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Nickel remediation by AM-colonized sunflower

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Abstract This greenhouse study aimed to examine the contribution of arbuscular mycorrhizal (AM) colonization on the uptake of and tolerance to nickel (Ni) in sunflower (*Helianthus annuus* L.). We hypothesized that AM colonization increases Ni content and tolerance in sunflower grown under varying soil Ni concentrations. The combined effect of AM colonization and soil Ni input on the assimilation of nitrogen, in particular the activity of glutamine synthetase (GS), in sunflower plants was also investigated. A factorial experimental design was performed with sunflower cv. Lemon Queen, with or without the AM fungus, *Glomus intraradices* Schenck & Smith, and treated with 0, 100, 200, or 400 mg Ni kg^{-1} dry soil (DS). The AM colonization significantly enhanced plant growth and Ni content, especially at the lower soil Ni treatments. Furthermore, the AM plants exposed to the highest soil Ni level of 400 mg Ni kg^{-1} DS had a significantly higher shoot Ni extracted percentage than non-AM plants, suggesting that the AM symbiosis contributed to Ni uptake, then its translocation from roots to shoots. The AM colonization also significantly increased the GS activity in roots, this being likely an indicator of an enhanced Ni tolerance. These findings support the hypothesis that AM symbiosis contributes to an enhanced Ni plant uptake and tolerance and should be considered as part of phytoremediation strategies.

Keywords Arbuscular mycorrhiza · *Glomus intraradices* · *Helianthus annuus* · Metal · Phytoremediation

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Introduction

Nickel (Ni), an essential micronutrient in plants in the range of 0.5–1.0 mg g^{-1} dry mass (DM; Marschner 1995), causes in excess (>5 mg g^{-1} DM) severe physiological disturbances such as root growth inhibition and leaf interveinal chlorosis (Krämer et al. 1997), or endoplasmic reticulum swelling and chloroplastic starch accumulation (Slivinskaya 1991). By contrast, hyperaccumulating plant species, such as *Thlaspi montanum* (Reeves et al. 1983) and *Sebertia acuminata* (Sagner et al. 1998), possess a specialized physiology in order to cope with toxic concentrations (Krämer et al. 2000; McGrath et al. 2001). In many hyperaccumulators, Ni is stored in vacuoles or transported through the cytoplasm by complexing with organic acids such as malate and citrate or amino acids such as histidine and glutamine (Bhatia et al. 2005; Krämer et al. 2000). Saber et al. (1999) who reported increases in the content of malate and citrate in sunflower seedlings after exposure to Al, Cd, and Zn suggest a prominent role of these compounds in toxic metal plant tolerance.

The potential of the hyperaccumulator plant species in metal remediation is, however, limited by a number of factors such as a slow growth rate and a low biomass (Cunningham et al. 1995; Reeves and Baker 2000). Thus, high biomass plant species, like tobacco or sunflower, appear to be good candidates for phytoremediation purposes (Audet and Charest 2006; Davies et al. 2002). Several other studies involving sunflower as a phytoremediator determined its effectiveness with a number of toxic metals such as Cd, Cr, Se, Al, Zn, and Pb (Davies et al. 2002; Kastori et al. 1996; Saber et al. 1999; Turgut et al. 2004; Ximénez-Embún et al. 2004; Zornoza et al. 1999).

The use of arbuscular mycorrhizal (AM) fungi was proposed (Gildon and Tinker 1983) in remediation strategy

to alleviate plant stresses as the extraradical hyphae not only increase the uptake of but also bind to metals (Joner et al. 2000), thereby decreasing their bioavailability (Audet and Charest 2007, 2008). In the present study, we hypothesized that AM colonization increases the uptake of and tolerance to Ni in sunflower. To test this hypothesis, we studied the effect of AM colonization in sunflower plants grown in a greenhouse under increasing soil Ni concentrations and determined the biomass, mineral concentrations, and glutamine synthetase (GS) activity. Results from this study will help to advance knowledge on the impact of AM fungal association with Ni phytoremediator species such as sunflower.

