This is a post-print version of an article published in:

Audet, P., & Charest, C. (2006). Effects of AM colonization on "wild tobacco" plants grown in zinc-contaminated soil. *Mycorrhiza*, *16*, 277-283. doi: 10.1007/s00572-006-0045-x

The final publication is available at www.springerlink.com.

Effects of AM colonization on 'wild tobacco' plants grown in zinc-contaminated soil

by

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ABSTRACT

This greenhouse study aimed to determine the effect of colonization by the arbuscular mycorrhizal (AM) fungus (*Glomus intraradices* Schenck & Smith) on the 'wild' tobacco (*Nicotiana rustica* L. var. Azteca), under soil-zinc (Zn) conditions. Plants of *N. rustica* were grown in AM or non-AM inoculated substrate and subjected to four soil-[Zn] concentrations (0, 50, 100, and 250mg Zn kg^{-1} dry soil). The AM root colonization increased markedly from 14% to 81% with the increasing soil-[Zn] and the mycorrhizal structures were significantly more abundant at the highest soil-[Zn], suggesting that Zn may be involved directly or indirectly in AM root colonization. In addition, total Zn content or Zn concentrations in shoots and roots were shown to increase as soil-[Zn] increased in both AM and non-AM plants. As for the growth parameters studied, there were no significant differences between treatments despite the increase in Zn content or concentration. The AM roots subjected to the highest soil-[Zn] had a significant reduction by about 50% of total Zn content and Zn concentration compared to non-AM roots. The percent Zn extracted in shoots and roots decreased as soil-[Zn] increased, while the extracted-Zn % was lower in AM treatments for shoots at 100-[Zn] and roots at 50-[Zn]. Soil-pH was significantly lower in non-AM than AM treatments at the highest soil-[Zn]. In summary, AM plants (particularly roots) showed lower Zn content and concentration than non-AM plants. In this regard, the AM fungi have a protective role for the host host plant, thus playing an important role in soil-contaminated immobilization processes; and, therefore, be of value in phytoremediation, especially when heavy metals approach toxic levels in the soil.

Key words: *Glomus intraradices*, heavy metals, *Nicotiana rustica*, phytoremediation.

INTRODUCTION

Phytoremediation is defined as the use of plant systems to clean up contaminated environments (Meagher 2000). The ultimate goal of phytoremediation research, whether under laboratory or field conditions, is the restoration of polluted ecosystems, although it is equally important to examine the environmental fate and direct effects of contaminants on plant systems (Schutzendubel & Polle 2002). Contaminants reviewed thus far have been classified as either halogenated solvents, polycyclic aromatic hydrocarbons, or heavy metals. In particular, heavy metals have reached phytotoxic concentrations in some ecosystems as a result of metal galvanization, rubber vulcanization, and agricultural feed additives or fertilizers (Barceloux 1999, Wenger et al. 2002). Zinc (Zn), an essential micronutrient, has been shown to reach critical toxicity levels when leaf concentrations approach and/or surpass 400 mg kg^{-1} dry tissue, at which point leaf necrosis and ultimately plant death occurs (Chaney 1993, Marschner 1986). This is of concern considering that soil-Zn concentrations have been found beyond 1000 mg Zn kg^{-1} dry soil in some areas near industrial sites and agricultural fields.

Arbuscular mycorrhizal (AM) symbiosis is a very ancient interaction between plant roots and zygomycetes fungi (Morton & Benny 1990). It is believed that AM symbiosis occurs in over 90% of terrestrial plants; it significantly increases tolerance to drought, nutrient deficiency, cold or warm temperature, and, in some cases, heavy metal contamination (Charest et al. 1997, Davies et al. 2001, Paradis et al. 1995, Subramanian et al. 1997, Subramanian & Charest, 1998). Recent phytoremediation studies that incorporated the AM-variable have had mixed findings. Some studies have shown that AM colonization increases the uptake and accumulation of heavy metals in host plants (Davies et al. 2001, 2002; Hovsepyan & Greipsson 2004; Rufyikiri et al. 2002,

2003), while others suggested that AM fungi exude enzymes that participate in the immobilization process of soil contaminants, in which case accumulation in plants is reduced (Joner et al. 2000, Leyval et al. 1997, Weissenhorn et al. 1993).

