Zinc accumulation in *Solanum nigrum* is enhanced by different arbuscular mycorrhizal fungi

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Keywords: Heavy metal; Black nightshade; Arbuscular mycorrhizal fungi; Zinc; Phytoremediation; Phytoextraction

Abstract

Solanum nigrum was found to proliferate in sediments with high levels of metal pollution. The effect of Zn on plant growth and tissue metal accumulation was assessed. The response of the plant to the inoculation with four different isolates of arbuscular mycorrhizal fungi (AMF) (*Glomus* sp. BEG140, *Glomus claroideum*, *Glomus mosseae* and *Glomus intraradices*) was studied. While the isolates of AMF did not have a significant ($P < 0.05$) influence on mycorrhizal colonisation, increasing Zn concentration to high levels (500 and 1000 mg kg^{-1}) induced significant ($P < 0.05$) decrease of the AMF colonisation. In general, the presence of AMF did not affect the growth and biomass of *S. nigrum* individuals. However, the level of metal in the matrix affected *S. nigrum* growth; plants grown at 100 mg kg^{-1} had significantly ($P < 0.05$) lower leaf, stem, root and total biomass than control ones and plants growing at 500 and 1000 mg kg^{-1} had the significantly ($P < 0.05$) lowest biomass. Plants inoculated with the AMF *G. claroideum* and *G. intraradices* presented significantly ($P < 0.05$) higher Zn accumulation in all plant tissues. In general, the stem tissues had the higher Zn content while the leaves registered the lowest values, which indicate a high translocation of the metal. AMF inoculation had no significant ($P < 0.05$) influence on the metal translocation within the plant. This study suggests that inoculation with the AMF *G. claroideum* or *G. intraradices*, can enhance the Zn accumulation in the tissues of *S. nigrum*, not affecting the plant translocation capacities.

Introduction

Excessive toxic metal levels in soils pose significant hazards to human and animal health as well as to the ecosystem in general (Blaylock and Huang, 2000). Deposition of heavy metal of anthropogenic sources has increased as a result of the industrial revolution, and it is necessary to remediate contaminated soils. This can be done by ex situ techniques, which require the removal of the polluted matrix for treatment, or by in situ methods, which remediate without the flaws of high cost and impact on the ecosystem. A growing, low cost, in situ remediation method is phytoremediation – the use of plants to remove, degrade or immobilize contaminants. Because the costs of growing a crop are minimal when compared to those of soil removal and replacement, the use of plants to remediate hazardous soils is seen as having great potential (Chaney et al., 1997).

Several studies have been conducted to identify plant species capable of accumulating heavy metals, such as zinc. *Sedum alfredii* (Ye et al., 2003), *Armeria maritima* and *Arabidopsis halleri* (Schwartz et al., 2001) and *Thlaspi caerulescens* (Whiting et al., 2001) are well known for their hyperaccumulating capacities. Plant species colonising contaminated soils are potential metal accumulators and may be very useful for phytoremediation strategies (Álvarez et al., 2003). *Solanum nigrum* (black nightshade) is a plant species indigenous to a metal polluted site in an industrialised area of Northern Portugal containing sediments with

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levels of metals above the limits established by EC Directive 86/278/EC, zinc being the main occurring metal (Atkins, 1999; Oliveira et al., 2001). Despite this contamination scenario, *S. nigrum* is present in a large amount and was found to contain up to 1130 mg Zn/kg tissue (Marques et al., 2003). The use of the roots of black nightshade for polychlorinated biphenyls degradation (Mackova et al., 1997) and copper and cadmium accumulation (Macek et al., 1994; Chen et al., 1996) has been reported. However, Zn accumulation studies with this plant are not known.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in most terrestrial ecosystem, forming symbiotic associations with the roots of the majority of plant species (Smith and Read, 1997). The fungal hyphae can extend into the soil and uptake large amounts of nutrients, including metals, to the host root (Marschner, 1998). AMF have been reported as valuable in the adaptation of plants to heavy metal contaminated environments. Citterio et al. (2005) reported enhanced growth and metal root to stem translocation on *Cannabis sativa* plants inoculated with the AMF *Glomus mosseae*; Chen et al. (2005) observed that a mixed AMF inoculum enhanced lead uptake and growth of *Kummerowia striata*, *Ixeris denticulate* and *Echinochloa crusgalli* var *mitis*; Hildebrandt et al. (1999) concluded that AMF improved the tolerance of *Viola calaminaria* to Zn and Pb in polluted soils; AMF have also been reported to enhance the uptake of Cd by *Trifolium subterraneum* (Joner and Leyval, 1997) and by *Pinus sylvestris* (Ahonen-Jonnarth and Finlay, 2001).

The aim of the present study was to assess the influence of Zn and of different AMF isolates on the growth of *S. nigrum*, and on metal uptake and accumulation.

Materials and methods

Preparation of sand

Sand (pH = 7, 0.5–1.0 mm, acquired from Areipor, Bucelas, Portugal) was immersed in 0.1 M HCl for 24 h, rinsed with deionised water, sterilised twice at 120 °C for 70 min and dried in a drying oven at 40 °C for 4 d. The sand was then amended with zinc as $ZnSO_4$. Solutions of the salt were prepared with deionised water and applied to the sand to obtain matrix concentrations of 0, 100, 500 and 1000 mg Zn/kg dry sand (mg kg^{-1}).