Materials and methods

Growth conditions

A greenhouse experiment was performed using a factorial block design (1 plant sp.×2 M×4 Ni×8 reps) with plants of sunflower, *Helianthus annuus* L. cv. Lemon Queen (OSC Seeds, Waterloo, ON, Canada), grown without or with the AM fungal inoculum, *Glomus intraradices* Schenck & Smith (DAOM 181602; Mycorhize Pro, Premier Tech, Rivière-du-Loup, QC, Canada) in soil treated with 0, 100, 200, or 400 mg Ni kg^{-1} dry soil (DS), applied once as $NiCl_2{\cdot}6H_2O$, 56 days after sowing (AS). Seeds were previously surface-sterilized twice with 25% commercial NaOCl for 5 min, rinsed with distilled H_2O and sown (~12 mm deep) in a 1:1 (*v*/*v*) sand/soil mixture (pH 5.0, electrical conductivity (EC) 0.10 mS cm^{-1}, organic matter 6.12%, sand 95%, silt <1%, clay 4%, N-NO3 4 ppm, total Kjeldahl nitrogen 0.12%, total organic carbon 2.67%, P 7 ppm, K 35 ppm, Mg 109 ppm, Ni 8 μg g^{-1}, Ca 4,100 μg g^{-1}, Na 697 μg g^{-1}, Cu 10 μg g^{-1}, Fe 7,160 μg g^{-1}, Mn 137 μg g^{-1}, Mo<1 μg g^{-1}, Zn 22 μg g^{-1}), previously autoclaved for 20 min at 121°C and 15-psi. Plots (7.6 L) were filled with 1/3 of this mixture, 1/3 of a dry substrate containing 15 AM propagules g^{-1}, or a same substrate without any propagules, and topped with 1/3 of soil mixture (total 3.74 kg DS). Plants were thinned to one seedling per pot 7 days AS and grown in the greenhouse at 24/20°C (day/night), 43% relative humidity, and a 16-h photoperiod under high-pressure sodium lamps (PL Light Systems, Beamsville, ON, Canada), with an average light intensity of 175 mmol s^{-1} $m^{-2}mA^{-1}$ measured below the canopy cover. Plants were fertilized with a NH_4NO_3-type Long Ashton Nutrient Solution (Hewitt and Smith 1975), once a week (250 mL) for 2 weeks, then biweekly (500 mL) for 6 weeks. Then, all the plants were harvested after 10 weeks of growth. Roots from three replicates were separated from shoots and used for the determination of AM root colonization. Leaves and flowers were separated from stems, and each plant organ was individually weighed for fresh mass, then oven-dried at 70°C for 72 h, for dry mass determination. The flowers, leaves, stems, and roots from the remaining five replicates were shock-frozen in liquid nitrogen and freeze-dried for 72 h, at −50°C and 3.7×10^{-1} mbar (Uni-trap Model 10–100, Virtis Inc., Gardiner, NY, USA), and used for the analysis of glutamine synthetase activity.

Root colonization

The AM root colonization was determined according to Dalpé (1993). Three replicates per treatment and 50 1-cm root sections (ten per slide) from each replicate were observed under compound microscope at ×100 and ×400. Mycorrhizal colonization was estimated as the percentage of the total root segments containing at least one of the AM fungal structures (e.g., arbuscules, hyphae, spores, or vesicles).

GS activity (EC 6.3.1.2)

Freeze-dried roots or leaves (200 mg) were ground with mortar and pestle, over ice, with sand, 2% polyvinyl pyrrolidone, and 10 mL of extraction buffer, pH 8.0, containing 25 mM Tris-HCl, 1 mM EDTA-diNa salt, 1 mM dithiothreitol, 1 mM reduced glutathione, 10 mM $MgSO_4$, 5 mM glutamate, and 0.01% Triton. After centrifugation at 12,100×g for 25 min at 4°C, the supernatants were used for the analysis of GS activity, expressed as γ-glutamylhydroxamate (GH) produced per gram per hour and determined by the synthetase assay according to Toussaint et al. (2004).