The rationale of this study was that AM fungi might be beneficial for plants under excess-nutrient stress as they are known to help under various other stresses, including increased mineral uptake when essential nutrients are limiting. The 'wild' tobacco, *Nicotiana rustica* L. var. Azteca, was selected because species of the Solanaceae family, including tomato and tobacco cultivars, are known as phytoaccumulators (Wenger et al. 2002). The main objective was to evaluate the role of an AM fungus, *Glomus intraradices* Schenck & Smith, as well as the efficacy of 'wild' tobacco in the phytoremediation process. Our working hypotheses were that the AM root colonization in host plants leads 1) to their enhanced tolerance to increasing soil-[Zn], and 2) to an increased uptake and content in their tissues. The derived predictions were that physiological parameters (e.g. shoot height, shoot & root dry mass, and leaf soluble chlorophyll and protein concentrations) and Zn content would be greater in AM than non-AM plants.

MATERIALS & METHODS

Growth Conditions

The factorial block design (1 plant *sp*. x 2 AM x 4 Zn) used in this greenhouse experiment consisted of AM and non-AM colonized plants subjected to four soil-Zn concentrations (0, 50, 100, and 250 mg Zn kg^{-1} dry soil) with 7 blocks per treatment for a total of 56 plants (1 plant per pot). Plants of *Nicotiana rustica* L. var. Azteca were grown from seeds (Ethnogens Seeds, Wichita, KS, USA), for 10 weeks in a soil mixture (sand:potting soil, 1:1 v/v) (Table 1). This soil mixture was thoroughly homogenized with an industrial mixer, autoclaved (20 min at 121˚C), and allowed to cool at ambient temperature before potting. Half of the 7.5 L pots (AM) were inoculated with 3500 spore propagules of *Glomus intraradices* Schenck & Smith (Premier Tech) incorporated as a 3 cm-thick layer of inoculum substrate, whereas an equivalent volume of control substrate (not containing spores) was incorporated in the control pots (non-AM). The greenhouse conditions consisted of 22˚C: 35˚C (night:day), a 16-h photoperiod, and 40% relative humidity. The average light intensity measured at five different locations in the greenhouse with a light meter (LICOR LI-250A quantum sensor, Lincoln, NE, USA) was 364.2 µmol m^{-2} sec^{-1}. All the plants were watered as needed and fertilized bi-weekly (400 mL per week) from week 4 to week 10 using a modified Long-Ashton nutrient solution (K_2SO_4 0.348 g L^{-1}, $CaCl_2$ anhydride 0.444 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.368 g L^{-1}, $NaH_2PO_4 \cdot H_2O$ 0.208 g L^{-1}, NH_4NO_3 0.402 g L^{-1}, $MnSO_4 \cdot 4H_2O$ 2.25 mg L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.25 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.3 mg L^{-1}, H_3BO_3 3.0 mg L^{-1}, NaCl 5.0 mg L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.12 mg L^{-1}, and EDTA-Fe 5.7 ppm). A total of 0.84 mg Zn were added to each pot including the control treatments over the growth period due to fertilization. Care was taken in watering and fertilizing to avoid any nutrient leaching. The Zn

was added between the 7th and 8th week in 100 mL doses from a $ZnSO_4 \cdot 7H_2O$ stock solution (45 mmol L^{-1}) until the desired soil-[Zn] was reached; except for the 0-[Zn] control treatment, the 50-[Zn] was applied in one dose, the 100-[Zn] in two doses, and the 250-[Zn] in five doses.

Harvest

All the plants were harvested 10 weeks after seeding. The fresh plant tissues were measured separately as shoots and roots. All the roots were thoroughly cleaned with tap water. Plants from three blocks were randomly selected for the determination of percent AM root colonization. Plant tissues from the four remaining blocks were oven dried at 70˚C for 72h, then later weighed and sampled for mineral analyses. The soil pH measurements were done before and after harvesting; the pH was read in dH_2O with a 1: 2.5 soil:water ratio (Jackson 1973).

