

# *Solanum nigrum* grown in contaminated soil: Effect of arbuscular mycorrhizal fungi on zinc accumulation and histolocalisation

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*Zn accumulation by S. nigrum is enhanced by AMF and the metal storage in the tissues at the root level occurs mainly in the cell walls and in the intercellular spaces.*

## Abstract

Zn tissue accumulation in *Solanum nigrum* grown in a non-contaminated and a naturally contaminated Zn matrix and the effect of inoculation with different arbuscular mycorrhizal fungi (AMF) on metal uptake were assessed. *S. nigrum* grown in the contaminated soil always presented higher Zn accumulation in the tissues, accumulating up to 1622 mg Zn kg<sup>-1</sup>. The presence of both *Glomus claroideum* and *Glomus intraradices* enhanced the uptake and accumulation of Zn by *S. nigrum* (up to 83 and 49% higher Zn accumulation, respectively). The main deposits of the metal were found in the intercellular spaces and in the cell walls of the root tissues, as revealed by autometallography, with the inoculation with different AMF species causing no differences in the location of Zn accumulation. These findings indicate that *S. nigrum* inoculated with selected heavy metal tolerant AMF presents extracting and accumulating capacities, constituting a potentially suitable remediation method for Zn polluted soils.

*Keywords:* *Solanum nigrum*; Arbuscular mycorrhizal fungi; Zn; Phytoremediation; Autometallography

## Introduction

The contamination of nature compartments by heavy metals has become a serious environmental problem. The worldwide release of heavy metals has reached 1,350,000 tons Zn in 2002 (Singh et al., 2003). The menace posed by toxic metals should be solved, due to environmental and human health safety reasons. Due to the high cost of conventional clean-up technologies, there is an increasing interest in the remediation

of contaminated sites using biological environmental friendly techniques. One such method is phytoremediation—the use of plants to remove, degrade or immobilize contaminants. Over 400 hyperaccumulator species—plants that accumulate up to 1% of the contaminant in the aboveground harvestable dry tissues (McGrath and Zhao, 2003)—have been reported, but generally these species bring up the problem of low biomass yield and growth rate (Baker et al., 2000). For this, fast growing, high biomass species colonising contaminated soils may present themselves as potential plants for the implementation of phytoremediation strategies.

*Solanum nigrum* L. (black nightshade) is a pioneer species growing in a polluted site in northern Portugal. This industrialised area presents levels of Zn that remain above the limits established by EC Directive 86/278/EC (Atkins, 1999).

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Collected plants at the site have been shown to accumulate up to 1130 mg Zn kg<sup>-1</sup> tissue (Marques et al., 2003). *Solanum nigrum* was also found to proliferate in other metal polluted sites; Khan et al. (1998) found *S. nigrum* growing in a steelworks waste site in Australia, accumulating up to 82 mg Zn, 15 mg Pb, 3 mg Cu and 17 mg Cd per kg dry tissue. Remediation experiments using *S. nigrum* are scarce. Macek et al. (1994) studied Cd accumulation by *S. nigrum* hairy-root cultures and reported metal accumulation up to 24 mg Cd kg<sup>-1</sup> dry plant tissue. *S. nigrum* has also been shown to accumulate up to 3800 mg Zn kg<sup>-1</sup> dry tissue in the roots, with no visible toxicity signs, when grown in sand spiked with 100 mg Zn kg<sup>-1</sup> (Marques et al., in press).

Arbuscular mycorrhizal fungi (AMF) are important root symbionts that may play a central role in a phytoremediation process (Merharg and Cairney, 2000). While plants provide important compounds for AMF survival, these fungi expand the contact surface between plants and soil, contributing to an enhanced plant uptake of macronutrients (Li et al., 1991) and micronutrients such as Zn (Bürkert and Robson, 1994). Although Zn is a micronutrient for plants, at higher concentrations it may become toxic (Borkert et al., 1998; Ebbs and Kochian, 1997). The contribution of AMF to plant adaptation in highly Zn contaminated sites varies. Huang et al. (2002) reported an exclusion strategy, showing lower Zn accumulation by AMF colonised *Zea mays*, while Jamal et al. (2002) reported an accentuation of Zn and Ni uptake in *Glycine max* and *Lens culinaris*.

The effect of metal pollution in plants growing in an artificially contaminated matrix may not be the same as for the individuals growing in a naturally contaminated soil, as differences in the biological availability of the metal, exposure to metal mixtures and adaptation to metal stress may occur (Lock et al., 2003). The use of naturally contaminated soils instead of spiked soils can shorten the differences between lab and field experiments in the description of natural processes. It has been shown that data resulting from such studies are in line with those obtained from field conditions (Bernhard et al., 2005).

The knowledge of the metal distribution in cell compartments is important for understanding the tolerance mechanisms in plant species. Metal accumulation in plant roots may reflect metal merely absorbed onto the root surface rather than within the root tissue (Weis and Weis, 2004). Studies on the histolocalisation of heavy metals in plants are rare (Heumann, 2002).

The aim of this study was to assess the influence of AMF on plant biomass production and Zn uptake and accumulation in plants growing in a naturally contaminated soil, and to determine the location of the accumulated Zn in plant tissues by autometallography.

## Materials and methods

### Site description, soil sampling and sand preparation

The soil used in this study was collected at a site located in Northern Portugal, Esteiro de Estarreja. It is a small and almost stagnated watercourse,

contaminated with the discharges of an adjacent wastewater conduct originating from the industrial complex that surrounds the area. The solid residues produced in the chemical complex, essentially contaminated with zinc, lead, arsenic, mercury, vanadium, and fluorides, were also discharged nearby the stream. Profiting from the high permeability of the site, the percolates proceeding from this improvised residue deposit infiltrated into the soil of the area. These emissions no longer occur, but high levels of metal contamination, especially Zn, are present (Atkins, 1999; Oliveira et al., 2001). The banks of the stream, ca. 2 m wide, are periodically flooded, from late October to late February, and the ditch of the stream remains almost dry during the remaining months. According to Atkins (1999), the metal contamination throughout the banks and between banks is very heterogeneous, with the area near the former exit of the wastewaters being the most polluted one. Soil was collected randomly from the banks of the contaminated stream, to a 20 cm depth, in the dry season.