Mineral analyses

Mineral elements (P, K, Ca, Mg, Mn, Cu, Zn, Al, Fe, and Mo) extracted from a concentrated H_2SO_4 solution and Ni extracted from a concentrated HNO_3 and 30% H_2O_2 solution were analyzed via atomic plasma emission spectroscopy in the "Laboratoire de Chimie Organique et Inorganique, Direction de la Recherche Forestière", MRN, QC, Canada. The relative extracted Ni percentage was determined by dividing the total Ni content in organ or plant tissue by the total Ni added to the soil and multiplying by 100 (Audet and Charest 2006).

Statistical analyses

Two-way analyses of variance (ANOVAs) were performed on all parameters using S-Plus (S-Plus 6.2, Insightful Corp.

2003), and 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 the assumptions were not met, nonparametric Kruskal–Wallis analyses were done. Differences were considered significant when their probability by chance alone was less than 0.05. Numerical differences with probability between 0.05 and 0.1 are presented for their biological meaning. When this occurs, the *P* values are given.

Results

Mycorrhizal colonization

Sunflower roots were colonized by 78%, 76%, 64%, and 64% at 0, 100, 200, and 400 mg kg^{-1} DS, respectively.

Growth parameters

The effects of AM ($P \leq 0.05$) and soil Ni ($P \leq 0.001$) treatments and their interaction ($P \leq 0.01$) were significant on dry mass which was higher in AM than non-AM plants at 0 and 100 soil Ni input (Fig. 1).

Nickel content and extracted percentage

The Ni content (Fig. 2) in all the plant organs was significantly ($P \leq 0.001$) increased with increasing soil Ni input. The AM treatment significantly increased the root ($P \leq 0.05$) and total ($P \leq 0.001$) Ni content by three- and twofold higher, respectively, at 100 soil Ni. Although not significant, the AM treatment marginally ($P=0.065$) increased the leaf Ni content by 1.4 times at the 400 soil Ni input. In addition, the AM treatment significantly increased the Ni extracted percentage in roots ($P \leq 0.01$) and whole