Preparation of mycorrhizal inocula

The four isolates of AMF used in this study have been isolated from heavy metal contaminated soils in central Europe and are in the AMF collection of the Department of Mycorrhizal Symbioses from the Institute of Botany, Academy of Sciences of the Czech Republic. Each of the four AMF isolates was individually grown in zeolite (clinoptilolite 1.0–2.5 mm, Chemko, Slovakia) for 12 months, prior to the beginning of the experiment, in multispore pot cultures with both *Zea mays* L. and *Trifolium pratense* L. as host plants, under the same greenhouse conditions. An inoculum suspension of each isolate was prepared by wet sieving (710 mm) 450 cm^3 of zeolite from pot cultures with deionised water to a final volume of 150 ml, and was used to inoculate six pots of each Zn treatment. Each pot of the mycorrhizal treatments received 10 ml of the inoculum suspension containing colonised root fragments, hyphae and spores. The suspension was pipetted at 2 cm below the sand matrix surface. Plants inoculated with the mixture of the four AMF received an inoculum suspension composed of equal parts of the four inocula. Pots from the control treatments received the same volume of the inoculum suspension autoclaved twice (121 °C for 25 min).

Experimental design

The experiment was a factorial design with four matrix Zn levels (0, 100, 500 and 1000 mg Zn/kg sand) and six mycorrhizal fungi treatments (no AMF, *Glomus* sp. BEG140, *Glomus claroideum*, *Glomus mosseae*, *Glomus intraradices* and a mixture of all the AMF isolates). Each treatment was replicated six times.

Solanum nigrum L. seeds were surface sterilised with 0.5% NaOCl for 10 min and were subsequently washed with sterilised water. Seeds were then germinated in non-contaminated sand in the greenhouse. After 3 weeks, three equally developed seedlings were transplanted into plastic pots containing 500 g of sterilised sand. Pots were randomised on the greenhouse, process that was repeated every three weeks. The plants were maintained in controlled conditions (12 h photoperiod, 450 μmol m^{-2} s^{-1} photosythetically active radiation, 18–38 °C temperature range, 16–71% relative humidity range), watered daily and supplemented with modified Hoagland solution containing 1/4 of the original P content and free of Zn (Hoagland and Arnon, 1950) once a week.

Sampling

The harvesting was made 6 weeks after the seedlings transplant for individuals grown at 500 and 1000 mg kg^{-1} and after 25 weeks for the remaining treatments. Plant roots were washed free of sand with deionised water. For assessing AMF colonisation, an adequate amount of fresh fine roots was collected from the plants sampled at each selected spot. Roots, stems and leaves were separated, oven dried at 70 °C for 2 d, after which biomass was determined by weighing the dried plant material.

Plant analyses

For assessing AMF colonisation fresh fine root samples were cut into approximately 1 cm pieces, heated in a pressure pan at 120 °C in 10% KOH and stained using an adaptation of the Phillips and Hayman (1970) protocol including a longer incubation in 2% HCl (Oliveira et al.,

2001). Stained root samples were examined microscopically to assess the percentage of mycorrhizal colonisation using the grid-line intersect method (Giovannetti and Mosse, 1980). For Zn content analysis, dried roots, stems and leaves were grinded and sieved to <1 mm. The resulting samples were then digested at high temperature (up to 205 °C) with a mixture of concentrated nitric, perchloric and sulphuric acids (40:4:1). Zinc content was determined using FA-AAS of the digests (Wallinga et al., 1989). BCR (Community Bureau of Reference) reference sample CRM 279 (sea lettuce) was analysed using the above described total zinc determination analytical method. The value obtained by FA-AAS (52.1 ± 1.1 mg kg^{-1}) confirmed the accuracy and precision of the method by comparison with the certified value (51.3 ± 1.2 mg kg^{-1}).

Statistical analysis

Statistical analysis was performed using the SPSS software program (SPSS Inc., Chicago, IL Version 12.0). The data were analysed by analysis of variance (ANOVA). To detect the statistical significance of differences ($P < 0.05$) between means, the Tukey test was performed.

Chemicals

The chemicals used were of analytical-grade and were obtained from Pronalab (liquid reagents), Merck (solid reagents) and Sigma (Trypan blue stain).

Results and discussion

Mycorrhizal colonisation

No AMF colonization was found in the non-inoculated control treatments. Few significant ($P < 0.05$) differences in colonisation were found when inoculation was made with different AMF for each Zn treatment, as revealed by one-way ANOVA applied to each Zn concentration in the sand (Table 1). Two-way ANOVA was performed for the dependent variable AMF colonisation versus the independent variables Zn concentration on the matrix and AMF isolate. The AMF species had no significant ($P < 0.05$) effect on the colonisation, with the percentage of colonisation registered for inoculated individuals being significantly ($P < 0.05$) different from the control but not between themselves. The metal concentration induced significant ($P < 0.05$) differences in the fungi colonisation. Percentage mycorrhizal colonisation was significantly ($P < 0.05$) higher in plants grown in the control matrix and the significantly ($P < 0.05$) lowest colonisations were registered for individuals grown in 1000 mg kg^{-1} contaminated sand, showing that high Zn concentrations reduced mycorrhizal colonisation in *S. nigrum*. Other studies have also shown that metals can have a negative effect on mycorrhizal colonisation. Chao and Wang (1990) reported a reduction on the AMF colonisation of *Zea mays* as a consequence of the addition of Zn, amongst other heavy metals. However, the effects of heavy metals on mycorrhizal colonisation can also be neutral – Diaz et al. (1996) reported no correlation between the presence of Zn and mycorrhizal colonisation of *Lygeum spartum* and *Anthyllis cytisoides* –, or positive – Hildebrandt et al. (1999) found higher AMF colonisation in plants of *Viola calaminaria* grown in strongly Zn contaminated soils.

