_{540}
0.0	0.000
0.5	0.122
1.0	0.248
1.5	0.380
2.0	0.509
2.5	0.635
3.0	0.774

Regression output

Constant	0.000
R squared	0.999
No. of observations	7
X Coefficients	0.255

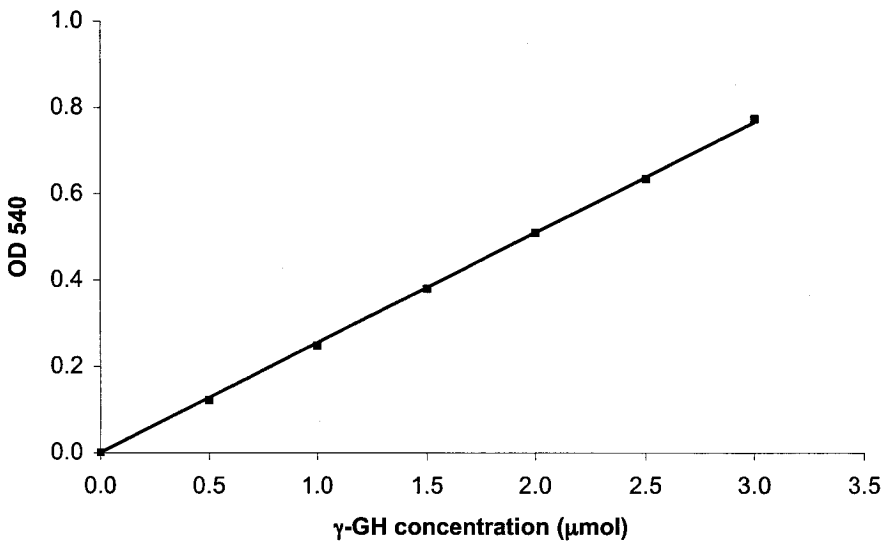
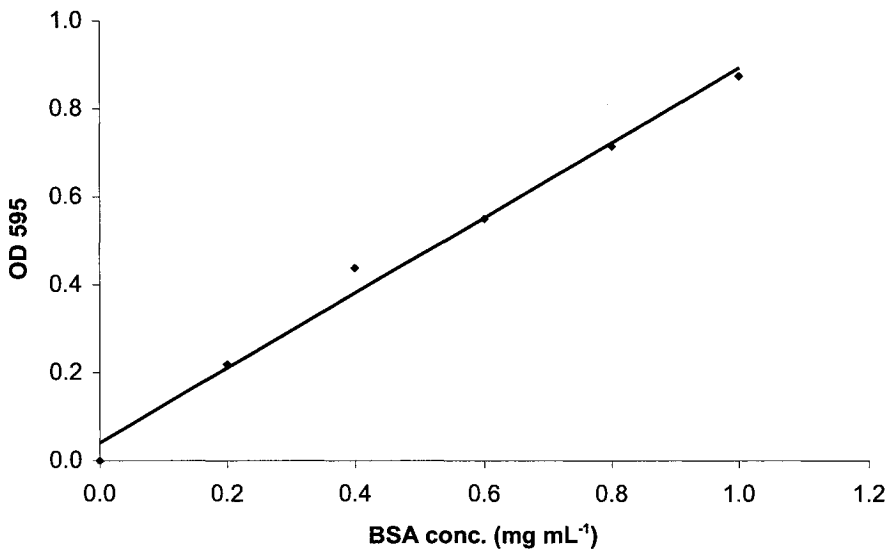


Figure A.4 Standard curve of BSA (bovine serum albumin) for the determination of protein concentrations.