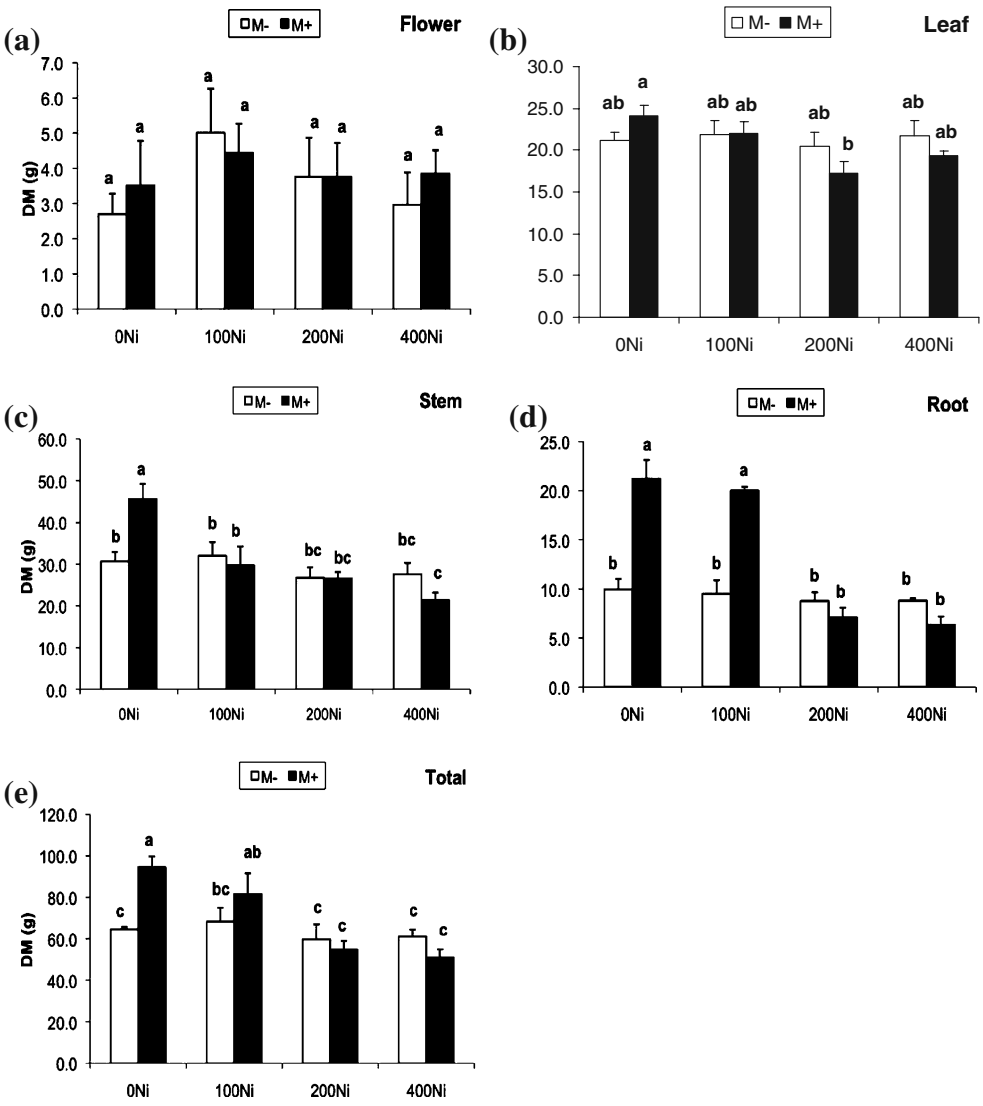

Fig. 1 Dry mass (*DM*) means ($n=5$ to 8) and SEs for flower (**a**), leaf (**b**), stem (**c**), root (**d**), and total plant (**e**) of sunflower with (M+) or without (M−) AM treatment in Ni (0, 100, 200, and 400 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for each organ or plant. *Different letters* refer to significant differences according to Tukey's test at $P<0.05$