Plant biomass

Few significant ($P < 0.05$) differences on plant biomass were found when inoculation was made with different AMF, for each Zn concentration in the matrix, as shown by one-way ANOVA (Table 2). Two-way ANOVA was performed for the dependent variable biomass (roots, stems, leaves and total plant) versus the independent variables Zn concentration on the matrix and AMF isolate. Inoculation with different AMF isolates affected significantly ($P < 0.05$) the biomass of the stems of *S. nigrum*, with few significant ($P < 0.05$) differences detected (Table 2). When *S. nigrum* was inoculated with *G.* sp. BEG140 or with *G. intraradices,* a significant ($P < 0.05$) difference was found in the biomass of the plant stems, translated in significant ($P < 0.05$) differences in the total biomass of the plant in some cases. However, in general, none of these showed significant ($P < 0.05$) differences from the non-inoculated control. Although the relationships between plants and AMF are generally reported as mutualistic

Table 1
Percentage of mycorrhizal colonisation (%) for each treatment

AMF	Zn (mg/kg)			
	0	100	500	1000
G. sp. BEG140	9.3 ± 0.8^{c}	9.1 ± 0.5^{bc}	4.9 ± 0.5^{bc}	4.2 ± 0.6^{ab}
G. claroideum	8.4 ± 0.6^{bc}	7 ± 1^{d}	7.1 ± 0.3^{a}	5.0 ± 0.4^{a}
G. mosseae	9.6 ± 0.4^{bc}	8.4 ± 0.4^{c}	5.7 ± 0.8^{b}	4.7 ± 0.8^{a}
G. intraradices	11 ± 2^{ab}	10.2 ± 0.6^{ab}	5.7 ± 0.6^{b}	3 ± 2^{ab}
Mixture	12 ± 2^{a}	10.5 ± 0.7^{a}	4.5 ± 0.6^{c}	3.5 ± 0.7^{b}

Results are expressed as percentual means ± SD ($n = 6$). Non-inoculated controls are not presented and were not considered for this statistical analysis. One way ANOVA was performed for each Zn concentration in the sand. Means in the same column with different letters are significantly different from each other ($P < 0.05$) according to the Tukey test.

Table 2
The effects of Zn concentration of the matrix and AMF species on *S. nigrum* biomass

Zn in the sand (mg kg^{-1})	AMF species	Zn (mg kg^{-1} dry tissue)							
		Leaves		Stems		Roots		Total	
0	No fungi	1.5797 ± 0.3195^{a}	($n = 6$)	3.4945 ± 1.0194^{ab}	($n = 6$)	2.2430 ± 1.0612^{a}	($n = 6$)	7.3172 ± 1.8881^{a}	($n = 6$)
	G. sp. BEG140	1.8965 ± 0.6186^{a}	($n = 6$)	3.7018 ± 1.4697^{ab}	($n = 6$)	2.0750 ± 0.4300^{a}	($n = 6$)	7.6734 ± 1.9718^{a}	($n = 6$)
	G. claroideum	1.9261 ± 0.4561^{a}	($n = 6$)	4.3592 ± 0.5679^{b}	($n = 6$)	2.4744 ± 0.6489^{a}	($n = 6$)	8.7597 ± 1.4441^{a}	($n = 6$)
	G. mosseae	1.6061 ± 0.4784^{a}	($n = 5$)	2.2599 ± 0.8883^{a}	($n = 5$)	2.3532 ± 0.9068^{a}	($n = 6$)	6.5362 ± 0.5726^{a}	($n = 5$)
	G. intraradices	2.0368 ± 0.3072^{a}	($n = 6$)	1.7235 ± 0.6276^{b}	($n = 6$)	2.4075 ± 0.7231^{a}	($n = 6$)	9.1678 ± 1.5337^{a}	($n = 6$)
	Mixture	1.6945 ± 0.3476^{a}	($n = 5$)	3.7041 ± 0.3130^{ab}	($n = 5$)	1.7915 ± 0.5219^{a}	($n = 5$)	7.1902 ± 0.7319^{a}	($n = 5$)
100	No fungi	0.8436 ± 0.2357^{a}	($n = 4$)	2.1119 ± 0.4496^{a}	($n = 4$)	1.3876 ± 0.1472^{a}	($n = 4$)	4.3431 ± 0.5243^{a}	($n = 4$)
	G. sp. BEG140	0.7101 ± 0.1861^{a}	($n = 4$)	0.3917 ± 0.2257^{b}	($n = 4$)	0.6266 ± 0.1883^{b}	($n = 4$)	1.7285 ± 0.5332^{b}	($n = 4$)
	G. claroideum	0.7299 ± 0.2640^{a}	($n = 4$)	0.5918 ± 0.2571^{b}	($n = 4$)	0.7861 ± 0.3318^{b}	($n = 4$)	2.1078 ± 0.6957^{b}	($n = 4$)
	G. mosseae	1.3262 ± 0.1059^{b}	($n = 5$)	2.5016 ± 0.1405^{a}	($n = 5$)	1.5801 ± 0.2322^{a}	($n = 4$)	5.3796 ± 0.1588^{a}	($n = 4$)
	G. intraradices	1.4148 ± 0.2368^{b}	($n = 4$)	2.3008 ± 0.2661^{a}	($n = 5$)	1.3037 ± 0.2301^{a}	($n = 4$)	5.0269 ± 0.7382^{a}	($n = 4$)
	Mixture	1.1117 ± 0.1753^{ab}	($n = 4$)	2.3158 ± 0.6570^{a}	($n = 5$)	1.3899 ± 0.1062^{c}	($n = 5$)	4.7725 ± 0.7755^{a}	($n = 4$)
500	No fungi	0.3781 ± 0.1115^{ab}	($n = 5$)	0.1974 ± 0.0093^{a}	($n = 6$)	0.2072 ± 0.0091^{a}	($n = 6$)	0.8208 ± 0.1350^{a}	($n = 6$)
	G. sp. BEG140	0.2543 ± 0.0692^{a}	($n = 6$)	0.1717 ± 0.0317^{ab}	($n = 6$)	0.1565 ± 0.0274^{b}	($n = 6$)	0.8191 ± 0.0912^{ab}	($n = 6$)
	G. claroideum	0.4486 ± 0.0851^{b}	($n = 6$)	0.1313 ± 0.0236^{b}	($n = 6$)	0.1591 ± 0.0326^{b}	($n = 6$)	0.6642 ± 0.0882^{ab}	($n = 6$)
	G. mosseae	0.3205 ± 0.0491^{ab}	($n = 6$)	0.1361 ± 0.0293^{b}	($n = 6$)	0.0955 ± 0.0035^{c}	($n = 6$)	0.5356 ± 0.0748^{b}	($n = 6$)
	G. intraradices	0.3969 ± 0.0685^{b}	($n = 6$)	0.1538 ± 0.0358^{ab}	($n = 6$)	0.1078 ± 0.0137^{c}	($n = 6$)	0.7133 ± 0.0652^{ab}	($n = 6$)
	Mixture	0.4062 ± 0.0781^{b}	($n = 6$)	0.1158 ± 0.0252^{b}	($n = 6$)	0.1046 ± 0.0093^{c}	($n = 6$)	0.6712 ± 0.0769^{ab}	($n = 6$)
1000	No fungi	0.4741 ± 0.0690^{ab}	($n = 6$)	0.2449 ± 0.0323^{a}	($n = 6$)	0.1763 ± 0.0314^{a}	($n = 6$)	0.8910 ± 0.0704^{ab}	($n = 6$)
	G. sp. BEG140	0.3929 ± 0.0752^{ab}	($n = 6$)	0.2039 ± 0.0670^{a}	($n = 6$)	0.1943 ± 0.0147^{a}	($n = 6$)	0.8442 ± 0.0866^{ab}	($n = 6$)
	G. claroideum	0.4900 ± 0.1191^{ab}	($n = 6$)	0.2623 ± 0.0404^{a}	($n = 6$)	0.1756 ± 0.0259^{a}	($n = 6$)	0.9165 ± 0.1831^{ab}	($n = 6$)
	G. mosseae	0.5175 ± 0.0480^{b}	($n = 6$)	0.3694 ± 0.0930^{a}	($n = 6$)	0.2004 ± 0.0881^{a}	($n = 6$)	1.1529 ± 0.0775^{a}	($n = 6$)
	G. intraradices	0.2389 ± 0.0988^{c}	($n = 6$)	0.1919 ± 0.0169^{a}	($n = 6$)	0.2187 ± 0.0639^{a}	($n = 6$)	0.6898 ± 0.0968^{b}	($n = 6$)
	Mixture	0.3617 ± 0.0954^{cb}	($n = 6$)	0.2010 ± 0.0318^{a}	($n = 6$)	0.1578 ± 0.0847^{a}	($n = 6$)	0.8398 ± 0.1628^{ab}	($n = 6$)
Zn in the sand (A)		$(F = 205)^{***}$		$(F = 620)^{***}$		$(F = 179)^{***}$		$(F = 340)^{***}$	
AMF species inoculated (B)		NS ($F = 1.45$)		$(F = 27.1)^{***}$		NS ($F = 1.69$)		$(F = 6.92)^{***}$	
A × B		NS ($F = 1.33$)		$(F = 15.1)^{***}$		$(F = 2.01)^{*}$		$(F = 5.22)^{***}$	