BSA conc. (mg mL^{-1})	OD_{595}
0.0	0.000
0.2	0.220
0.4	0.439
0.6	0.550
0.8	0.715
1.0	0.876

Regression output

Constant	0.040
R squared	0.990
No. of observations	6
X Coefficients	0.854



Soluble protein concentrations

The soil [Ni] was marginally significant (P = 0.052) on the leaf protein concentration without any significant differences according to the Tukey's test (Appendix 8 and 9). However, the protein concentrations tended to increase in the leaves of plants supplied with NH_4^+. The protein concentrations in roots were significantly affected by the fertilizer ($P < 0.01$) and soil [Ni] ($P < 0.05$), and by the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.05$). According to the Tukey's test, the root protein concentration was significantly higher in plants supplied with NH_4^+ than NO_3^-. The soil [Ni] tended to increase the concentration for both N-type fertilizers, except for M+Ni+ supplied with NH_4^+.

Table A.3 ANOVA results for protein concentrations in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in Ni soil treatment.

Parameters	Leaf	Root
F	NS	**
M	NS	NS
Ni	MN	*
F X M	NS	NS
F X Ni	NS	NS
M X Ni	NS	NS
F X M X Ni	NS	*

* $P < 0.05$; ** $P < 0.01$; MN, $0.05 < P < 0.1$; NS, not significant

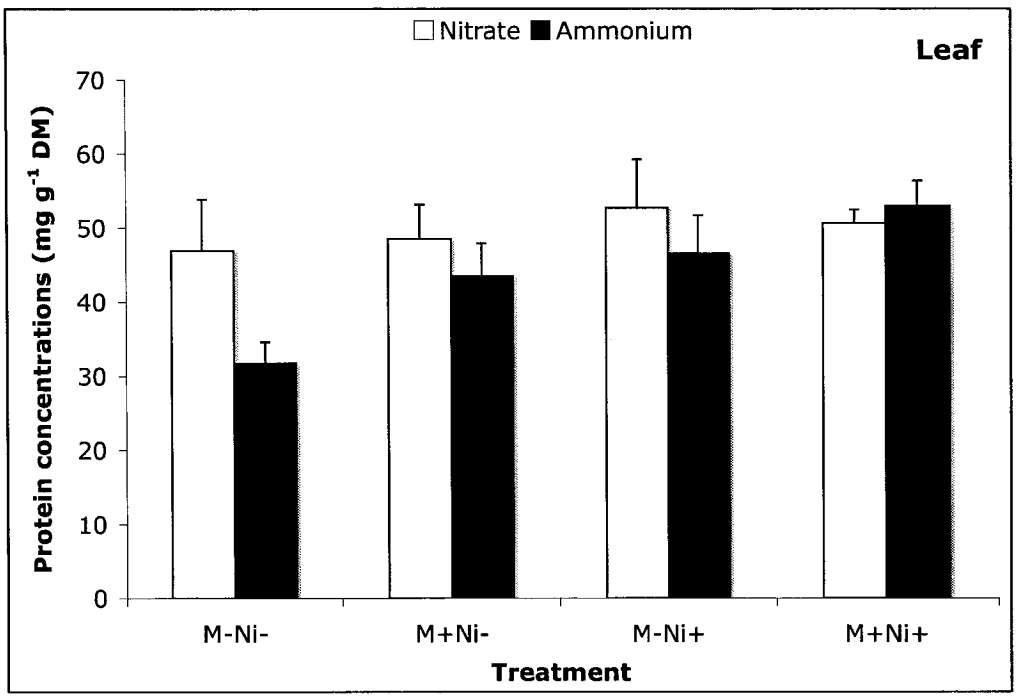
Table A.4 Three-way ANOVA on leaf and root protein concentrations.