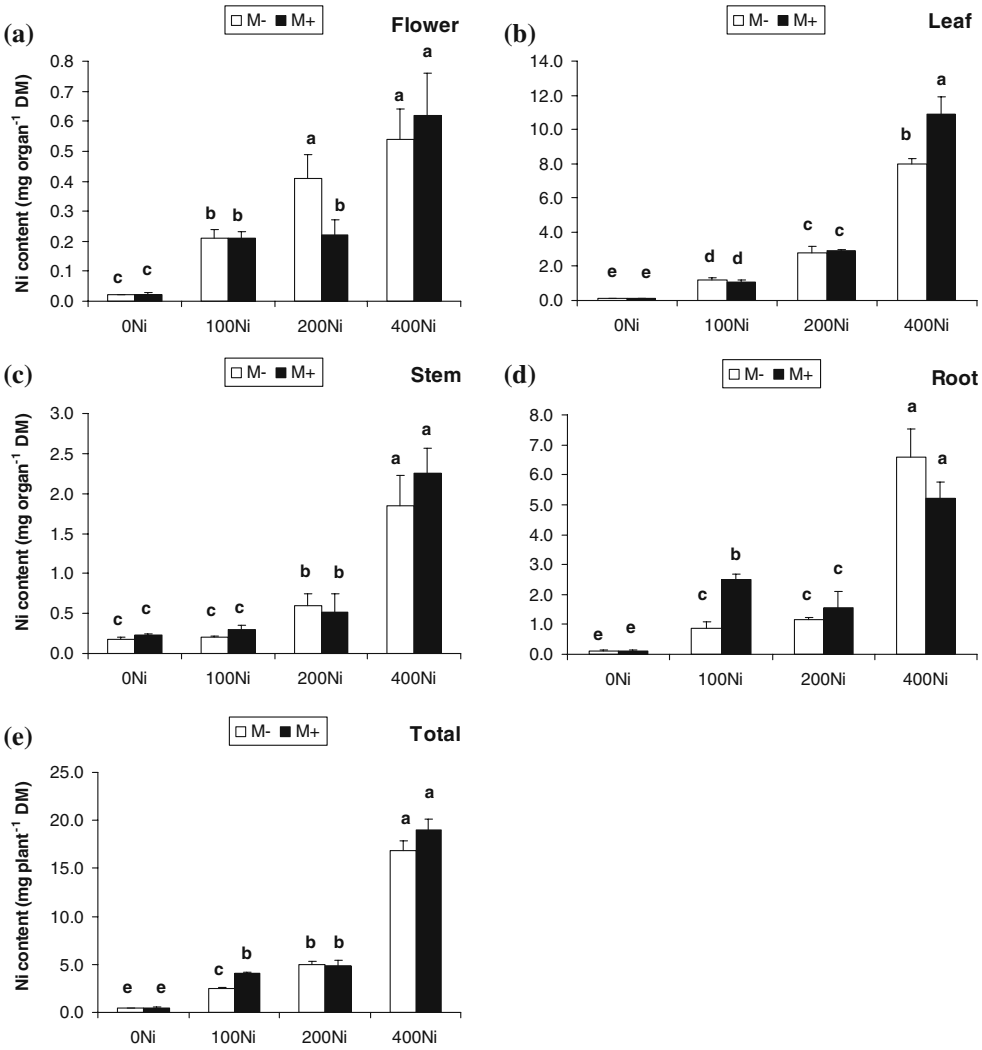

Fig. 2 Ni content (milligrams Ni per organ or per plant DM) means (n=3 to 4) and SEs in flower (**a**), leaf (**b**), stem (**c**), root (**d**), and total plant (**e**) of sunflower with (M+) or without (M−) AM treatment in Ni (0, 100, 200, and 400 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for each organ or plant. *Different letters* refer to significant differences according to Tukey's test at P<0.05

plants (P≤0.001) at 100 Ni and in shoots (P≤0.05) at 400 Ni by 3, 1.6, and 1.3 times, respectively (Fig. 3).

Mineral concentrations

In shoots (Tables 1 and 3), the AM treatment led to significant effects on the concentrations of Ca, Mg, and Mn (P≤0.05) and Cu (P≤0.001) and a marginally nonsignificant effect (P=0.079) on Zn. The soil Ni treatment had also significant effects on the concentrations of Ca (P≤0.05) and Cu (P≤0.001) and marginally nonsignificant effects on Mg (P=0.099), Mn (P=0.051), and Fe (P=0.062) in shoots.

In roots (Tables 2 and 3), the AM treatment had significant effects on the concentrations of P, K, Cu, and Fe (P≤0.05), Mn (P≤0.01), and Ca, Zn, and Al (P≤0.001). The soil Ni input also had a significant effect on the concentration of Ca (P≤0.01) and marginally nonsignificant effects on Zn (P=0.087) and Al (P=0.098). There were also significant or marginally nonsignificant interactions on the concentrations of Al (P≤0.05) and Ca (P=0.095). At 0 soil Ni input, the Cu concentration was twofold higher in AM than non-AM plants. The Ca and Al concentrations were also significantly (P≤0.001) higher in AM than non-AM plants at 100 soil Ni by 1.4 and 3.4 times, respectively. The Zn concentrations were significantly (P≤0.001) higher in AM than non-AM plants by 1.6 to 2.8 times at all soil Ni levels. 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.

