# EDDS and EDTA-enhanced zinc accumulation by *solanum nigrum* inoculated with arbuscular mycorrhizal fungi grown in contaminated soil

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## Abstract

The effect of two different chelating agents [EDTA and EDDS (*S*,*S*-ethylenediaminedissucinic acid)] on Zn tissue accumulation in *Solanum nigrum* L. grown in a naturally contaminated soil was assessed. Under those conditions, the response of the plant to the inoculation with two different isolates of arbuscular mycorrhizal fungi (AMF) – *Glomus claroideum* and *Glomus intraradices* – was also studied. Plants grown in the local contaminated soil (Zn levels of 433 mg $kg^{-1}$) accumulated up to 1191 mg $kg^{-1}$ of Zn in the roots, 3747 mg $kg^{-1}$ in the stems and 3409 mg $kg^{-1}$ in the leaves. *S. nigrum* plants grown in the same soil spiked with extra Zn (Zn levels of 964 mg $kg^{-1}$) accumulated up to 4735, 8267 and 7948 mg Zn $kg^{-1}$ in the leaves, stems and roots, respectively. The addition of EDTA promoted an increase in the concentration of Zn accumulated by *S. nigrum* of up to 231% in the leaves, 93% in the stems and 81% in the roots, while EDDS application enhanced the accumulation in leaves, stems and roots up to 140, 124 and 104%, respectively. In the stems, the presence of Zn was predominantly detected in the cortex collenchyma cells, the starch sheath and the internal phloem and xylem parenchyma, and the addition of chelating agents did not seem to have an effect on the localisation of accumulation sites. The devise of a chelate-enhanced phytoextraction strategy, using chelating agents and AMF, is discussed.

## Introduction

The remediation of heavy metal contaminated soils is a challenging task due to the persistent characteristics of the elements, to which conventional technologies present costly and, sometimes, disturbing solutions. An emerging technology, phytoextraction – the use of plants to extract toxic compounds from soils (Blaylock and Huang, 2000) appears as a low cost and environmental-friendly solution for the remediation of soils contaminated with heavy metals. Although several conditions must be met for the adequate effectiveness of phytoextraction to be achieved, the bioavailability of metals to the plant root is considered to be a critical requirement for metal uptake to occur (Kayser et al., 2000). The addition of chelating agents can increase metal availability: the chelating agent complexes the free metal ion in solution, allowing further dissolution of the sorbed phase until an equilibrium between the free metal and the insoluble phases occurs (Norwell, 1991). Among the chelating agents used in studies of phytoextraction, EDTA has been the most widely cited because it is highly effective in mobilising metals in

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soils. Lai and Chen (2004) reported EDTA-enhanced Zn shoot uptake by *Dianthus chinensis* and *Vetiver zizanoides* grown in heavy metal contaminated soil. *Brassica juncea* has shown increased Cu accumulation when EDTA was applied to the growing matrix (Wu et al., 2004) and the same effect was reported for Cd and Cr uptake by *Helianthus annus* (Turgut et al., 2005). However, EDTA and EDTA-heavy metals complexes can be toxic to plants and soil microorganisms, and can also persist in the environment due to their low level of biodegradability (Bucheli-Witschel and Egli, 2001; Grčman et al., 2003). The use of other chelating agents, such as EDDS, which is more readily biodegradable and constitutes a low toxic alternative to enhance the phytoextraction of heavy metals, has been proposed (Meers et al., 2005; Luo et al., 2006). As an example, Grčman et al. (2003) reported up to 4.7- and 3.5-fold EDDS-increased Zn and Cd uptakes in *Brassica rapa*.

Biotic factors also affect metal uptake by the plants. It is a generally held view that the majority of plants growing under natural conditions form symbiotic associations with arbuscular mycorrhizal fungi (AMF) which can enhance plant growth an improve plant reproduction (Smith and Read, 1997). The contribution of AMF to enhanced metal uptake and accumulation by plants in heavy metal contaminated sites has been reported (Joner and Leyval, 1997). The arbuscular mycorrhizal status changes the chemical composition of root exudates and influences soil pH (Citterio et al., 2005) – these factors, alone or in combination, can also influence metal mobility or availability, and thus, the potential of phytoextraction of metal contaminated soil can be enhanced by inoculating accumulator plants with the most appropriate AMF. *Solanum nigrum* (black nightshade) is an indigenous plant species to a metal polluted site in an industrialised area of Northern Portugal containing sediments with levels of metals above the limits established by European Council Directive 86/278/CEE of 12 June 1986, the main occurring metal in those sediments being zinc (Oliveira et al., 2001). Despite this contamination scenario, *S. nigrum* is abundant on the site (Marques et al., 2003). Previous studies with this plant showed that *S. nigrum* was able to accumulate up to 3800 mg Zn $kg^{-1}$ dry tissue in the roots with no visible toxicity signs, and the inoculation with the AMF *Glomus intraradices* and *Glomus claroideum* contributed to an increase in Zn accumulation in plant tissues (Marques et al., 2006). In the present study, the influence of the addition of the chelating agents EDDS and EDTA, and their relationship with the presence of AMF isolates, on the bioavailability of Zn in contaminated soils, was assessed under greenhouse conditions. Metal uptake and Zn accumulation and localisation in *S. nigrum* is reported.