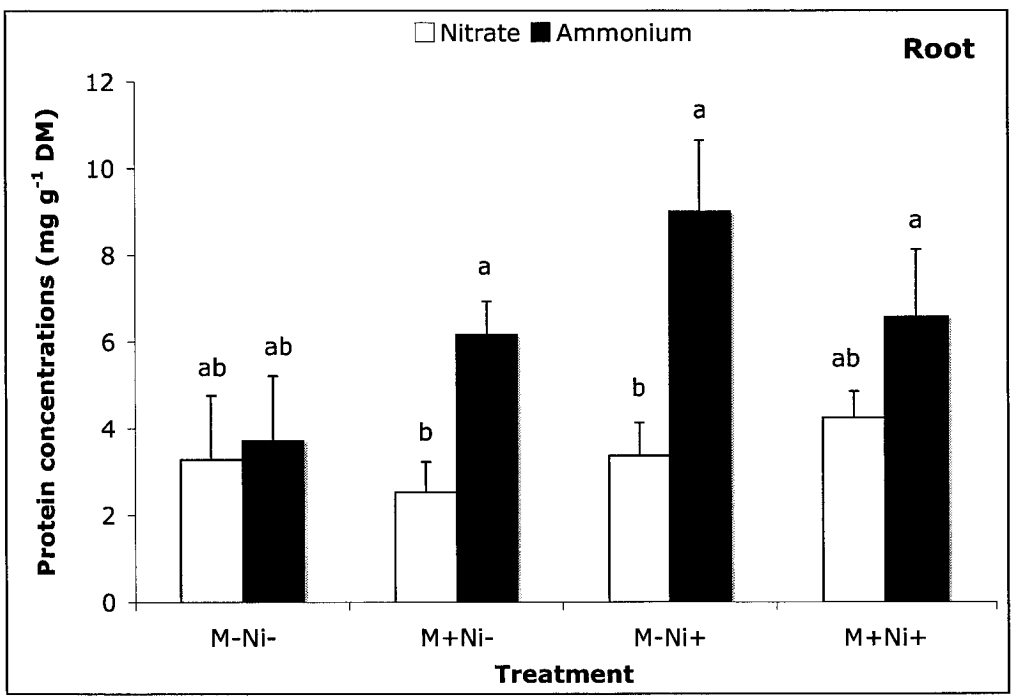
Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	286.21	286.21	2.36	1	56.36	56.36	14.33
M	1	156.59	156.59	1.29	1	0.08	0.08	0.02
Ni	1	505.74	505.74	4.17	1	23.37	23.37	5.94
F X M	1	174.49	174.49	1.44	1	0.10	0.10	0.02
F X Ni	1	134.90	134.90	1.11	1	3.74	3.74	0.95
M X Ni	1	39.77	39.77	0.36	1	2.38	2.38	0.61
F X M X Ni	1	1.24	1.24	0.01	1	17.87	17.87	4.541

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Figure A.5 Protein concentration (mg g^{-1} DM) in leaves (top) and roots (bottom) of sunflower plants supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for leaves and roots. Means (n = 3 to 6) and SEs are shown and different letters refer to significant differences according to the Tukey's test at $P < 0.05$. When no letters are present, then no significant differences were detected.


Nitrate
Ammonium
Leaf
Protein concentrations (mg g-1 DM)
70
60
50
40
30
20
10
0
M-Ni-
M+Ni-
M-Ni+
M+Ni+
Treatment


Nitrate
Ammonium
Root
Protein concentrations (mg g-1 DM)
12
10
8
6
4
2
0
ab
ab
b
a
b
a
ab
a
M-Ni-
M+Ni-
M-Ni+
M+Ni+
Treatment

Specific activity

The leaf NR specific activity was significantly affected by the fertilizer ($P < 0.001$) and AM ($P < 0.01$) treatments (Appendix 10 and 11). The interactions of fertilizer with AM ($P < 0.001$) and with soil [Ni] ($P < 0.001$), and of AM with soil [Ni] ($P < 0.01$) were also significant. According to the Tukey's test, it tended to be higher in plants supplied with NH_4^+ than NO_3^-, except for M+Ni+. The leaf NR specific activity was also significantly higher in AM than non-AM plants with or without Ni supplied with NO_3^-, but was significantly lower in AM than non-AM plants without Ni supplied with NH_4^+. The interactions of fertilizer with AM ($P < 0.05$) and with soil [Ni] ($P < 0.05$) were significant on the root NR specific activity. Although not significant, it tended to be lower in AM than non-AM plants supplied with NH_4^+, but higher in AM than non-AM plants supplied with NO_3^-.