Glutamine synthetase

The AM (P≤0.05) and soil Ni (P≤0.001) effects and their interaction (P≤0.01) were significant on GS activity in the leaves where (Fig. 4a) it increased with increasing soil Ni regardless the AM treatment. However, with the AM treatment, the leaf GS activity was significantly twofold higher at 200 and 400 Ni than at 0 and 100 soil Ni. In roots (Fig. 4b), the AM treatment (P≤0.001) and its interaction with soil Ni input (P≤0.01) were also significant on GS

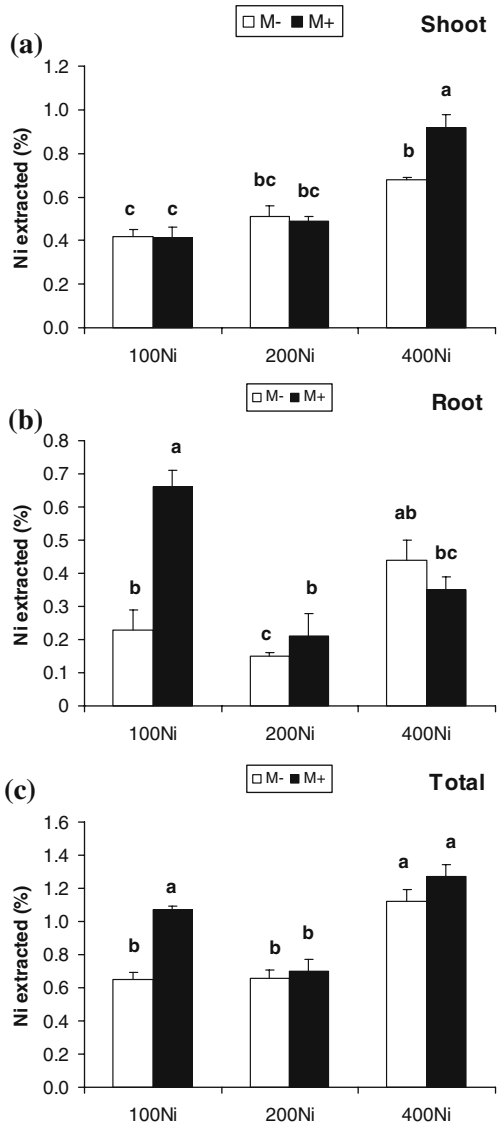

Fig. 3 Ni extracted percentage means (n=3) and SEs in flower (**a**), leaf (**b**), stem (**c**), root (**d**), and total plant (**e**) of sunflower with (M+) or without (M−) AM treatment in Ni (0, 100, 200, and 400 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for root, shoot, or total. *Different letters* refer to significant differences according to Tukey's test at P<0.05

activity. At 0 and 100 soil Ni, the GS activity was significantly higher in AM than non-AM roots by four and two times, respectively, and approximately the same at 200 and 400.

Discussion

In this study, the AM colonization of sunflower did contribute to Ni uptake as shown by a significant twofold increase in total Ni extracted percentage in AM than non-AM plants, of which 62% of the Ni content was found in

Table 1 Mineral concentration (milligrams per gram DM) means (n=3) and SEs in leaves of sunflower grown with (M+) or without (M−) AM treatment in soil Ni treatment (0, 100, 200, and 400 mg kg^{-1} DS)

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

Within a column, values not sharing the same letter indicate significant differences according to the Tukey's test at P<0.05. Aluminum data are not shown as concentrations were below detectable limit

Table 2 Mineral concentration (milligrams per gram DM) means (n=3) and SEs in roots of sunflower with (M+) or without (M−) AM treatment in soil Ni treatment (0, 100, 200, and 400 mg kg^{-1} DS)