Leaf Protein & Chlorophyll Analyses

One gram of fresh leaf tissue from each replicate (3 per treatment) was used for soluble protein analysis according to Bradford (1976). Soluble proteins were extracted with 10 mL of a 25 mM Tris-HCl buffer solution (pH 8.0) and 2 mL 2% polyvinylpyrollidone (PVP) using a mortar and pestle on ice. The extracts were centrifuged at 13000 g for 20 min at 4˚C. The readings were done at 595nm and the protein concentrations measured from a BSA (bovine serum albumin, 99% protein) standard curve.

Chlorophyll concentrations from each replicate (3 per treatment) were determined according to Bruinsma (1963). One gram of fresh leaf tissue was submerged into 100 mL of 95% ethanol and kept in dark at room temperature until the tissue was discolored; the readings were taken at 649 nm and 665 nm.

Soil Mineral Analysis

Minerals extracted from a H_2SO_4 solution (e.g. B, Ca, Co, I, Mn, Mo, Na, Ni & Zn), $NaHCO_3$ solution (e.g. P), and $C_2H_7NO_2$ solution (e.g. K & Mg) for pre-contaminated soil were analysed via Atomic Absorbtion Spectrometry (Accutest laboratories Ltd, ON, Canada).

Plant Mineral Analyses

Minerals (e.g. Al, Ca, Cu, Fe, K, Mn, Mo, P & Zn) extracted from a H_2SO_4 solution for both dry shoots & roots (n=4) were analysed via Atomic Absorption Spectrometry ("Laboratoire de Chimie Organique et Inorganique, Direction de la recherche forestière", MRN, QC, Canada). The Zn content was calculated from the Zn concentration multiplied by the shoot and root dry mass, separately. The extracted Zn percentage (%) was determined by dividing the Zn content by the total Zn added to the soil and multiplying by 100. Although extracted Zn percentage for the 0-[Zn] treatments may be calculated using trace Zn concentration values for pre-contaminated soil, the 0-[Zn] treatment was not included in the statistical tests since values exceeded 100%.

Root Colonization

Roots were cleaned and stained using aniline blue 0.02% dye solution (6.78 mM aniline blue; 500 mL glycerol; 450 mL H_2Od; 50 mL 1% HCl) according to Dalpé (1993). Fifty 1-cm long root segments per replicate (n=3) were examined at x100 and x400 under a compound microscope for the presence of vesicles, arbuscules, or hyphae (and possibly spores). Mycorrhizal colonization was estimated as the percentage of the total root-segments containing vesicles, arbuscules, and hyphae individually, as well as the percentage of roots containing at least one of these AM fungal structures.

Statistical Analyses

One- and two-tailed parametric analyses of variance (ANOVA) were performed, coupled with Bonferonni and Tukey's studentized range tests. Kolmogorov-Schmirnoff and Levene's tests were used respectively to verify both normality and evenness of variability (Zar 1999). All of the p-values were determined using S-Plus 6.2 (Insightful Corp. 2003). A block design was used in order to reduce the statistical error of the tests attributed to the greenhouse variability.

RESULTS

There were no significant differences in shoot height, shoot and root dry masses (DM), or leaf chlorophyll and soluble protein concentrations observed among any of the treatments. Shoot and root DM ranged from 31.2 to 37.3 g and 11.2 to 13.9 g, respectively. Shoot height varied from 75 to 92 cm. Leaf protein and chlorophyll concentrations ranged from 2.45 to 2.92 mg g^{-1} fresh mass (FM) and 13.1 to 17.2 mg g^{-1} FM, respectively. No visual stress (e.g. leaf necrosis, chlorosis, wilting, or senescence) was observed over the course of the 10-week growth period. The soil pH decreased ranged from 5.83 to 4.97 as soil-[Zn] increased. Notably, the soil pH of the non-AM treatment at 250-[Zn] was significantly more acidic than for all other treatments.