The leaf GS specific activity was significantly affected by soil [Ni] ($P < 0.05$) and marginally significantly ($P = 0.096$) by the interaction of fertilizer with AM (Appendix 10 and 11). Although not significant, it tended to be lower in the NO_3^--fed non-AM plants with than without soil [Ni], but tended to be higher in the AM than non-AM plants with soil [Ni]. The interaction of fertilizer with soil [Ni] was marginally significant ($P = 0.069$) on the root GS specific activity. Although not significant, it tended to decrease in the NO_3^--fed plants with than without soil [Ni]. The leaf GDH specific activity was significantly affected by the fertilizer ($P < 0.01$), AM ($P < 0.01$) and soil [Ni] ($P < 0.05$) treatments (Appendix 10 and 11). The interaction of fertilizer with soil [Ni] ($P < 0.01$) and the tripartite interaction of fertilizer, AM and soil [Ni] ($P < 0.05$) were also significant. According to the Tukey's test, it was significantly lower in the leaves of NO_3^--fed AM than non-AM plants without soil [Ni], and was also significantly lower in NH_4^+-fed AM than non-AM plants with soil [Ni]. The Tukey's test also indicated that the leaf GDH specific activity was significantly higher in AM plants without soil Ni, but was significantly lower in the AM plants with Ni when supplied with NH_4^+ than NO_3^-. The root GDH specific activity was significantly affected by the fertilizer ($P < 0.001$) and AM ($P < 0.05$) treatments. The interaction of fertilizer with soil [Ni] was also marginally significant ($P = 0.079$). According to the Tukey's test, it tended to be

higher in plants supplied with NO_3^- than NH_4^+. The root GDH specific activity tended to be higher in AM than non-AM plants and tended to decrease with the soil [Ni] when supplied with NO_3^-.

Table A.5 Specific activity means (n = 3 to 6) and (SEs) for NR (μmol mg^{-1} proteins h^{-1}), GS (μmol γ-GH produced mg^{-1} proteins h^{-1}) and GDH (μmol mg^{-1} proteins h^{-1}) in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer and grown with (M+) or without (M-) AM fungi in Ni (0 or 100 mg kg^{-1} DS) soil treatment.

Treatment	N-type	NR		GS		GDH	
		Leaf	Root	Leaf	Root	Leaf	Root
M-Ni-	NO_3^-	0.011^{d} (0.000)	0.009 (0.000)	1.13 (0.18)	11.7 (6.60)	0.16^{ab} (0.01)	2.09^{ab} (1.15)
	NH_4^+	0.029^{a} (0.004)	0.016^{a} (0.006)	1.11^{a} (0.11)	3.52^{a} (1.66)	0.10^{b} (0.01)	0.57^{b} (0.29)
M+Ni-	NO_3^-	0.015^{c} (0.000)	0.017^{a} (0.003)	1.24^{a} (0.03)	5.10^{a} (0.62)	0.06^{c} (0.01)	2.94^{a} (0.45)
	NH_4^+	0.021^{bc} (0.001)	0.006^{a} (0.001)	1.28^{a} (0.21)	2.07^{a} (0.25)	0.11^{b} (0.01)	0.76^{b} (0.08)
M-Ni+	NO_3^-	0.011^{d} (0.0.03)	0.007^{a} (0.003)	0.64^{a} (0.09)	3.07^{a} (0.83)	0.23^{a} (0.02)	0.96^{b} (0.16)
	NH_4^+	0.019^{bc} (0.001)	0.018^{a} (0.000)	1.19^{a} (0.21)	3.86^{a} (0.81)	0.15^{ab} (0.03)	0.52^{b} (0.14)
M+Ni+	NO_3^-	0.025^{b} (0.001)	0.005^{a} (0.001)	1.10^{a} (0.03)	3.06^{a} (0.49)	0.19^{ab} (0.03)	1.48^{ab} (0.09)
	NH_4^+	0.020^{bc} (0.002)	0.008^{a} (0.000)	0.95^{a} (0.14)	4.29^{a} (1.59)	0.04^{c} (0.02)	1.12^{ab} (0.40)

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at $P < 0.05$.