Sand (pH 7, 0.5–1.0 mm, 0.45% loss on ignition, acquired from Areipor, Bucelas, Portugal) is an inert substrate, with only vestigial levels of Zn, and was thus used as a control matrix. Sand was immersed in 0.1 M HCl for 24 h and rinsed with deionised water.

Sand and the collected soil were sterilised at 120 °C for 70 min in two consecutive days and dried in a drying oven at 40 °C for 4 days.

### 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 mycorrhizal fungi 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 controlled growth room conditions (12 h photoperiod, 450 µmol m<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiation, 18–38 °C temperature range, 16–71% relative humidity range). An inoculum suspension of each isolate was prepared by wet sieving (710 µm) 450 cm<sup>3</sup> of zeolite from pot cultures with deionised water to a final volume of 150 ml. 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 two matrix Zn levels (sand, considered as the control, and contaminated soil) 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* seeds were surface sterilised with 0.5% (v/v) NaOCl for 10 min and were subsequently washed with sterilised deionised water. Seeds were then germinated in plastic pots with about 500 g sterilised soil or sand. Each pot received 15 seeds. Pots were randomised in the greenhouse, a process that was repeated every 3 weeks. After sowing, seedlings were reduced to three per pot. The plants were maintained in a controlled growth room (12 h photoperiod, 450 µmol m<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiation, 18–38 °C temperature range, 16–71% relative humidity range), were watered daily. Plants growing in the sand matrix were supplemented with modified Hoagland solution containing 1/4 of the original P content and free of Zn (Hoagland and Arnon, 1950) once a week.

### Plants sampling

The harvesting was made 28 weeks after seedling. Plant roots were washed free of sand or soil with deionised water. For assessing AMF colonisation, a subsample 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 days, after which biomass was determined by weighing the dried plant material.

### Mycorrhizal colonisation

To assess AMF colonisation, the fresh fine root sub-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).

### Zn accumulation in the plant

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). Zn 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 Zn determination analytical method. The value obtained by FA-AAS ( $52.1 \pm 1.1$  mg Zn kg<sup>-1</sup> sample) confirmed the accuracy and precision of the method by comparison with the certified value ( $51.3 \pm 1.2$  mg Zn kg<sup>-1</sup> sample).

### Autometallography

Autometallography was performed on small pieces of roots according to Heumann (2002), except that phosphate buffer was substituted for piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (Salema and Brandão, 1973) and using Na<sub>2</sub>S as S donor (Danscher and Montagnese, 1994). The samples without osmium tetroxide treatment were embedded in EPON. Ultrathin sections were prepared using a glass or diamond knife on an ultramicrotome (Supernova Reichert), collected on nickel grids and contrasted with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (Zeiss EM 10C). Semi-thin sections for light microscopy were stained with saphranine and photographed on an Optiphot2 microscope.

### Soil analysis

Soil samples were oven dried at 40 °C for 48 h and passed through a 2 mm sieve. The soil pH was measured using a 1:2.5 (w/v) soil water ratio. Water content was determined by drying pre-oven dried (40 °C) soil at 105 °C until constant mass was achieved. Organic matter content was determined by loss on ignition. Samples for total phosphorous and nitrogen were digested at high temperatures (up to 330 °C) with a selenium and salicylic and sulphuric acids mixture and determined by colorimetry. Soil samples were digested at high temperatures (up to 140 °C) with concentrated nitric and hydrochloric acids (1:1), for total Zn determination. All previous methods were based on Houba et al. (1995). The water (De Koe, 1994), exchangeable (Thomas, 1982) and available (Thomas, 1982) Zn fractions were determined using, respectively, 1:5 soil water (De Koe, 1994), 1:5 soil 1 M NH<sub>4</sub>-Ac (De Koe, 1994), and 1:10 soil 0.05 M EDTA (Houba et al., 1995) ratios (w/v). The resulting solutions were incubated for 2 h at 20 °C, after which they were filtered through a 0.45 µm cellulose acetate filter. The Zn content of the resulting digests and extracts were determined using flame atomic absorption spectroscopy (FA-AAS) in an Unicam 960 spectrophotometer (Houba et al., 1995). BCR (Community Bureau of Reference) reference sample CRM 141 R (calcareous loam soil) was analysed through the above-referred total Zn determination analytical method. The value obtained by FA-AAS ( $284 \pm 1$  mg Zn kg<sup>-1</sup> sample) confirmed the accuracy and precision of the method by comparison with the certified value ( $283 \pm 5$  mg Zn kg<sup>-1</sup> sample).

### Statistical analysis

Statistical analysis was performed using the SPSS software program (SPSS Inc., Chicago, IL Version 12.0). The data were analysed by one-way 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 analytical-grade and were obtained from Sigma (Trypan Blue colorant, PIPES, citric acid, arabic gum and AgNO<sub>3</sub>), AGA (ethanol), TAAB (glutaraldehyde and EPON), Agar Scientific (saphranine), Aldrich (Na<sub>2</sub>S), Pronalab (remaining liquid reagents) and Merck (remaining solid reagents).

## Results and discussion

### Soil properties

Properties of the soil collected at the banks of Esteiro de Estarreja are given in Table 1. The soil used in this study presented a Zn level of about 426 mg Zn kg<sup>-1</sup> dry soil. According to Kabata-Pendias and Pendias (1984), a total fraction of 70–400 mg kg<sup>-1</sup> Zn in the soil would already be considered as toxic to plants. In addition, the observed Zn level in the soil is slightly higher than what is set in legislation for agricultural soils, 150–300 mg kg<sup>-1</sup>, for soils with pH ranging from 6 to 7 (EC Directive 86/278/CEE from June 12th), and falls within the range of contamination seen as potential for the application of phytoremediation based clean-up strategies, as one of the disadvantages of the technique is the possible toxicity of high levels of metals present in the soil to plants (Glass, 2000). The metal phytoavailability—the fraction of the total contaminant mass in soil considered to be available for the receptor plant (Luthy et al., 2003)—has emerged as an important paradigm, replacing the old belief that biological response by receptor organisms could be predicted by the total concentration in the soil (Adriano et al., 2004). In general, aqueous extraction seems to provide an estimate of the amount of bioavailable metal in the soil solution. Estimates of the total bioavailable metal, which includes not only the metal available in soil solution but also the metal ions bound to soil exchange sites, are obtained by extracting the soil with organic compounds. In the present study, the Zn content extractable by water, ammonium acetate or EDTA represent 2, 4 or 28% of the total, respectively (Table 1). Therefore, in metal