One-way ANOVA was performed for each Zn concentration in the sand. Means in the same concentration group with different letters are significantly different from each other ($P < 0.05$) according to the Tukey test.
Two-way ANOVA was performed to determine the influence of AMF and of Zn concentration in the matrix. The test results are shown with the test statistic and as: NS – non-significant at the level $P < 0.05$; * – significant at the level $P < 0.05$; ** – significant at the level $P < 0.01$; *** – significant at the level $P < 0.001$.

(Smith and Read, 1997), with some studies reporting enhanced plant growth under mycorrhizal colonisation (Chen et al., 2005), neutral or even negative plant responses have been observed (Johnson et al., 1997). One of the primary benefits of AMF in metal contaminated environments, related with improvements in plant growth, may be the maintenance of nutrient acquisition under metal exposure (Meharg and Cairney, 2000). Plant biomass reduction is generally observed in plants following Zn excess (Kochian, 1993), which was also found in our study, as increasing Zn concentration significantly reduced ($P < 0.05$) the biomass of all *S. nigrum* sections. Plants grown at 500 and 1000 mg kg^{-1} had the significantly ($P < 0.05$) lowest biomass for all plant sections. The fact that no significant ($P < 0.05$) differences in the biomass of the plants grown in the 500 and 1000 mg kg^{-1} Zn concentration were obtained may be due to the death of the individuals present in those pots (for this reason plants were harvested 6 weeks after transplanting). However, it should be stressed that these are levels present in a sand matrix, resulting in high availability of the metal to the plant.

Zn concentration in the plant

S. nigrum accumulated up to 1450, 3240 and 3810 mg Zn kg^{-1} in the leaves, stems and roots, respectively, with no visual toxicity signs. When exposed to higher concentrations, despite the toxicity observed, the plant was able to accumulate up to 20470, 49040 and 29010 mg Zn kg^{-1} in the leaves, stems and roots, respectively. AMF inoculation had an effect on the uptake of Zn by *S. nigrum* (Table 3). Significant ($P < 0.05$) differences in Zn accumulation were found in plants inoculated with AMF, as shown by one-way ANOVA applied to each Zn concentration in the sand. The inoculation with *G. intraradices* and *G. claroideum* led to significantly higher ($P < 0.05$) Zn accumulation in all plant tissues (Table 3). Two-way ANOVA was performed for the dependent variable Zn accumulation by *S. nigrum* versus the independent variables Zn concentration on the matrix and AMF isolate. All the AMF induced significantly ($P < 0.05$) higher Zn accumulations in the stem tissue, with the exception of *G.* sp. BEG140. Zn accumulation in the root and leaf