Table A.6 ANOVA results for specific activities for NR, GS, and GDH in leaves and roots of sunflower supplied with a NO_3^-- or NH_4^+-type fertilizer (F) and grown with or without AM fungi (M) in Ni soil treatment.

Parameters	NR activity		GS activity		GDH activity	
	Leaf	Root	Leaf	Root	Leaf	Root
F	***	NS	NS	NS	**	***
M	**	NS	NS	NS	**	*
Ni	NS	NS	*	NS	*	NS
F X M	***	*	MN	NS	NS	NS
F X Ni	***	*	NS	MN	**	MN
M X Ni	**	NS	NS	NS	NS	NS
F X M X Ni	NS	NS	NS	NS	*	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MN, $0.05 < P < 0.1$; NS, not significant

Table A.7 Three-way ANOVA on leaf and root specific activity for NR.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	124.10	124.10	24.23	1	0.03	0.03	1.86
M	1	29.75	29.75	5.81	1	0.42	0.42	23.94
Ni	1	86.72	86.72	16.93	1	0.07	0.07	4.22
F X M	1	37.16	37.16	7.26	1	0.02	0.02	1.40
F X Ni	1	145.71	145.71	28.45	1	0.20	0.20	11.49
M X Ni	1	37.34	37.34	7.29	1	0.00	0.00	0.17
F X M X Ni	1	1.32	1.32	0.26	1	0.05	0.05	3.06

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table A.8 Three-way ANOVA on leaf and root specific activity for GS.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	124.10	124.10	24.23	1	0.03	0.03	1.86
M	1	29.75	29.75	5.81	1	0.42	0.42	23.94
Ni	1	86.72	86.72	16.93	1	0.07	0.07	4.22
F X M	1	37.16	37.16	7.26	1	0.02	0.02	1.40
F X Ni	1	145.71	145.71	28.45	1	0.20	0.20	11.49
M X Ni	1	37.34	37.34	7.29	1	0.00	0.00	0.17
F X M X Ni	1	1.32	1.32	0.26	1	0.05	0.05	3.06

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Table A.9 Three-way ANOVA on leaf and root specific activity for GDH.

Parameters	Leaf				Root			
	df	SS	MS	F-value	df	SS	MS	F-value
F	1	124.10	124.10	24.23	1	0.03	0.03	1.86
M	1	29.75	29.75	5.81	1	0.42	0.42	23.94
Ni	1	86.72	86.72	16.93	1	0.07	0.07	4.22
F X M	1	37.16	37.16	7.26	1	0.02	0.02	1.40
F X Ni	1	145.71	145.71	28.45	1	0.20	0.20	11.49
M X Ni	1	37.34	37.34	7.29	1	0.00	0.00	0.17
F X M X Ni	1	1.32	1.32	0.26	1	0.05	0.05	3.06

df = degrees of freedom, SS = sum of squares, MS = mean square, F-value = F distribution

F = fertilizer, M = AM fungi, Ni = nickel

Figure A.6 Chromatogram of sesquiterpene lactones from AM colonized sunflower leaf extracts: putative STLs A, B, C and D at retention times of 8.66, 9.88, 19.27 and 24.55 minutes, respectively.