Table 1  
Soil properties

pH	6.24 ± 0.03
Water content (%)	1.14 ± 0.05
Organic content (%)	6.8 ± 0.3
N (mg kg <sup>-1</sup> soil)	3960 ± 129
P (mg kg <sup>-1</sup> soil)	179 ± 16
Total Zn (mg kg <sup>-1</sup> soil)	426 ± 2
EDTA extractable Zn (mg kg <sup>-1</sup> soil)	121 ± 3
NH <sub>4</sub> -Ac extractable Zn (mg kg <sup>-1</sup> soil)	15.3 ± 0.7
H <sub>2</sub> O extractable Zn (mg kg <sup>-1</sup> soil)	8.1 ± 0.6

Results are expressed as means ± SD ( $n = 3$ ).

bioavailability investigations the use of different soil extraction techniques is recommended (Lasat, 2002). The non-acidic characteristics of the soil used in this study also indicate that the metal is not all readily available for plant uptake, as Zn solubility decreases with pH increase (Lucas and Davis, 1961).

The acid washed sand used as a control in the experiments was also tested for total, water, extractable and available Zn—using the same procedures described for the analysis of Zn contents in the soil—and no metal was detectable in either of the fractions.

#### Mycorrhizal colonisation

No AMF colonisation was found in the plants inoculated with a sterilised mixture (controls). Two-way ANOVA was performed for the dependent variable AMF colonisation versus the independent variables matrix (sand and contaminated soil) and inoculated fungi (*Glomus* sp. BEG140, *Glomus claroideum*, *Glomus mosseae*, *Glomus intraradices* and the mixture of all the AMF isolates) (Fig. 1). Soil contamination influenced ( $P < 0.05$ ) positively mycorrhizal colonisation in *S. nigrum* and the AMF species only had a significant ( $P < 0.05$ ) influence on the colonisation in the case of plants inoculated with *G. intraradices*—presenting significantly ( $P < 0.05$ ) higher infection rates than the rest—and the mixture of all the fungi—presenting the significantly ( $P < 0.05$ ) highest colonisation rates of *S. nigrum*.

A positive relation between soil contamination and AMF presence has also been shown in other studies. Tonin et al. (2001) found that soil polluted with Cd and Zn enhanced the mycorrhizal colonisation of roots of *Trifolium repens*. Root colonisation of *Zea mays* was also found to be higher in a Cd, Cu, Pb and Zn polluted soil than in an uncontaminated one (Weissenhorn et al., 1995). Whitfield et al. (2004) reported enhanced mycorrhizal colonisation of *Thymus polytrichus* when grown in Cd, Pb and Zn enriched soil. In the present study, this positive relation may be due to the use of AMF

isolates indigenous from metal contaminated sites, which can develop heavy metal tolerance by means of adaptation towards heavy metal contamination (Leung et al., 2006).

#### Plant biomass

Plants grown in contaminated soil had higher stem and lower root masses (Table 2). The higher nutrient content of the contaminated soil may explain higher stem biomasses but plant biomass reduction can be expected in plants in the presence of Zn (Kochian, 1993), especially in the case of the roots, as they are absorptive organs, and are thus subjected to earlier and higher accumulation (Xiong, 1998). MacFarlane and Burchett, (2002) also reported a reduction in the root biomass and an increase in the stem biomass of *Avicennia marina* plants exposed to Zn.

It has been reported by many researchers that AMF colonisation reduces weight, branching, length, and fineness of roots (Hetrick et al., 1988; Koide, 1985; Price et al., 1989). This is possibly due to an AMF-mediated increase of P uptake. Since the plant is able to uptake more P only with the aid of AMF external hyphae, a greater area for nutrient absorption is not required, thus the root fresh and dry weights may be reduced (Smith and Read, 1997). However, in the present study, AMF colonisation did not influence the biomass yield of *S. nigrum* (Table 2), and few significant ( $P > 0.05$ ) differences were found between the biomass of stems, roots or leaves of *S. nigrum* when inoculated with the different AMF species. Nevertheless, none of these treatments presented significant ( $P < 0.05$ ) differences comparing with the non-inoculated control, suggesting that, in general, the presence of AMF did not influence the growth and biomass formation of *S. nigrum* individuals. Although the relationships between plants and AMF are generally reported as mutualistic (Smith and Read, 1997), neutral plant responses, as observed in this study, have been observed by other authors (Johnson et al., 1997).

#### Zn concentration in the plant

The matrix contamination had an influence in the Zn content in the tissues of *S. nigrum*, with the tissues of plants grown in contaminated soil always presenting higher Zn levels (Table 3). *S. nigrum* plants grown in the contaminated soil accumulated up to 1622 mg kg<sup>-1</sup> of Zn in the roots, 411 mg kg<sup>-1</sup> in the stems and 253 mg kg<sup>-1</sup> in the leaves. The levels registered for the roots are considered as phytotoxic for plants—500–1500 mg kg<sup>-1</sup>, according to Chaney (1989)—but all the tissues showed Zn levels above what is considered as normal levels in plant tissues, 10–100 mg kg<sup>-1</sup>, according to Frisberg et al. (1996), independently of the inoculation with AMF.

As regards the leaf tissues, the AMF inoculated plants did not present significant ( $P < 0.05$ ) differences from the non-mycorrhizal individuals in the case of control sand matrix. Plants grown in contaminated soil and germinated in a *G. claroideum* or *G. intraradices* inoculated matrix had significantly ( $P < 0.05$ ) higher Zn accumulation in the leaf tissues than the ones with other or no AMF colonisation (Table 3).

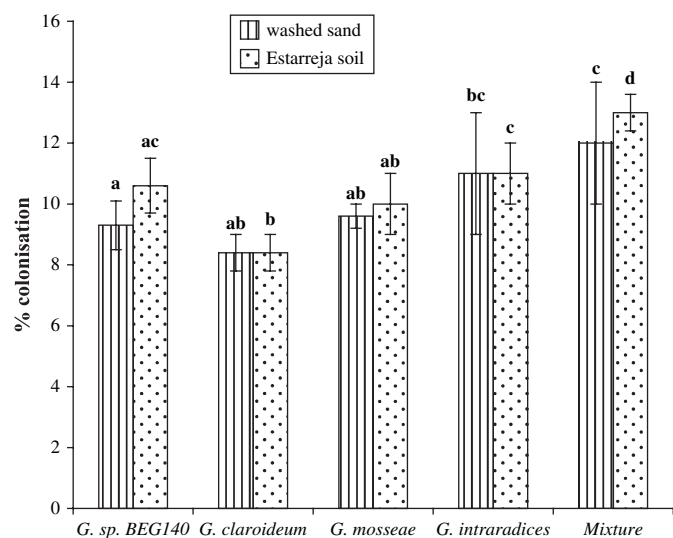


Fig. 1. Percentage of mycorrhizal colonisation (%) for each treatment. Results are expressed as percentual means. The error bar represents the SD ( $n = 6$ ).