Table 3
The effects of Zn concentration of the matrix and AMF species on *S. nigrum* Zn accumulation

Zn in the sand (mg kg^{-1})	AMF species	Zn (mg kg^{-1} dry tissue)							
		Leaves		Stems		Roots		Total	
0	No fungi	26 ± 2^{a}	($n = 6$)	53 ± 6^{a}	($n = 6$)	117 ± 27^{ab}	($n = 6$)	66 ± 8^{a}	($n = 6$)
	G. sp. BEG140	26 ± 8^{a}	($n = 6$)	57 ± 11^{a}	($n = 6$)	139 ± 23^{ab}	($n = 6$)	71 ± 8^{a}	($n = 6$)
	G. claroideum	24 ± 3^{a}	($n = 6$)	60 ± 15^{a}	($n = 6$)	111 ± 8^{a}	($n = 6$)	66 ± 8^{a}	($n = 6$)
	G. mosseae	27 ± 10^{a}	($n = 5$)	53 ± 11^{a}	($n = 5$)	118 ± 10^{ab}	($n = 6$)	72 ± 10^{a}	($n = 5$)
	G. intraradices	28 ± 2^{a}	($n = 6$)	53 ± 15^{a}	($n = 6$)	144 ± 16^{b}	($n = 6$)	71 ± 10^{a}	($n = 6$)
	Mixture	25 ± 6^{a}	($n = 5$)	54 ± 4^{a}	($n = 5$)	126 ± 7^{ab}	($n = 5$)	65 ± 7^{a}	($n = 5$)
100	No fungi	1064 ± 87^{ab}	($n = 4$)	2105 ± 248^{a}	($n = 4$)	2670 ± 155^{a}	($n = 4$)	2077 ± 183^{a}	($n = 4$)
	G. sp. BEG140	1137 ± 181^{b}	($n = 4$)	2739 ± 194^{b}	($n = 4$)	2342 ± 202^{a}	($n = 4$)	1932 ± 68^{a}	($n = 4$)
	G. claroideum	1449 ± 61^{c}	($n = 4$)	3244 ± 33^{c}	($n = 4$)	3666 ± 122^{b}	($n = 4$)	2174 ± 292^{a}	($n = 4$)
	G. mosseae	837 ± 46^{d}	($n = 5$)	1932 ± 127^{a}	($n = 5$)	2362 ± 331^{a}	($n = 4$)	1862 ± 142^{a}	($n = 4$)
	G. intraradices	1417 ± 31^{c}	($n = 4$)	3134 ± 190^{bc}	($n = 5$)	3811 ± 114^{b}	($n = 4$)	2856 ± 121^{b}	($n = 4$)
	Mixture	880 ± 83^{ad}	($n = 4$)	1965 ± 65^{a}	($n = 5$)	2474 ± 344^{a}	($n = 5$)	1818 ± 76^{a}	($n = 4$)
500	No fungi	12030 ± 1278^{a}	($n = 5$)	18920 ± 1358^{a}	($n = 3$)	9880 ± 534^{a}	($n = 3$)	12770 ± 569^{a}	($n = 3$)
	G. sp. BEG140	14490 ± 1224^{b}	($n = 6$)	19620 ± 1410^{ab}	($n = 3$)	9280 ± 1063^{a}	($n = 3$)	14580 ± 1047^{ab}	($n = 3$)
	G. claroideum	20730 ± 2883^{c}	($n = 6$)	31560 ± 1183^{c}	($n = 3$)	15010 ± 1715^{c}	($n = 3$)	21500 ± 2094^{c}	($n = 3$)
	G. mosseae	11320 ± 845^{a}	($n = 6$)	22100 ± 1255^{ab}	($n = 3$)	11300 ± 981^{ab}	($n = 3$)	13550 ± 352^{ab}	($n = 3$)
	G. intraradices	18210 ± 1500^{c}	($n = 6$)	32920 ± 1918^{c}	($n = 3$)	13790 ± 902^{bc}	($n = 3$)	20990 ± 964^{c}	($n = 3$)
	Mixture	12620 ± 1191^{ab}	($n = 6$)	22200 ± 941^{b}	($n = 3$)	22200 ± 941^{d}	($n = 3$)	15830 ± 1159^{b}	($n = 3$)
1000	No fungi	14470 ± 1090^{ab}	($n = 6$)	31540 ± 1217^{a}	($n = 3$)	16440 ± 1665^{a}	($n = 3$)	18750 ± 814^{a}	($n = 3$)
	G. sp. BEG140	16360 ± 1072^{b}	($n = 6$)	30490 ± 1544^{a}	($n = 3$)	23390 ± 2921^{bc}	($n = 3$)	21250 ± 1498^{ab}	($n = 3$)
	G. claroideum	20470 ± 1829^{c}	($n = 6$)	49040 ± 2323^{b}	($n = 3$)	27620 ± 1598^{c}	($n = 3$)	30560 ± 1658^{c}	($n = 3$)
	G. mosseae	12910 ± 1331^{a}	($n = 6$)	39410 ± 1340^{c}	($n = 3$)	19130 ± 1736^{ab}	($n = 3$)	23960 ± 1137^{b}	($n = 3$)
	G. intraradices	20710 ± 1875^{c}	($n = 6$)	47710 ± 1618^{b}	($n = 3$)	29010 ± 1469^{c}	($n = 3$)	30740 ± 2085^{c}	($n = 3$)
	Mixture	13500 ± 1331^{a}	($n = 6$)	30740 ± 2144^{a}	($n = 3$)	17560 ± 1336^{a}	($n = 3$)	18480 ± 820^{a}	($n = 3$)
Zn in the sand (A)		$(F = 11085)^{***}$		$(F = 13343)^{***}$		$(F = 13731)^{***}$		$(F = 17592)^{***}$	
AMF species inoculated (B)		$(F = 48)^{***}$		$(F = 37)^{***}$		$(F = 50)^{***}$		$(F = 55)^{***}$	
A × B		$(F = 13)^{***}$		$(F = 5)^{***}$		$(F = 7)^{***}$		$(F = 8)^{***}$	