The total Zn content and Zn concentrations significantly increased as soil-[Zn] increased, in the shoots (Fig.1a) and in the roots (Fig.1b) of both AM and non-AM plants. More specifically, the Zn content and Zn concentrations were significantly lower in the roots of AM (24.5 mg Zn per total root mass & 2.07 mg g^{-1} DM) than non-AM plants (43.1 & 3.13) at the highest soil-[Zn], respectively; notably, this was not observed in the shoots. In shoots, P & Mn concentration decreased as soil-[Zn] increased, while Mg concentration was lowest at the highest

soil-[Zn] (Table 2). In the roots, Al concentration was lower in the AM than non-AM treatment at the 100-[Zn], Mn was lower in AM than non-AM treatments at 100- and 250-[Zn], while Cu was the lowest in both AM and non-AM plants at 100- and 200-[Zn] (Table 3). Minerals tested but not mentioned showed no significant differences between the treatments in shoots & roots. The extracted-Zn percentage (%) significantly decreased in both shoots (from 18.5% to 6.57%) and roots (from 11.6% to 2.44%) as soil-[Zn] increased (Table 4). In the shoots, at 100-[Zn] the extracted-Zn % was significantly higher in non-AM than AM treatments. In the roots, at 50-[Zn] the extracted-Zn % was significantly higher in non-AM than AM treatments. The 250-[Zn] treatment showed the lowest extracted-Zn % in the AM roots.

Root AM colonization significantly increased from 14% to 81% as soil-[Zn] increased (Table 5). The hyphae (extra- or intraradical), arbuscules, and vesicles were all observed in the AM roots at all the Zn concentrations. Each of these mycorrhizal structures was significantly more abundant at 250-[Zn] than any other soil-[Zn] level.

DISCUSSION

Of particular interest in this study, the AM colonization level was observed to increase by five-fold when soil-Zn concentration was increased, thus suggesting that Zn affects directly the root colonization, and/or indirectly the mycorrhizospheric conditions. In other words, Zn may have caused stress in the host plant, in the AM fungus, or both, and therefore induced AM root colonization. To our knowledge, this is the first time that the effect of Zn contamination inducing AM root colonization has been shown. The increase in AM root colonization is likely the result of numerous factors including increases in soil-Zn concentration and the subsequent

decrease in soil pH. Rufyikiri et al. (2003), who assessed root- and hypha-induced substrate-pH modifications, suggested that roots lead to an acidification while mycelium to an alkalinization of the growth medium. This phenomenon may explain the significantly more acidic soil pH in non-AM than AM treatments at 250-[Zn]. Yet, the differences in soil pH could also be attributed to the metal-binding capacity of AM fungi despite some active pH modification by the hyphal exudates (Rufyikiri et al. 2003). Phosphorus-Zn interactions may also have contributed to the increase in root colonization subsequent to increasing soil-[Zn], especially considering the large body of literature pertaining to enhanced P-nutrition & plant growth linked to mycorrhizal colonization (Christie et al. 2004**). The fact that P concentration decreased as soil-[Zn] increased does agrees with the study of Shetty et al. (1995) who stated that Zn and P are mutually antagonistic; yet, no differences were found between AM and non-AM treatments to suggested a significant interaction. In our study, the significant increase in abundance of AM structures, especially hyphae and arbuscules, suggests that the plant-fungal symbiosis was the most active under the highest soil-Zn concentration. Thus, the host plant seemed to invest more in the AM symbiosis than in heavy metal sequestration under soil-Zn excess; this finding is interesting as lower total Zn content and Zn concentration were measured in the roots of AM than non-AM plants at the highest soil-[Zn]. Janoušková and Vosatká (2005) have recently reported increased colonization of *in vitro* carrot roots by *Glomus intraradices* when Cd concentration increased. Furthermore, these authors found a two-fold lower Cd concentration and a third lower total Cd content in AM than non-AM roots, both trends being found for Zn in the present study. Increased root colonization of clover by *G. mosseae* as Cd concentration increased has also been reported by Joner and Leyval (1997). It is possible that *G. intraradices* and *G. mosseae* are