Table 2  
Influence of different AMF species on *Solanum nigrum* biomass (g) grown in control and contaminated matrixes

AMF	Control sand			Contaminated soil		
	Root	Stem	Leaves	Root	Stem	Leaves
No fungi	2.2430 ± 1.0612 (6) <sup>a</sup>	3.4945 ± 1.0194 (6) <sup>ab</sup>	1.5797 ± 0.3195 (6) <sup>a</sup>	1.8328 ± 0.1934 (6) <sup>a</sup>	8.4576 ± 0.6972 (4) <sup>a</sup>	1.7949 ± 0.4762 (6) <sup>a</sup>
<i>Glomus</i> sp. BEG140	2.0750 ± 0.4300 (6) <sup>a</sup>	3.7018 ± 1.4697 (6) <sup>ab</sup>	1.8966 ± 0.6186 (6) <sup>a</sup>	1.8542 ± 0.4604 (6) <sup>a</sup>	5.6559 ± 1.3067 (5) <sup>b</sup>	1.5519 ± 0.5587 (6) <sup>a</sup>
<i>G. claroideum</i>	2.4744 ± 0.6489 (6) <sup>a</sup>	4.3592 ± 0.5679 (6) <sup>b</sup>	1.9261 ± 0.4561 (6) <sup>a</sup>	1.9748 ± 0.2625 (4) <sup>a</sup>	4.4544 ± 0.6065 (4) <sup>b</sup>	1.4866 ± 0.3446 (6) <sup>a</sup>
<i>G. mosseae</i>	2.3532 ± 0.9068 (6) <sup>a</sup>	2.2600 ± 0.8803 (5) <sup>a</sup>	1.6061 ± 0.4784 (5) <sup>a</sup>	1.8954 ± 0.4786 (6) <sup>a</sup>	3.8577 ± 0.7515 (4) <sup>b</sup>	1.8759 ± 0.4797 (6) <sup>a</sup>
<i>G. intraradices</i>	2.4075 ± 0.7231 (6) <sup>a</sup>	4.7235 ± 0.6276 (6) <sup>b</sup>	2.0368 ± 0.3072 (6) <sup>a</sup>	1.8197 ± 0.2458 (4) <sup>a</sup>	8.5712 ± 0.5386 (4) <sup>a</sup>	1.6893 ± 0.3904 (6) <sup>a</sup>
Mixture	1.7915 ± 0.5219 (5) <sup>a</sup>	3.7041 ± 0.3130 (5) <sup>ab</sup>	1.6946 ± 0.3476 (5) <sup>a</sup>	1.7942 ± 0.1879 (6) <sup>a</sup>	8.2986 ± 1.3144 (4) <sup>a</sup>	1.8044 ± 0.3219 (6) <sup>a</sup>

Results are expressed as means ± SD (*n*). Means in the same column with different letters are significantly different from each other ( $P < 0.05$ ) according to the Tukey test.

*S. nigrum* individuals grown in soil collected from Estarreja and inoculated with AMF had a significantly ( $P < 0.05$ ) higher Zn accumulation in the stems—except in the case of the AMF mixture inoculation—when comparing the non-inoculated plants. Plants inoculated with *G. claroideum* or *G. intraradices* presented significantly ( $P < 0.05$ ) higher Zn levels in its stems (Table 3). These differences were not observed in plants grown in the control matrix.

For Zn accumulation in the roots, in the control sand, no significant ( $P < 0.05$ ) difference was found with the AMF inoculations, but for the contaminated soil, plants inoculated with *G. claroideum* or *G. intraradices* presented significantly ( $P < 0.05$ ) higher Zn accumulation in its tissue (Table 3).

These results suggest that the inoculation with *G. claroideum* or *G. intraradices* led to higher Zn accumulation in the plant tissues, when germination and growth occurred in the Estarreja soil, a naturally Zn contaminated matrix. These two *Glomus* species induced similar Zn accumulation in *S. nigrum*, however plants inoculated with *G. claroideum* had significantly ( $P < 0.05$ ) lower root colonisation. It seems, therefore, that Zn accumulation is not related to the percentage of root colonisation by AMF, which was also reported by Toller et al. (2005) for sorghum treated with different mycorrhizal consortia. When the same AMF treatments were tested in other experiments using *S. nigrum* growing in an artificially contaminated sand matrix, exposed to Zn levels up to 1000 mg kg<sup>-1</sup> of dried sand, the inoculation with *G. intraradices* and *G. claroideum* also resulted in increased Zn accumulations in the tissues, up to 62% higher than those obtained for the non-inoculated individuals (Marques et al., in press).

A higher metal accumulation in AMF inoculated plants has been reported for other species. Mycorrhizal *Trifolium repens* and *Zea mays* growing in a Zn, Cu, Cd and Pb contaminated soil sequestered more metals in the root and shoot compared to non-mycorrhizal plants (Joner and Leyval, 2001). Jamal et al. (2002) reported that AMF inoculation enhanced Zn accumulation in *G. max* and *L. culinaris* plants growing in a contaminated soil. Some reports even indicate higher concentrations of trace metals in plants due to AMF, resulting in levels that would usually be toxic to the plant (Joner and Leyval, 1997). One of the possible explanations for this increase in Zn accumulation in the mycorrhizal plants may be the stimulation caused by mycorrhization in the plant phenolic defence system resulting in the formation of thiols, like glutathione (Schützendubel and Andrea, 2002). This compound is structurally related to a form of peptides called phytochelatins. Such phytochelatins are able to bind heavy metals and detoxify contaminated soils (Cobbett and Goldsbrough, 2002), establishing higher uptake and accumulation in the plant. Higher phytochelatins levels can result in an increase in the accumulation of Zn (Faucheur et al., 2005). For all the AMF treatments the root tissues had, on average, the higher Zn content, followed by the stems, with the leaves registering the lowest values. This observation of the relative concentrations may suggest low translocation, which is contradicted when looking at absolute levels (accumulation × biomass), as the biomass of the aboveground part of the plants is always higher. A good candidate for a phytoextraction strategy would be a species that has a translocation factor higher than one (McGrath and Zhao, 2003). These absolute translocation rates