One-way ANOVA was performed for each Zn concentration in the sand. Means in the same concentration group with different letters are significantly different from each other ($P < 0.05$) according to the Tukey test.
Two-way ANOVA was performed to determine the influence of AMFs and of Zn concentration in the matrix. The test results are shown with the test statistic and as: NS – non-significant at the level $P < 0.05$; * – significant at the level $P < 0.05$; ** – significant at the level $P < 0.01$; *** – significant at the level $P < 0.001$.

tissues was significantly ($P < 0.05$) higher when *S. nigrum* was inoculated with *G. claroideum*, *G. intraradices* and the fungi mixture. The different reports found on literature suggest that AMF can help plants adapting to metal contaminated soils, either by excluding the metals or enhancing their uptake by the plant. Increasing Zn uptake by mycorrhizal *Trifolium repens* has been reported by Joner and Leyval (2001) and higher Zn accumulation was found on roots and stems of *Sorghum bicolour* plants grown in soil inoculated with AMF, including *G. intraradices* (Toller et al., 2005). However, Diaz et al. (1996) has shown a reduction in Zn accumulation in *G. mosseae* colonised *Zea mays* plants. In the present study, inoculation of *S. nigrum* with *G. claroideum* or *G. intraradices* led to higher Zn accumulation in all the different tissues of the plant.

The concentration of Zn in the sand matrix affected the Zn content in the tissues of *S. nigrum* (Table 3). Plants growing at 500 mg kg^{-1} had significantly ($P < 0.05$) higher Zn accumulation in root, stem, leaves and in the total than plants grown at the 100 mg kg^{-1} sand matrix (Table 3). The individuals grown in the 1000 mg kg^{-1} Zn sand matrix were those that registered the significantly ($P < 0.05$) highest accumulation in all plant sections, independently of the AMF treatment. The levels of Zn in the tissues of *S. nigrum*, with the exception of the individuals grown in control sand, are above what is considered as normal levels of Zn in plant tissues – 10–100 mg kg^{-1}, according to Frisberg et al. (1996) – and even higher than the normal values reported by Reeves and Baker (2000) for plants growing in metal-enriched soils (20–400 mg kg^{-1}). *S. nigrum* appears to have accumulating characteristics, according to the high Zn levels observed in its tissues. Nevertheless, it cannot fall into the category of the hyperaccumulators, as in the case of Zn an uptake of 1% without toxicity signs would be required (McGrath and Zhao, 2003) – in our study, at those levels, the plants were visually damaged and no further development occurred after transplanting (at 500 and 1000 mg kg^{-1} of Zn in the sand). However, the use of *S. nigrum* should not be disregarded from a phytoremediation approach of a metal contaminated site, mainly due to its high metal extraction and accumulation capacities, and its high translocation properties.

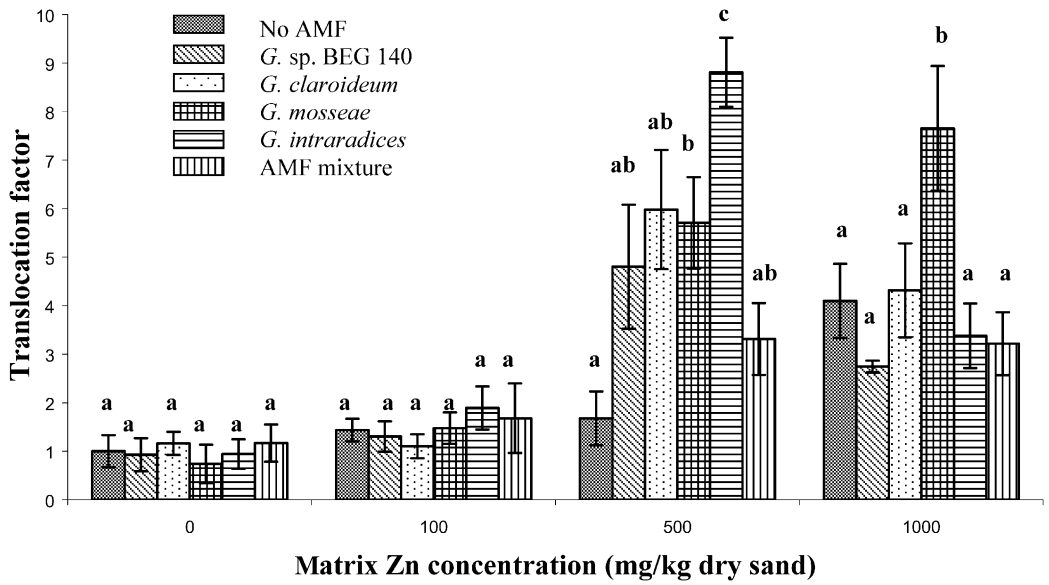

Fig. 1. Translocation factors of *S. nigrum* under different AMF and Zn matrix concentration (average values are shown). Bars upon the columns represent ±SD. One-way ANOVA was performed for each Zn concentration in the sand. Means in the same concentration group with different letters are significantly different from each other ($P < 0.05$) according to the Tukey test.