tolerant to Zn and Cd soil excess, a property that has been described for Cd-tolerant *G. mosseae* found at heavy-metal polluted sites (Weissenhorn et al. 1993). A number of studies have reported that AM colonization can increase the uptake of Zn and other trace minerals by host plants (Davies 1987, Gildon & Tinker 1983); yet few studies have observed the induction of AM root colonization when Zn is in excess. Gildon & Tinker (1981) found a 35% colonization of *G. mosseae* in clover roots from a contaminated site. However, in a pot study, Gildon & Tinker (1983) did not observe any colonization of onion roots by *G. mosseae* at a soil-Zn concentration of 75 mg Zn kg^{-1} dry soil, that is a concentration three times lower than the highest soil-[Zn] used in the present study. These differences between field and greenhouse experiments may be attributed to differences in plant species, in plant species mycorrhizal dependency, in plant nutrient requirements, and/or in AM fungus strain susceptibility or resistance under heavy metal stress.

Our hypothesis that Zn uptake is more enhanced in AM than non-AM plants must be rejected on the basis that total Zn content and Zn concentration in roots at the highest soil-[Zn] level were lower in AM than non-AM plants. This is in agreement with the study of Bradley et al. (1981, 1982) who reported that plants of *Calluna vulgaris* L. grown in soil-Zn and -Cu excess showed significantly lower total Zn and Cu contents in the shoots of mycorrhizal than non-mycorrhizal plants. It was also shown that Zn immobilization in the fungal mycelium of *G. mosseae* and *G. versiforme* led to lower Zn uptake in clover plants; in fact, ten times higher Zn concentration was measured in the mycelium than in the host plant (Chen et al. 2001). As found in the present study, Li & Christie (2000) also observed lower Zn concentration in AM than non-AM plants, particularly as soil-Zn application rate increased. Accordingly, the mycorrhizal fungus may have immobilized soil contaminants and prevented these from being taken up by the

host plant, especially under increasingly toxic soil-Zn concentrations (Leyval et al. 1997, Weissenhorn et al. 1995). Recent molecular analyses have identified a plasma membrane Zn transporter from *Medicago truncatula*, and a putative Zn transporter from *G. intraradices* (Burleigh et al. 2003, González-Guerrero et al. 2005). The regulation of these transporters may be important in explaining the lower total Zn content and concentration in AM than non-AM roots found in this study.

Our results also showed that the extracted-Zn percentage (%) decreases as soil-Zn level increases, while the highest extraction % was less than 20% of the total Zn added. In our study, the shoots and roots of AM plants for some soil-[Zn] treatments showed lower extracted Zn % than non-AM plants, a decrease ranging from 25% to 50%. Notably, the lowest percent Zn extracted was found in the AM roots at 250-[Zn], corresponding to the highest level of root colonization. These same trends can also be extrapolated from other phytoremediation studies (Davies et al. 2001, Joner & Leyval 1997, Leyval et al. 1997, Rufyikiri et al. 2003, Wenger et al. 2000). Based on our Zn removal yield results, additional studies are needed to assess the effectiveness of phytoextraction in soil remediation processes. Despite this, it has been suggested that AM fungi may eliminate the bioavailability of otherwise toxic contaminants and consequently buffer the soil for plants and other microorganisms (Joner et al. 2000, Leyval et al. 1997). By contrast, Davies et al. (2001, 2002) and Rufyikiri et al. (2002, 2003) showed, respectively, colonization by *G. intraradices* enhanced uptake and accumulation of chromium in sunflower and of uranium in cultivated *in vitro* carrot roots by *G. intraradices.* Although AM fungi may enhance heavy metal phytoextraction for some plant species, our findings agree with the hypothesis of metal-binding AM fungal capacity as suggested by others (Chen et al. 2001, Joner et al. 2000, Leyval et al. 1997, Li & Christie 2000). In fact, it can be presumed that AM colonization benefits host plants by increasing the uptake of limiting trace minerals, and possibly

decreasing their uptake when in excess. Moreover, the results from all the physiological parameters studied indicate that both AM and non-AM 'wild' tobacco plants are equally tolerant to increasing soil Zn concentrations. Therefore, the hypothesis that tolerance to increasing soil-[Zn] is enhanced in AM than non-AM plants must be rejected. However, the lack of differences for physiological parameters may be accounted by the fact that the Zn was added late in the growth phase and that much growth had occurred prior to Zn stress. In this case, the increases in % AM colonization must have transpired as a rapid burst only after the addition of Zn. Also, it may well be that the Zn toxicity level is greater than the highest soil-Zn concentration used in this study. In this regard, Wenger et al. (2002) reported plant mass decreases in cultivated tobacco and maize when grown in 750 mg Zn kg^{-1} dry soil, a concentration three times higher than the highest concentration used in the present study. However, as Zn was applied to the soil at different growth phases & under different conditions, this limits the breadth of comparison with our study.