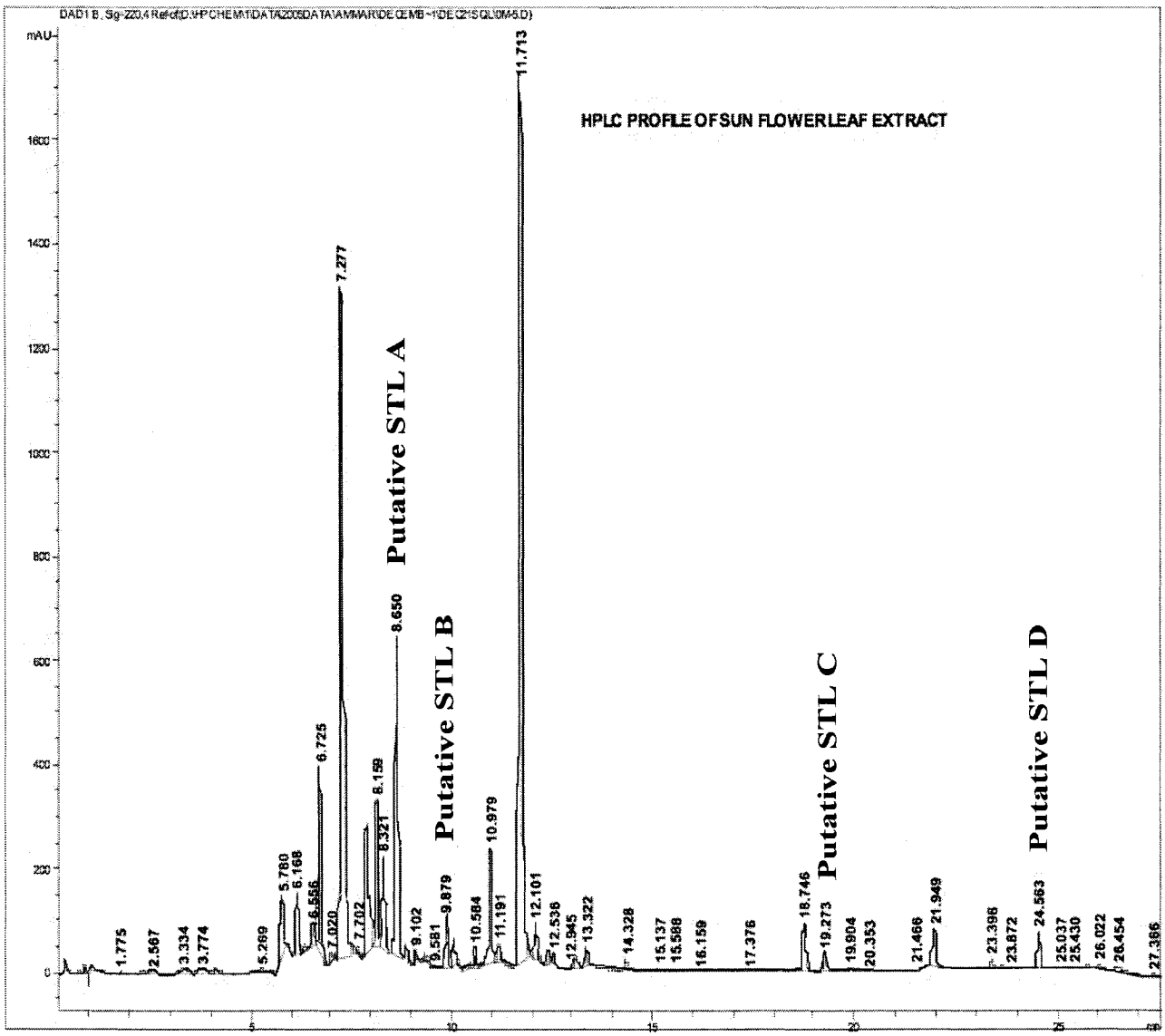


Figure A.7 UV absorption spectra of parthenolide standard at 220 nm.