Table 3  
Influence of different AMF species on *Solanum nigrum* Zn tissue concentration (mg kg<sup>-1</sup> dry weight) grown in control and contaminated matrixes

AMF	Control sand			Contaminated soil		
	Root	Stem	Leaves	Root	Stem	Leaves
No fungi	117 ± 26 (6) <sup>a</sup>	53 ± 6 (6) <sup>a</sup>	26 ± 2 (6) <sup>ab</sup>	1029 ± 64 (6) <sup>a</sup>	285 ± 16 (4) <sup>a</sup>	114 ± 16 (6) <sup>a</sup>
<i>Glomus</i> sp. BEG140	139 ± 22 (6) <sup>a</sup>	57 ± 10 (6) <sup>a</sup>	26 ± 8 (6) <sup>ab</sup>	1095 ± 96 (6) <sup>a</sup>	363 ± 11 (5) <sup>bc</sup>	131 ± 20 (6) <sup>a</sup>
<i>G. claroideum</i>	110 ± 8 (6) <sup>a</sup>	60 ± 14 (6) <sup>a</sup>	24 ± 3 (6) <sup>a</sup>	1622 ± 106 (4) <sup>b</sup>	411 ± 30 (4) <sup>d</sup>	250 ± 30 (6) <sup>b</sup>
<i>G. mosseae</i>	118 ± 10 (6) <sup>a</sup>	53 ± 11 (5) <sup>a</sup>	27 ± 10 (5) <sup>ab</sup>	1027 ± 102 (6) <sup>c</sup>	349 ± 6 (4) <sup>b</sup>	188 ± 11 (6) <sup>a</sup>
<i>G. intraradices</i>	144 ± 14 (6) <sup>a</sup>	54 ± 15 (6) <sup>a</sup>	28 ± 2 (6) <sup>b</sup>	1586 ± 59 (4) <sup>d</sup>	397 ± 14 (4) <sup>cd</sup>	253 ± 19 (6) <sup>b</sup>
Mixture	126 ± 7 (5) <sup>a</sup>	54 ± 4 (5) <sup>a</sup>	25 ± 5 (5) <sup>ab</sup>	1139 ± 109 (6) <sup>a</sup>	251 ± 11 (4) <sup>c</sup>	136 ± 11 (6) <sup>a</sup>

Results are expressed as means ± SD (*n*). Means in the same column with different letters are significantly different from each other ( $P < 0.05$ ) according to the Tukey test.

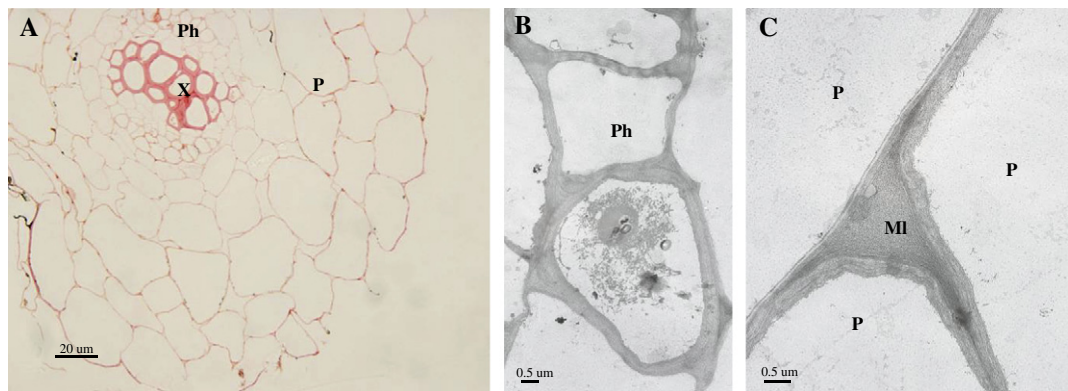


Fig. 2. Root section of *Solanum nigrum* inoculated with *Glomus* sp. BEG140 grown in control sand. After autometallography was performed on the root pieces, no labelling was detected in control plants. (A) General structure of the root as seen in cross section under the light microscope; cortical parenchyma cells (P), phloem (Ph), xylem (X). (B) Electron microscopy image of a detail of the phloem cells (Ph). (C) Region of contact of three cortical parenchyma cells (P), middle lamellae (MI).

of *S. nigrum* vary according to the growth matrix. When growth occurred in sand, the translocation rates were 0.86, 0.90, 1.13, 0.59, 0.90 and 1.07 when the AMF treatment was, respectively, no fungi, *Glomus* sp. BEG140, *G. claroideum*, *G. mosseae*, *G. intraradices* and a mixture of all fungi. The rates obtained for plants grown in contaminated soil were generally higher than those registered for plants grown in the control sand, when the same AMF treatment was applied; 1.39, 1.11, 0.69, 0.87, 1.33 and 1.14 when the AMF treatment was, respectively, no fungi, *Glomus* sp. BEG140, *G. claroideum*, *G. mosseae*, *G. intraradices* and a mixture of all fungi. The application of AMF to plants grown in contaminated soil always resulted in a decrease of the translocation rate. This decrease may indicate that the tested AMF act as a zinc barrier in *S. nigrum* growing in a naturally contaminated matrix by immobilising heavy metals in the roots, restricting their translocation to stems (Kaldorf et al., 1999).

*S. nigrum* appears to have extracting and accumulating characteristics, according to the high Zn levels observed in its tissues when exposed to the Zn contamination of the substrate, with no apparent toxicity signs.

### Zn localisation

Autometallography is a technique used to define the location of metals in the tissues and was first applied by Timm, 1958 (Heumann, 2002). The method has evolved and has become extremely specific for Zn ions (Ibata and Otsuka, 1969; Danscher and Zimmer, 1978). In environmental toxicology, autometallography has been used essentially as an option to observe the sites of metal accumulation in animal tissues (Soto et al., 1996). The application to plant tissues is scarce and until now few studies on the localisation of Zn have been reported, namely with the Zn tolerant species *Armeria maritime* (Heumann, 2002).