In summary, this study has contributed significant evidence with regard to the effects of an AM fungus on the 'wild' tobacco plants subjected to soil-[Zn] excess. Further investigation on the effects of heavy metals on AM fungi in interaction with host plants subjected to heavy metals will be of interest and should contribute to a better understanding of the mycorrhizospheric network.

ACKNOWLEDGMENTS

This research was funded by a grant from the Natural Sciences and Engineering Research Council

of Canada (NSERC) to C.C.

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Table 1. Soil composition & characteristics prior to Zn-contamination.

Parameter	
Total Kjedahl Nitrogen	0.12%
Total Organic Carbon	2.67%
Ca	4100 ug g^{-1}
Na	697 ug g^{-1}
Co	10 ug g^{-1}
I	7160 ug g^{-1}
Mn	137 ug g^{-1}
Mo	<1 ug g^{-1}
Ni	8 ug g^{-1}
Zn	22 ug g^{-1}
P	7 ppm
K	35 ppm
Mg	109 ppm
B	1.1 ppm
Organic Matter	6%
Sand (>0.050mm)	89%
Silt (0.002-0.050mm)	<1%
Clay (<0.002mm)	4%

Table 2. Mineral concentrations (mg.g^{-1} dry mass) in shoots of *N. rustica* under increasing soil-[Zn]. Means (n=4) and S.E. () are shown.

Zn (mg.kg^{-1} dry soil)	Inoculum	Zn	P	Mg	Mn
0	non-AM	0.10^{d} (0.01)	7.24^{a} (0.87)	8.10^{a} (0.83)	0.61^{a} (0.15)
	AM	0.12^{d} (0.0)	7.13^{a} (0.80)	9.88^{a} (1.03)	0.67^{a} (0.15)
50	non-AM	1.70^{b} (0.09)	6.51^{ab} (0.22)	8.88^{a} (0.38)	0.48^{ab} (0.18)
	AM	1.32^{b} (0.13)	6.09^{ab} (0.78)	9.43^{a} (0.79)	0.67^{a} (0.06)
100	non-AM	1.61^{b} (0.09)	5.64^{b} (0.65)	10.4^{a} (2.18)	0.63^{a} (0.15)
	AM	2.30^{b} (0.17)	6.68^{a} (0.35)	6.37^{b} (0.32)	0.31^{b} (0.08)
250	non-AM	3.41^{a} (0.41)	5.47^{b} (0.35)	6.55^{b} (0.58)	0.40^{b} (0.12)
	AM	3.20^{a} (0.17)	5.39^{b} (0.46)	6.38^{b} (0.59)	0.30^{b} (0.09)
F-values and level of significance	Zn	123***	3.56*	2.77ms	2.82ms
	Inoculum	0.05ns	0.05ns	0.48ns	0.27ns
	Zn X Inoculum	4.34***	0.60ns	3.15*	2.55ms
	Block	3.42ns	2.05ns	1.32ns	6.82ns

'ns' not significant; 'ms' marginally significant ($p<0.1$); *$p<0.05$; ***$p<0.001$; different letters in columns designate treatments that are significantly different according to Bonferonni test.

Table 3. Mineral concentrations (mg.g^{-1} dry mass) in roots of *N. rustica* under increasing soil-[Zn]. Means (n=4) and S.E. () are shown.