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

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

AM roots as compared to 35% in non-AM roots. This may aid in plant tolerance as most of the Ni absorbed could be sequestered in roots thereby minimizing the disruption of biochemical processes in shoots. Our results agree with others which showed that AM fungi participate to soil remediation by sequestering toxic metals in fungal tissues, then decreasing their bioavailability (Audet and Charest 2007, 2008; Leyval et al. 1997). Other studies have reported enhanced uptake of Cu, Ni, Pb, and Zn in AM roots of the perennial grass (Killham and Firestone 1983) and of Zn in wild tobacco (Audet and Charest 2006). In our study, at the two higher soil Ni input of 200 and 400 mg, the Ni content was two to three times greater in shoots than roots, regardless of the AM factor. However, the Ni extracted percentage (0.92%) at 400 mg was significantly higher by 35% in the shoots of AM than non-AM plants. This suggests that a relatively high Ni proportion was transferred to the above-ground organs, a trait especially important in phytoextraction (Li et al. 2003). This Ni extracted percentage is quite substantial as most plants have less than 0.01% Ni under noncontaminated soils (Cunningham et al. 1995; Marschner 1995). For comparison, the hyperaccumulator species *Berkheya coddii* can accumulate in its leaves up to 4% Ni under noncontaminated soils (Li et al. 2003; Turnau and Mesjasz-Przybylowicz 2003) and *S. acuminata* up to 26% in dry latex (Sagner et al. 1998). Turnau and Mesjasz-Przybylowicz (2003) also reported higher Ni content in the AM (1.3%) than non-AM (0.5%) plants of *B. coddii*. Our results suggest that the greater Ni content into shoots was enhanced by the AM colonization in combination with the large biomass of sunflower and the growth dilution effect.

Decreased root growth has been found to be one of the first signs of metal toxicity (Ahonen-Jonnarth and Finlay 2001). This was also observed in our study, as root was the most affected organ in the two highest soil Ni input of 200 and 400 mg, resulting in decreased root and total plant mass and root/shoot ratio (data not shown). It was also reported in sunflower a decrease of total dry mass owing to a combination of Cd, Cr, and Ni and of root and stem dry mass due to Ni (Turgut et al. 2004; Zornoza et al. 1999). Yet, in the present study, the AM colonization significantly increased root mass at 0 and 100 mg soil Ni, resulting in higher total sunflower plant mass. This suggests that at low or moderate soil Ni input, the AM contribution to plant growth and tolerance is more prominent. This is also shown by higher concentrations of Ca, Mg, Mn, and Cu in leaves and of P, Ca, Mn, Zn, Cu, Fe, and Al in roots in AM than non-AM plants, thus indicating 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 that AM colonization enhanced the concentrations of Cu,

Table 3 ANOVA results for mineral concentrations in leaves and roots of sunflower according to the AM (M) and soil Ni treatments

	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×Ni	NS	NS	NS	NS	NS	NS	NS	NS	NS
Root									
M	*	*	***	NS	**	***	*	*	***
Ni	NS	NS	**	NS	NS	MN	NS	NS	MN
M×Ni	NS	NS	MN	NS	NS	NS	NS	NS	*

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

*$P\leq0.05$; **$P\leq0.01$; ***$P\leq0.001$

Zn, and Fe (Liu et al. 2000), P, Cu, and Zn (Chen et al. 2001), and P, K, Al, and Fe (Davies et al. 2002) in maize, red clover, and sunflower, respectively. It is likely that the AM association contributed to an enhanced mineral acquisition in order to alleviate Ni toxicity. Jones and Hutchinson (1988) concluded that the high root Ni content in mycorrhizal birch seedlings, correlated with increased P content, suggests a detoxifying mechanism through polyphosphate-Ni binding. This may be the case in our study since P concentrations were higher in AM than non-AM plants. Furthermore, we observed that the concentrations of Cu in the leaves of AM plants increased with increasing soil Ni, especially at the highest soil input of 400 mg, with similar trends for Ca, Mg, Mn, and Fe. At 100 mg soil Ni, the concentrations of Ca, Zn, and Al were all significantly higher in roots of AM than non-AM plants. Similar results were reported by Yang et al. (1985) in which Ni correlated positively with Co, Cr, Mn, Na, and Zn in leaves of hyperaccumulator plant species among the Flacourtiaceae family.