In the present study, autometallography was performed on root samples of all treatments. Figs. 2, 3 and 4 show representative results. Dark staining and electron-dense grains resulting from the metal presence are visible, especially on treatments resulting from plant growth on contaminated soil (Figs. 3 and 4), in comparison with root sections of *S. nigrum* plants grown in control sand (Fig. 2). This corroborates the data indicating that plants grown in a substrate rich in Zn accumulated higher

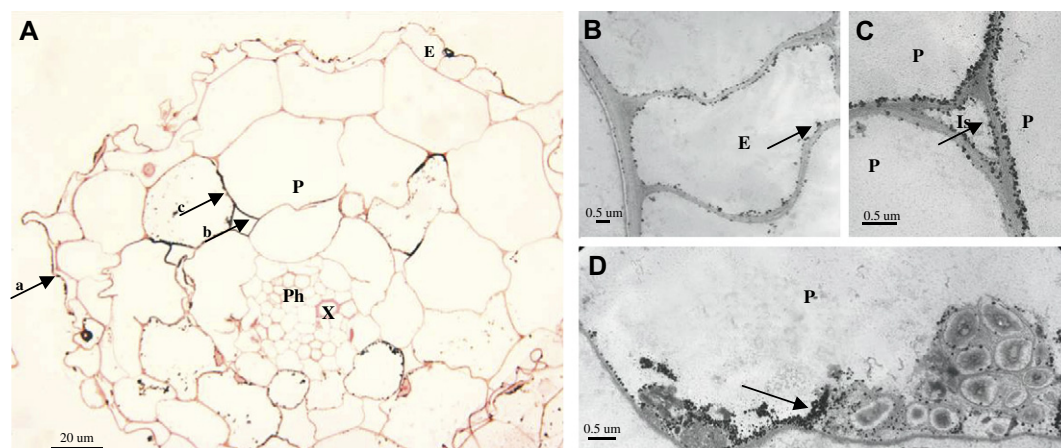


Fig. 3. Root sections of *Solanum nigrum* inoculated with *Glomus* sp. BEG140 grown in contaminated soil. (A) Light microscopy image of a root cross section; xylem (X), phloem (Ph), cortical parenchyma cells (P) and the epidermal cells (E). The arrows indicate Zn deposits, as revealed by the dark staining in the epidermis (a), the parenchyma cells of the cortex (c) and intercellular spaces (b). (B), (C) and (D) Electron microscopy analysis reveals Zn deposition (arrows) in the epidermal cells (E), in intercellular spaces (Is) and in the cytoplasm of cortical parenchyma cells (P).

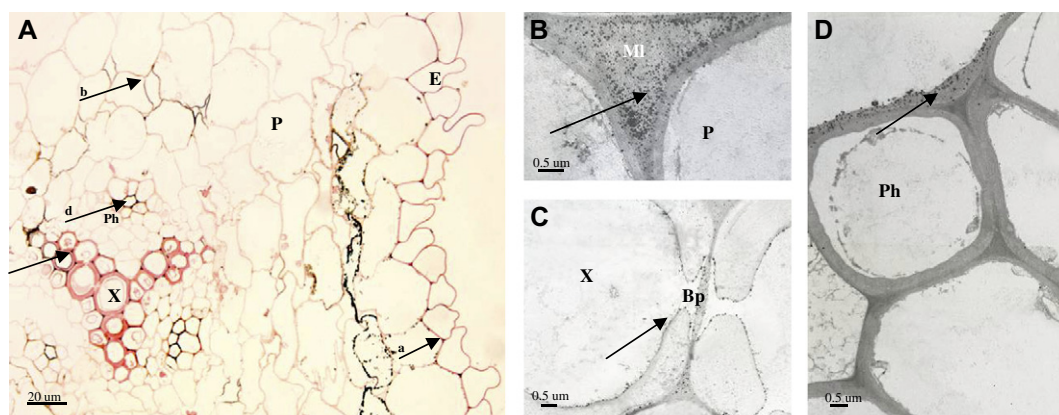


Fig. 4. Root sections of non-inoculated *Solanum nigrum* grown in contaminated soil. (A) General root structure showing the presence of Zn as indicated by the dark staining (arrows) in the epidermis (a), cortical parenchyma cells (b), and xylem (c) phloemic tissues (d). (B) Region of contact of three parenchyma cells (P) revealing Zn accumulation in the cell wall (arrow). (C) Detail of xylem conducting cells (X) and a bordered pit pair (Bp) showing Zn accumulation (arrow) in the lumen of the cells adjacent to the cell walls. (D) Phloem parenchyma cells also reveal Zn deposits (arrow); phloem conducting element (Ph).

levels of the metal in its tissues than the plants grown in control sand. The deposits are present in the intercellular spaces and cell walls of the cortical parenchyma cells (Figs. 3C and 4B), and in the secondary cell wall of xylem conducting elements (Fig. 4C). Labelling was also found intracellularly (Fig. 3A), in particular in the epidermis (Fig. 3B), cortical parenchyma (Fig. 3D) and phloem cells (Fig. 4D). Similar localisation of metal accumulation has been found in the root tissues of *S. nigrum* plants growing in culture medium artificially contaminated with Zn—up to 100 mg Zn l<sup>-1</sup> (unpublished data). This is also consistent with reports found for other plants accumulating Zn, as *Thlaspi caerulescens* (Frey et al., 2000), *A. maritime* ssp. *halleri* (Heumann, 2002), *Silene vulgaris* (Harmens et al., 1993) and *Avicenia marina* (MacFarlane and Burchett, 2002). According to Memon et al. (2001), Zn location at the cell wall level is one of the resistance mechanisms of tolerant plants, as it allows avoiding the Zn in the most sensitive areas, where the metabolic activities take place. The accumulation of Zn in the cellular walls and intercellular spaces was observed in plants for all the AMF treatments, as for the non-inoculated control.

The levels indicated as metal concentrations in the roots, as analysed via AAS, reflect metal absorbed within the root tissue and not metal just merely absorbed onto the root surface, as proposed by Weis and Weis (2004).

Autometallography was shown to be a good method for complementing studies on metal tissue accumulation brought by physical methods (atomic absorption spectroscopy after tissue acid digestion), allowing to localise the metals in the plant tissues, and contributing to the understanding of the mechanism through which *S. nigrum* avoids the Zn toxic effects in its roots.