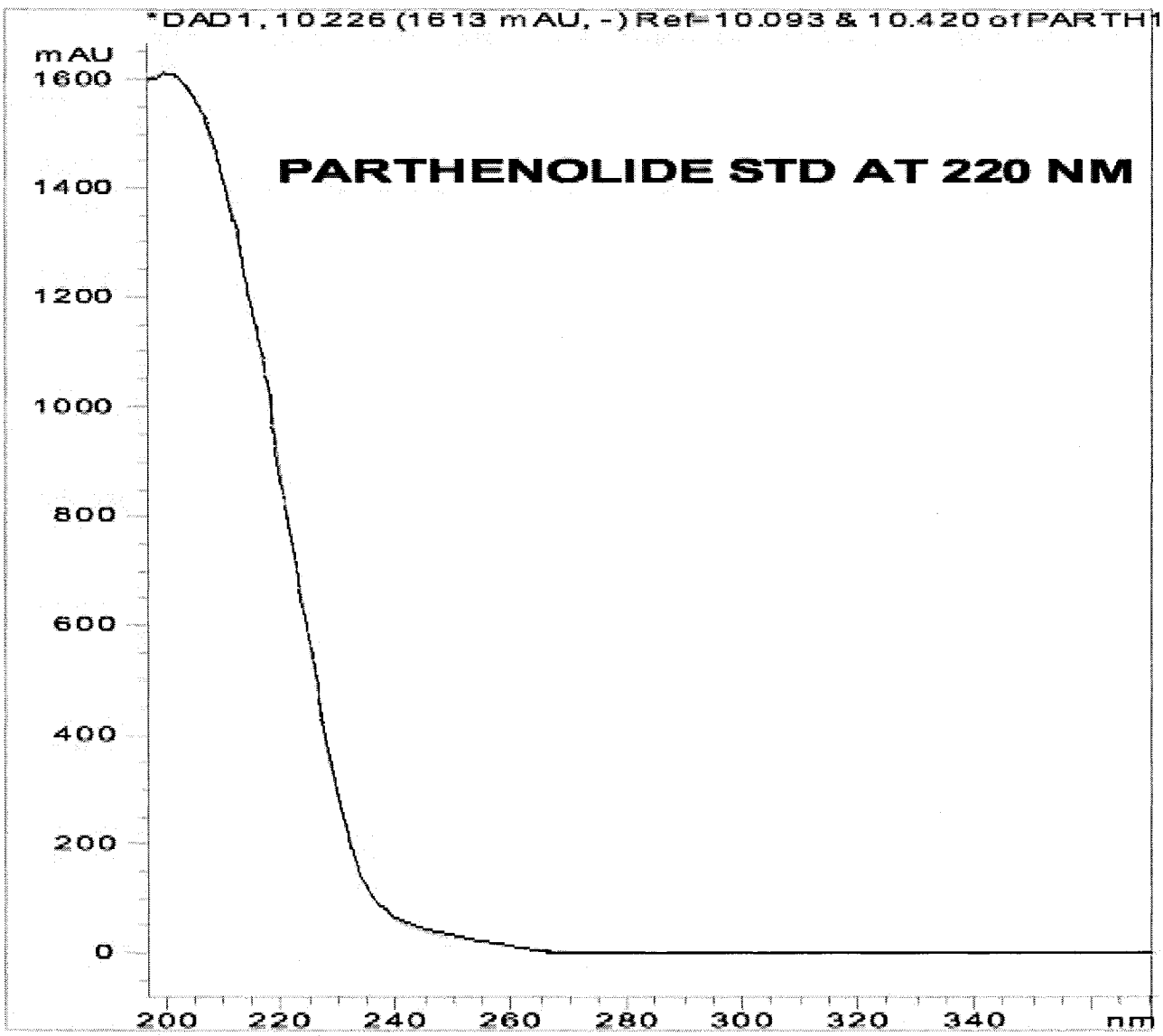


Figure A.8 MS spectrum of parthenolide standard at 220 nm.