Zn (mg.kg^{-1} dry soil)	Inoculum	Zn	Mn	Cu	Al
0	non-AM	0.12^{d} (0.02)	0.25^{a} (0.03)	0.05^{a} (0.05)	0.73^{a} (0.2)
	AM	0.38^{d} (0.25)	0.28^{a} (0.04)	0.05^{ab} (0.005)	0.63^{a} (0.17)
50	non-AM	1.71^{bc} (0.20)	0.21^{a} (0.03)	0.05^{a} (0.003)	0.93^{a} (0.33)
	AM	0.98^{cd} (0.27)	0.20^{a} (0.02)	0.04^{ab} (0.002)	0.55^{ab} (0.13)
100	non-AM	1.84^{b} (0.18)	0.20^{a} (0.04)	0.03^{ab} (0.003)	1.23^{a} (0.58)
	AM	2.23^{b} (0.25)	0.15^{ab} (0.04)	0.03^{b} (0.006)	0.23^{b} (0.09)
250	non-AM	3.13^{a} (0.16)	0.20^{a} (0.04)	0.03^{b} (0.003)	0.95^{a} (0.41)
	AM	2.07^{b} (0.20)	0.11^{b} (0.02)	0.03^{b} (0.00)	0.45^{ab} (0.13)
F-values and level of significance	Zn	43.6***	3.42*	9.2***	43***
	Inoculum	2.47ns	1.92ns	3.34ms	2.47ns
	Zn X Inoculum	4.59**	1.83ns	1.23ns	4.59*
	Block	4.59ns	2.28ns	0.56ns	0.41ns

'ns' not significant; 'ms' marginally significant; *p<0.05; **p<0.01; ***p<0.001; different letters in columns designate treatments that are significantly different according to Bonferonni test.

Table 4. Percent Zn extracted from total Zn added in shoots and roots of *N. rustica* under increasing soil-[Zn]. Percent values (n=4) and S.E. () are shown.

Zn ($mg.kg^{-1}$ dry soil)	Inoculum	Shoots	Roots
50	non-AM	14.7^{ab} (1.55)	11.6^{a} (2.36)
	AM	18.5^{a} (0.65)	5.98^{b} (1.60)
100	non-AM	12.2^{b} (1.10)	5.20^{b} (0.73)
	AM	8.05^{c} (0.43)	6.48^{b} (0.63)
250	non-AM	6.57^{c} (0.73)	4.31^{bc} (0.42)
	AM	6.75^{c} (0.48)	2.44^{c} (0.22)
F-values and level of significance	Zn	79.8***	10.0***
	Inoculum	3.99*	4.36*
	Zn X Inoculum	12.0***	4.07*
	Block	2.75ns	1.30ns