## Conclusions

The results obtained in our study suggest that, in the case of *S. nigrum*, the inoculation with *G. claroideum* and *G. intraradices*, resulted in higher Zn accumulation when the germination and growth was conducted in a Zn naturally contaminated soil. However, our data indicate that Zn uptake

is not related to the percentage of root colonisation by AMF. The inoculation with *G. claroideum* induced an increase of 58, 44 and 120% in the Zn accumulation levels for the roots, stems and leaves, respectively, when comparing to the non-inoculated plants. When inoculated with *G. intraradices*, the increase in the accumulation levels was of 54, 39 and 122% for roots, stems and leaves, respectively. Nevertheless, in general, the presence of AMF did not influence the growth and biomass formation of *S. nigrum*.

As seen by electron microscopy, electron-dense grains resulting from the metal presence were visible in the intercellular spaces and in the cellular walls of root sections of *S. nigrum* plants grown on contaminated media, showing the apoplast as a Zn reservoir. The inoculation with different AMF species caused no differences in the localisation of Zn in the intercellular spaces and cell walls of *S. nigrum* roots.

The use of *S. nigrum* inoculated with *G. claroideum* or *G. intraradices* appears to be a good option for the decontamination of Zn contaminated soil due to the enhanced accumulating capacities presented by these associations.

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## References

- Adriano, D.C., Wenzel, W.W., Vangronsveld, J., Bolan, N.S., 2004. Role of assisted natural remediation in environment cleanup. *Geoderma* 122, 121–142.

- 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, Lisbon. No. 595.
- Baker, A.M.J., McGrath, S.P., Reeves, R.D., Smith, J.A.C., 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biochemical resource for phytoremediation of metal polluted soils. In: Terry, N., Banuelos, G. (Eds.), *Phytoremediation of Contaminated Soil and Water*. Lewis Publishers, pp. 85–107.
- Borkert, C.M., Cox, F.R., Tucker, M.R., 1998. Zinc and copper toxicity in peanut, soybean, rice and corn in soil mixtures. *Communications on Soil Science and Plant Analysis* 29, 2991–3005.
- Bürkert, B., Robson, A., 1994. <sup>65</sup>Zn uptake in subterranean clover (*Trifolium subterraneum* L.) by three vesicular arbuscular mycorrhizal fungi in a root free sandy soil. *Soil Biology and Biochemistry* 26, 1117–1124.
- Bernhard, R., Verkleij, J.A.C., Nelissen, H.J.M., Vink, J.P.M., 2005. Plant specific responses to zinc contamination in a semi-field lysimeter and on hydroponics. *Environmental Pollution* 138, 100–108.
- Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* 54, 159–182.
- Chaney, R.L., 1989. Toxic element accumulation in soils and crops: protecting soil fertility and agricultural food-chains. In: Bar-Yosef, B., Barrow, N.J., Goldshmid, J. (Eds.), *Inorganic Contaminants in the Vadose Zone*. Springer-Verlag, Berlin, pp. 140–158.
- Danscher, G., Montagnese, C., 1994. Autometallographic localization of synaptic vesicular Zn and lysosomal gold, silver and mercury. *Journal of Histochemistry* 17, 15–21.
- Danscher, G., Zimmer, J., 1978. An improved Timm sulphide silver method of light and electron microscopic localization of heavy metals in biological tissues. *Histochemistry* 55, 27–40.
- De Koe, T., 1994. *Agrostis castellana* and *Agrostis delicatula* on heavy metal and arsenic enriched sites in NE Portugal. *The Science of the Total Environment* 145, 103–109.
- Ebbs, S.D., Kochian, L.V., 1997. Toxicity of zinc and copper to Brassica species: implications for phytoremediation. *Journal of Environmental Quality* 26, 776–781.
- Faucheur, S., Behra, R., Sigg, L., 2005. Thiol and metal contents in periphyton exposed to elevated copper and zinc concentrations: a field and microcosm study. *Environmental Science and Technology* 39, 8099–8107.
- Frey, B., Keller, C., Zierold, K., Schulin, R., 2000. Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* 23, 675–687.
- Frisberg, L., Nordberg, G.F., Kessler, E., Vouk, V.B., 1996. *Handbook of the Toxicology of Metals*, second ed., vols. I,II. Elsevier Science Publishers, Amsterdam.
- Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist* 84, 489–500.
- Glass, D.J., 2000. Economic potential of phytoremediation. In: Raskin, I., Ensley, B.D. (Eds.), *Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment*. John Wiley & Sons, Inc., pp. 18.
- Harmens, H., Gusmão, N.G.C.P.B., den Hartog, P.R., Verkleij, J.A.C., Ernst, W.H.O., 1993. Uptake and transport of Zn in Zn sensitive and Zn tolerant *Silene vulgaris*. *Journal of Plant Physiology* 141, 309–315.
- Hetrick, B.A.D., Kitt, D.G., Wilson, G.W.T., 1988. Mycorrhizal dependence and growth habit of warm-season and cool-season tall grass prairie plants. *Canadian Journal of Botany* 66, 1376–1380.
- Heumann, H.G., 2002. Ultrastructural localization of Zn in Zn-tolerant *Armeria maritima* ssp. *Halleri* by autometallography. *Journal of Plant Physiology* 159, 191–203.
- 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.
- Houba, V.J.G., van der Lee, J.J., Novozamsky, I., 1995. *Soil Analysis Procedures*. Department of Soil Science and Plant Nutrition. Wageningen Agricultural University, Syllabus, Wageningen.
- Huang, Y., Chen, Y.J., Tao, C., 2002. Uptake and distribution of Cu, Zn, Pb and Cd in maize related to metals speciation changes in rhizosphere. *Chinese Journal of Applied Ecology* 13, 860–862.
- Ibata, Y., Otsuka, N., 1969. Electron microscopic demonstration of Zn in the hippocampal formation using Timm's sulphide silver technique. *Journal of Histochemistry and Cytochemistry* 17, 171–175.
- Jamal, A., Ayub, N., Usman, M., Khan, A.G., 2002. Arbuscular mycorrhizal fungi enhance Zn and nickel uptake from contaminated soil by soybean and lentil. *International Journal of Phytoremediation* 4, 205–221.
- Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of Mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* 135, 575–585.
- Joner, E.J., Leyval, C., 1997. Uptake of Cd by roots and hyphae of *Glomus mosseae/Trifolium subterraneum* mycorrhiza from soil amended with high and low concentration of cadmium. *New Phytologist* 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. *Biology and Fertility of Soils* 33, 351–357.
- Kabata-Pendias, A., Pendias, H., 1984. *Trace Elements in Soils and Plants*. CRC Press, Florida.
- Kaldorf, M., Khun, A.J., Schröder, W.H., Hildebrandt, H., Bothe, H., 1999. Selective element deposits in maize colonised by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. *Journal of Plant Physiology* 154, 718–728.
- Khan, A.G., Chaudhry, T.M., Hayes, W.J., Khoo, C.S., Hill, L., Fernandez, R., Gallardo, P., 1998. Physical, chemical and biological characterization of a steelworks waste at Port Kembla, New South Wales, Australia. *Water, Air and Soil Pollution* 104, 389–402.
- 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.
- Koide, R.T., 1985. The nature of growth depressions in sunflower caused by vesicular arbuscular mycorrhizal infection. *New Phytologist* 99, 449–462.
- Lasat, M.M., 2002. Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality* 31, 109–120.
- Leung, H.M., Ye, Z.H., Wong, M.H., 2006. Interactions of mycorrhizal fungi with *Pteris vittata* (As hyperaccumulator) in As-contaminated soils. *Environmental Pollution* 139, 1–8.
- Li, X.-L., Marschner, H., George, E., 1991. Acquisition of phosphorous and copper by V.A.-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant and Soil* 136, 49–57.
- Lock, K., Janssens, F., Janssen, C.R., 2003. Effects of metal contamination on the activity and diversity of springtails in an ancient Pb-Zn mining area at Plombières, Belgium. *European Journal of Biology* 39, 25–29.
- Lucas, R.E., Davis, J.F., 1961. Relationships between pH values of organic soils and availabilities of 12 plant nutrients. *Soil Science* 92, 177–182.
- Luthy, R.G., King, R.M.A., Brown, S.L., Dzomback, D.A., Fendorf, S.E., Giesy, J.P., Hughes, J.B., Luona, S.N., Malone, L.A., Menzie, C.A., Roberts, S.M., Ruby, M.V., Schulte, T.W., Smets, B.F., Ehlers, L.E., 2003. Bioavailability of contaminants in soils and sediments: processes, tools and applications. National Research Council, Committee on Bioavailability of Contaminants in Soils and Sediments. National Academy Press, Washington, DC.
- Macek, T., Kotrba, P., Suchova, M., Skacel, F., Demnerova, K., Ruml, T., 1994. Accumulation of cadmium by hairy root cultures of *Solanum nigrum*. *Biotechnology Letters* 16, 621–624.
- MacFarlane, G.R., Burchett, M.D., 2002. Toxicity, growth and accumulation relationships of copper, lead and Zn in the grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Marine Environmental Research* 54, 65–84.
- Marques, A.P.G.C., Oliveira, R.S., Rangel, A.O.S.S., Castro P.M.L. Zinc accumulation in *Solanum nigrum* is enhanced by different arbuscular mycorrhizal fungi. *Chemosphere*, in press.
- 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.
- McGrath, S., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from contaminated soils. *Current Opinion in Biotechnology* 14, 227–282.