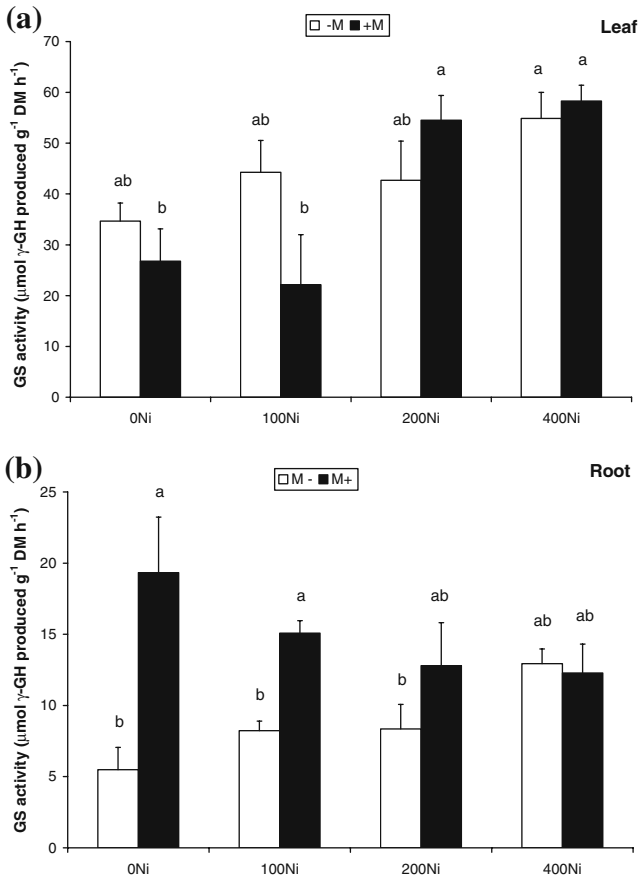

Fig. 4 Glutamine synthetase (*GS*) activity (micromoles γ-GH produced per gram DM per hour) in sunflower leaf (**a**) and root (**b**) with (M+) or without (M−) AM treatment in Ni (0, 100, 200, and 400 mg kg^{-1} DS) soil treatments. Statistical analyses were done separately for leaf and root. Means (n=4 to 5) and SEs are shown and *different letters* refer to significant differences according to Tukey's test at $P<0.05$

The AM symbiosis also enhanced N metabolism as shown by higher GS activity in AM than non-AM roots from 0 to 100 mg soil Ni input. An enhanced activity of glutamine synthesis may be important for Ni complexation as shown by Bhatia et al. (2005) who reported that glutamine is the dominant amide in the xylem sap of the Ni hyperaccumulator *Stackhousia tryonii*. In many hyperaccumulators, Ni has also been found chelated with malate or citrate (Bhatia et al. 2005), or histidine (Krämer et al. 2000), or stored in vacuoles; this indicates that metal is transported through the cytoplasm (Bhatia et al. 2005; Leopold et al. 1999). This ability to bind nickel allows protection to the cytosol and organelles as it results in decreased free ion activity (Baker 1981; Bhatia et al. 2005; Leopold et al. 1999). The increased GS activity, along with the increased biomass and mineral concentrations we detected in AM plants, suggests that AM fungi contribute to enhanced sunflower tolerance observed even at the greatest soil Ni levels and may have afforded the increased Ni uptake we noted.

In summary, our study showed that AM colonization not only augment plant Ni uptake in sunflower as observed by the increased Ni extracted percentage in shoots but also contribute to alleviate plant metal toxicity by enhancing plant growth through mineral acquisition and possibly by chelating with glutamine as observed by the enhanced GS activity. These findings support the hypothesis that AM symbiosis contributes to enhanced Ni uptake and Ni plant

tolerance and can be effectively incorporated in phytoremediation strategies on a large-scale level, as the AM fungi also contribute to revegetation of degraded lands.

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