Root to above ground parts translocation

Root translocation to the aboveground, easily harvestable, parts of the plant is a key factor for the choice of a species as a metal accumulator – especially for phytoextraction purposes. A good candidate for a phytoremediation strategy would be a species that has a good translocation of the contaminant from the root to the stems and leaves, which means a translocation factor, i.e., a ratio between root and aboveground concentrations, higher than 1 (McGrath and Zhao, 2003). Nevertheless, this kind of model, including only the concentration, is not taking into account other determining issues, such as the biomass of the different plant parts (Rossi et al., 2002). The translocation factors obtained in the present study are presented in Fig. 1. These values were determined using the ratio {[(Zn concentration in the stem) * (stem biomass) + (Zn concentration in the leaves) * (leaves biomass)]/[(Zn concentration in the root) * (root biomass)]}. The high translocation factors obtained indicate that *S. nigrum* might be a good Zn phytoextractor, as the main metal accumulation is occurring in the aboveground part of the plant. The effect of the different AMF species on the translocation factor for each Zn concentration in the sand was analysed by one-way ANOVA. Significant ($P < 0.05$) differences between AMF treatments were only obtained at high Zn concentrations (500 and 1000 mg kg^{-1}) (Fig. 1). Two-way ANOVA was performed for the dependent variable translocation factor versus the independent variables Zn concentration on the matrix and AMF species. The presence or species of inoculated AMF had a significant ($P < 0.05$) influence on the translocation of Zn from the root to the aboveground parts of *S. nigrum*. The inoculation with *G.* sp. BEG140 led to significantly ($P < 0.05$) lower translocation than the inoculation with *G. mosseae* or *G. intraradices*. The data analysis also showed that the metal concentration induced significant ($P < 0.05$) differences between all Zn treatments, with higher translocations found in the following order of Zn matrix concentration: 500 > 1000 > 100 > 0 mg kg^{-1}. This may indicate that the tested AMF do not act as a zinc barrier in the roots of *S. nigrum* by binding heavy metals in roots, not restricting their normal translocation to stems (Dueck et al., 1986; Dehn and Schuepp, 1989). This is contrary to what was reported by Chen et al. (2005) for mycorrhizal *Kummerowia striata*, *Ixeris denticulate* and *Echinocchloa crusgalli,* which presented poor heavy metal translocation to stems due to a binding of heavy metals in the roots. Khan et al. (2000) also reported very little translocation of Zn absorbed by mycorrhizal seedlings of maize to the stems. Studies conducted at molecular and physiological levels have also shown that high translocation rates may be related with special Zn transporters. Pence et al. (2000) have found that these Zn transporters are expressed to much higher levels in both roots and shoots of the hyperaccumulator *Thlaspi caerulescens* than in the non-accumulator *Thlaspi arvense*, resulting in increased Zn uptake and translocation.

Conclusions

The identity of the isolates of AMF inoculated into the growth substrates of *S. nigrum* had no influence on the colonisation of the plant roots and higher Zn matrix concentration in the sand resulted in lower mycorrhizal colonisation in *S. nigrum*. In general, the presence of AMF did not influence the biomass of *S. nigrum* individuals, but increasingly Zn matrix concentrations resulted in a decrease of the biomass and an increase in Zn accumulation in the tissues of

S. nigrum plants. The inoculation with *G. claroideum* and *G. intraradices* enhanced Zn accumulation in the tissues of *S. nigrum*. The high translocation factors indicate that the main reservoir of the metal is in the aboveground part of the plants, pointing out *S. nigrum* as a potentially good zinc phytoextractor.

The use of *S. nigrum* inoculated with *G. claroideum* or *G. intraradices* appears as a good option for the decontamination of polluted soils with available Zn levels up to 100 mg kg^{-1} via metal phytoextraction, with no resulting apparent toxicity to the plant. At higher contamination levels, higher translocation rates may occur, but with the cost of poor plant biomass development and probable early death of the individuals.

Acknowledgement

The authors wish to thank Câmara Municipal de Estarreja for the provision of access to the site. This work was supported by Fundação para a Ciência e a Tecnologia and Fundo Social Europeu (III Quadro Comunitário de apoio), research grants of Ana Marques (SFRH/BD/7030/2001) and Rui Oliveira (SFRH/BD/1464/2000). Miroslav Vosátka is acknowledged for providing the mycorrhizal fungi inocula. This work was funded by Project MICOMETA – POCI/AMB/60131/2004 (Fundação para a Ciência e Tecnologia).

References

Ahonen-Jonnarth, U., Finlay, R.D., 2001. Effect of elevated nickel and cadmim on growth and nutrient uptake of mycorrhizal and non-mycorrhizal *Pinus silvestris* seedlings. Plant Soil 236, 128–138.

Álvarez, E., Fernández-Marcos, M.L., Vaamonde, C., Fernández-Sanjurjo, M.J., 2003. Heavy metals in the dump of an abandoned mine in Galicia (NW Spain) and in the spontaneously occurring vegetation. Sci. Total Environ. 313, 185–197.

Atkins, W.S., 1999. Estratégia de Redução dos Impactes Ambientais Associados aos Resíduos Industriais Depositados no CQE. Estudo de Impacte Ambiental (Environmental Impact Study). IPAMB. No. 595, Lisbon.

Blaylock, M.J., Huang, J.W., 2000. Phytoextraction of metals. In: Raskin, I., Ensley, B.D. (Eds.), Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment. John Wiley & Sons Inc., New York, USA, pp. 193–229.

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J.M., 1997. Phytoremediation of soil metals. Curr. Opin. Biotech. 8, 279–284.

Chao, C.C., Wang, Y.P., 1990. Effects of heavy metals on the infection of vesicular arbuscular mycorrhizae and the growth of maize. J. Agric. Assoc. China 152, 34–45.

Chen, J.P., Chen, W.R., Hsu, R.C., 1996. Biosorption of copper from aqueous solutions by plant root tissues. J. Ferment. Bioeng. 81, 458–463.

Chen, X., Wu, C.H, Tang, J.J., Hu, S.J., 2005. Arbuscular mycorrhizae enhance metal uptake and growth of host plants under a sand culture experiment. Chemosphere 60, 665–671.