- Memon, A.R., Aktoprakligil, D., Ozdemir, A., Vertii, A., 2001. Heavy metal accumulation and detoxification mechanisms in plants. *Turkish Journal of Botany* 25, 111–122.
- Merharg, A.A., Cairney, J.W.G., 2000. Co-evolution of mycorrhizal symbionts and their hosts to metal-contaminated environments. *Advances in Ecological Research* 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.
- Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycology Society* 55, 158–161.
- Price, N.S., Roncadori, R.W., Hussey, R.S., 1989. Cotton root growth as influenced by phosphorus nutrition and vesicular-arbuscular mycorrhiza. *New Phytologist* 111, 61–66.
- Salema, R., Brandão, I., 1973. The use of PIPES buffer in the fixation of plant cells for electron microscope. *Journal of Submicroscopic Cytology* 5, 66–79.
- Schützendubel, A., Andrea, P., 2002. Plant responses to abiotic stresses: heavy metal induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* 53, 1351–1365.
- Singh, O.V., Labana, S., Pandey, G., Budhirahja, R., 2003. Phytoremediation: an overview of metallic ion decontamination from soil. *Applied Microbiology and Biotechnology* 61, 405–412.
- Smith, S.E., Read, D.J., 1997. *Mycorrhizal Symbiosis*, second ed. Academic Press, London.
- Soto, M., Cajaraville, M.P., Marigomez, I., 1996. Tissue and cell distribution of copper, zinc and cadmium in the mussel *Mytilus galloprovincialis*, determined by autometallography. *Tissue and Cell* 28, 557–568.
- Thomas, G.W., 1982. Exchangeable cations. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), *Methods of Soil Analysis Part 2, Agronomy Series No.9*. American Society of Agronomy and Soil Science. Soc. Am., Inc., Publishers, Madison, pp. 159–165.
- Timm, F., 1958. Zur histochemie der schermetalle. *Das sulphide-silberverfahren*. *Deutsche Zeitschrift für gerichtliche Medizin* 46, 706–711.
- 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, and Soil Pollution* 164, 155–172.
- Tonin, C., Vandenkoornhuyse, P., Joner, E.J., Straczeck, J., Leyval, C., 2001. Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover. *Mycorrhiza* 10, 161–168.
- 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.
- Weis, J.S., Weis, P., 2004. Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. *Environmental International* 30, 685–700.
- Weissenhorn, L., Leyval, C., Berthelin, J., 1995. Bioavailability of heavy metals and abundance of arbuscular mycorrhiza in soil polluted by atmospheric deposition from a smelter. *Biology and Fertility of Soils* 19, 22–28.
- Whitfield, L., Richards, A.J., Rimmer, D.L., 2004. Effects of mycorrhizal colonisation on *Thymus polytrichus* from heavy metal contaminated sites in northern England. *Mycorrhiza* 14, 47–54.
- Xiong, Z.T., 1998. Lead uptake on seed germination and plant growth in a Pb hyperaccumulator *Brassica pekinensis* Rupr. *Bulletin of Environmental Contamination and Toxicology* 60, 285–291.