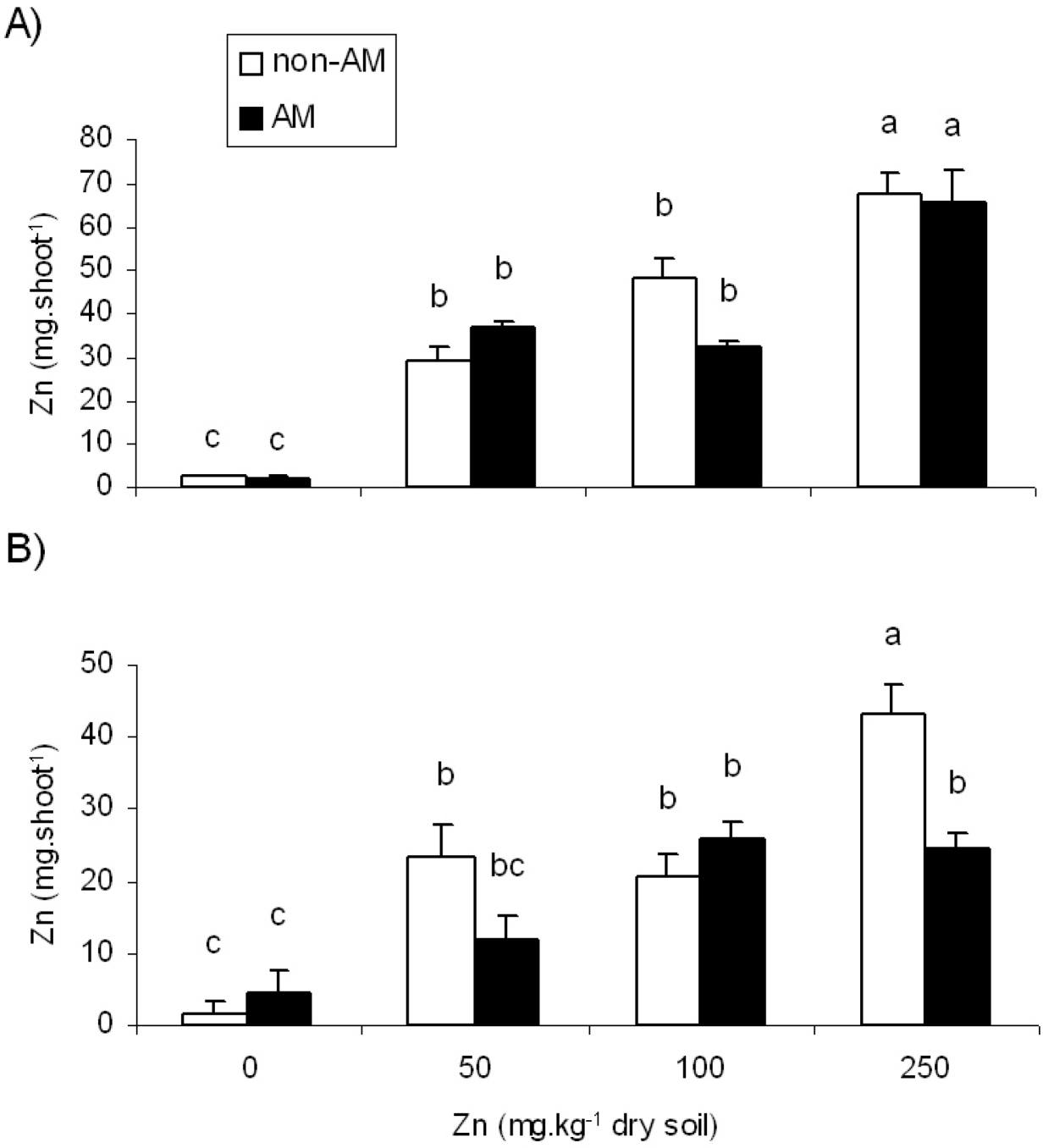
'ns' not significant; *$p<0.05$; ***$p<0.001$; different letters in columns designate treatments that are significantly different according to Bonferonni test.

Table 5. Percent of total roots colonized, arbuscules, vesicles, and hyphae in *N. rustica* under increasing soil-[Zn]. The percent values and S.E. () are indicated and represent the number of roots having AM root structures in relation to the total number of roots sampled x100 per treatment (n=3).

Zn ($mg.kg^{-1}$ dry soil)		Total Roots	Arbuscules	Vesicles	Hyphae
0		14.2^{c} (3.7)	6.66^{b} (4.2)	4.17^{b} (2.2)	10.0^{b} (0)
50		29.4^{b} (0.8)	15.0^{b} (5.2)	5.00^{b} (1.4)	15.8^{b} (5.5)
100		47.5^{b} (7.7)	13.6^{b} (3.3)	15.0^{b} (1.8)	23.0^{b} (4.4)
250		81.6^{a} (4.6)	58.9^{a} (3.9)	37.9^{a} (8.8)	74.2^{a} (12)
F-values and level of significance	Zn	62.3***	57.1***	53.6***	65.3***
	Block	1.11ns	0.62ns	2.81ns	0.87ns

'ns' not significant; ***$p<0.001$; different letters in columns designate treatments that are significantly different according to Bonferonni test.

Figure 1. Total Zn content (mg.plant^{-1}) in both shoots A) and roots B) of *N. rustica* under increasing soil-[Zn]. Means (n=4) and S.E. for non-AM (empty bars) and AM (solid bars) treatments are shown. Different letters designate treatments that are significantly different according to Bonferonni test ($p<0.05$).


A)
non-AM
AM
Zn (mg.shoot-1)
80
70
60
50
40
30
20
10
0
c
c
b
b
b
b
a
a
B)
Zn (mg.shoot-1)
50
40
30
20
10
0
c
c
b
bc
b
b
a
b
0
50
100
250
Zn (mg.kg-1 dry soil)