Citterio, S., Prato, N., Fumagalli, P., Aina, R., Massa, N., Santagostino, A., Sgorbati, S., Berta, G., 2005. The arbuscular mycorrhizal fungus *Glomus mosseae* induces growth and metal accumulation changes in *Cannabis sativa* L. Chemosphere 59, 21–29.

Dehn, B., Schuepp, H., 1989. Influence of VA mycorrhizae on the uptake and distribution of heavy metals in plants. Agric. Ecosyst. Environ. 29, 79–83.

Diaz, G., Azconaguilar, C., Honrubia, M., 1996. Influence of arbuscular mycorrhizae on heavy metal (Zn and Pb) uptake and growth of *Lygeum spartum* and *Anthyllis cytisoides*. Plant Soil 180, 241–249.

Dueck, T.A., Visser, P., Ernst, W.H.O., Schat, H., 1986. Vesicular–arbuscular mycorrhizae decrease zinc toxicity to grasses growing in zinc polluted soil. Soil Biol. Biochem. 18, 331–333.

Frisberg, L., Nordeber, G.F., Kessler, E., Vouk, V.B., 1996, second ed. Handbook of the Toxicology of Metals, vols. I and II Elsevier Science Publishers, Amsterdam.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–500.

Hildebrandt, U., Kaldorf, M., Bothe, H., 1999. The zinc violet and its colonisation by arbuscular mycorrhizal fungi. J. Plant Physiol. 154, 709–717.

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Circular 347. Agricultural Experiment Station, University of California, Berkeley.

Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytol. 135, 575–585.

Joner, E.J., Leyval, C., 1997. Uptake of 109Cd by roots and hyphae of a *Glomus mosseae*/*Trifolim subterraneum* mycorrhiza from soil amended with high and low concentration of cadmium. New Phytol. 135, 353–360.

Joner, E.J., Leyval, C., 2001. Time-course of heavy metal uptake in maize and clover as affected by root density and different mycorrhizal inoculation regimes. Biol. Fert. Soils 33, 351–357.

Khan, A.G., Kuek, C., Chaudhry, T.M., Khoo, C.S., Hayes, W.J., 2000. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere 41, 197–207.

Kochian, L.V., 1993. Zinc absorption from hydroponic solutions by plant roots. In: Robson, A.D. (Ed.), Zinc in Soils and Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 45–57.

Macek, T., Kotrba, P., Suchova, M., Skacel, F., Demnerova, K., Ruml, T., 1994. Accumulation of cadmium by hairy root cultures of *Solanum nigrum*. Biotechnol. Lett. 16, 621–624.

Mackova, M., Macek, T., Ocenaskova, J., Burkhard, J., Demnerova, K., Pazlarova, J., 1997. Biodegradation of polychlorinated biphenyls by plant cells. Int. Biodeter. Biodegr. 39, 317–325.

Marques, A.P.G.C., Rangel, A.O.S.S., Castro P.M.L., 2003. The uptake of heavy metals in plant species endemic to a polluted stream near an industrialized region of Portugal. In: Neves, M., Neves, A.C.V. (Eds.) Proceedings of the International Symposium Environment 2010 – Situation and Perspectives for the European Union. Universidade do Porto – Faculdade de Engenharia, Porto, Portugal, pp. A01.

Marschner, H., 1998. Role of root growth, arbuscular mycorrhiza and root exudates for the efficiency in nutrient acquisition. Field Crops Res. 56, 203–207.

McGrath, S., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from contaminated soils. Curr. Opin. Biotech. 14, 227–282.

Meharg, A.A., Cairney, J.W.G., 2000. Co-evolution of mycorrhizal simbionts and their hosts to metal contaminated environments. Adv. Ecol. Res. 30, 69–112.

Oliveira, R.S., Dodd, J.C., Castro, P.M.L., 2001. The mycorrhizal status of *Phragmites australis* in several polluted soils and sediments of an industrialized region of Northern Portugal. Mycorrhiza 10, 241–247.

Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L.D., Lasat, M.M., Garvin, D.F., Eide, D., Kochian, L.V., 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caeruluescens*. PNAS 97, 4956–4960.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. T. Brit. Mycol. Soc. 55, 158–161.

Reeves, R.D., Baker, A.J.M., 2000. Metal accumulating plants. In: Raskin, I., Ensley, B.D. (Eds.), Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment. John Wiley & Sons Inc., New York, USA, pp. 193–229.

Rossi, G., Figliola, A., Socciarelli, S., Pennelli, B., 2002. Capability of Brassica napus to accumulate cadmium, zinc and copper from soil. Acta Biotechnol. 1–2, 133–140.

Schwartz, C., Gérard, E., Perronet, K., Morel, J.L., 2001. Measurement of in situ phytoextraction of zinc by spontaneous metallophytes growing on a former smelter site. Sci. Total Environ. 279, 215–221.

Smith, S.E., Read, D.J., 1997. Micorrhizal Symbiosis. second ed. Academic Press Limited, London.

Toller, H.D., Morton, J.B., Cumming, J.R., 2005. Growth and metal accumulation of mycorrhizal sorghum exposed to elevated copper and zinc. Water Air Soil Pollut. 164, 155–172.

Wallinga, I., Vark, W., Houba, V.J.G., Lee, J.J., 1989. Plant Analysis Procedures, Department of Soil Science and Plant Nutrition, Wageningen Agricultural University, Syllabus, Wageningen.

Whiting, S.N., Leake, J.R., McGrath, S.P., Baker, A.J.M., 2001. Zn accumulation by *Thlaspi caerulescens* from soils with different Zn availability: a pot study. Plant Soil 236, 11–18.

Ye, H.B., Yang, X.E., He, B., Long, X., Shi, W.Y., 2003. Growth response and metal accumulation of *Sedum alfredii* to Cd/Zn complex-polluted ion levels. Acta Bot. Sin. 45, 1030–1036.