## Materials and methods

### Soil preparation

The soil used in this study was collected at a site located in Northern Portugal – Esteiro de Estarreja. The site 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, and high levels of metal contamination, especially Zn, are present (Oliveira et al., 2001). Soil was collected randomly from the banks of the contaminated stream, to a 20 cm depth, in the dry season. The collected soil was sterilised at 120 °C for 70 min in two consecutive days and dried in a drying oven at 40 °C for 4 d.

In order to obtain more information on the behaviour of *S. nigrum* when exposed to higher toxicity, soil with a second level of Zn contamination was prepared by adding a solution of $ZnSO_4$ to half of the mass of soil collected for the experiments, up to a concentration of 500 more mg Zn $kg^{-1}$ dry soil. The portion of metal-treated soil was wetted for 1 wk by adding deionised water to maintain 60% of the water holding capacity; the soil was then dried in the greenhouse for approximately 2 wk. This spiked soil was subjected to 3 cycles of wet and dry processes before amendments were added (Blaylock et al., 1997).

### Preparation of mycorrhizal inocula

The two 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 two 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 the 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 pipeted at 2 cm below the soil surface. Pots from 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 [contaminated soil from Estarreja (L) and the same soil spiked with extra Zn (L + Zn)], 3 chelating treatments (no agent, EDTA and EDDS) and 3 AMF treatments (no AMF, *G. claroideum* and *G. intraradices*). Each treatment was replicated five times.

Microbial populations from the local (L) soil were reintroduced to the equilibrated soil by adding 10 ml of soil filtrate (Whatman No. 1) to each pot. The filtrate was obtained from 200 g of non-sterile local soil (L) shaken

for 2 h in 2 l of sterile deionised water (Oliveira et al., 2006).

*Solanum nigrum* L. (obtained from Instituto Botânico do Porto) seeds were surface sterilised with 0.5% NaOCl for 10 min and were subsequently washed with sterilised water. Seeds were then germinated in the naturally contaminated Estarreja soil in the greenhouse. Three weeks after seeding, three equally developed seedlings were transplanted into plastic pots containing 300 g of each matrix. Pots were randomised on the greenhouse, process that was repeated every three weeks. Plants were maintained in a controlled growth room (12 h photoperiod, 450 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation, 18–38 °C temperature range, 16–71% relative humidity range) and were watered daily. After *S. nigrum* had been grown for 23 wk, solutions of EDDS and EDTA were added at a rate of 0.5 g $kg^{-1}$ soil to selected pots.

### Plant analyses

Harvest was made 10 d after treatment with EDDS and EDTA. Plant roots were washed free of soil with deionised water. For assessing AMF colonisation, a sub-sample of fresh fine roots was collected from plants sampled at each pot (see below). For assessing Zn localisation, subsamples of stem tissue were taken and were prepared for autometallography (see below). Roots, stems and leaves of all the plants from each pot were separated, oven dried at 70 °C for 2 d, after which biomass was determined by weighing the dried plant material from each pot.

For assessing AMF colonisation 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 under a stereomicroscope (Olympus, SZ60, Tokyo, Japan) 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 flame atomic absorption spectroscopy (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 (52.8 ± 0.9 mg Zn $kg^{-1}$ sample) confirmed the accuracy and precision of the method by comparison with the certified value (51.3 ± 1.2 mg Zn $kg^{-1}$ sample).

### Autometallography

Autometallography was performed on subsamples of stems according to Heumann (2002), using $Na_2S$ as S donor (Danscher and Montagnese, 1994). The samples were treated with osmium tetroxide (Heumann, 2002) and were embedded in epoxy Resin 812, according to the supplier's instructions. Semi-thin sections were prepared using a glass or diamond knife on an ultramicrotome (Supernova Reichert). Sections were always cut in a zone not deeper than 100 μm from the surface of the stem piece. The sections were stained with saphranine and photographed on an Olympus BX50 microscope coupled to an Olympus DP50 digital camera.

### Soil analysis

Samples from the soils to be used in the experiment were oven dried at 40 °C for 48 h and passed through a 1 mm sieve. Soil pH, water content, organic matter content, total phosphorous (P) and nitrogen (N) were determined. The water (De Koe, 1994), EDTA extractable and ammonium acetate ($NH_4$-Ac) extractable Zn fractions were determined, as well as the total Zn in the soil. All methods were based on Houba et al. (1995) and were previously described by Marques et al. (2007b). BCR 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 (292.3 ± 0.3 mg Zn $kg^{-1}$ sample) confirmed the accuracy and precision of the method by comparison with the certified value (283 ± 5 mg Zn $kg^{-1}$ sample).

At the time of plant harvest, soil samples were collected from each pot and oven-dried at 40 °C for 4 d. After harvesting, the Zn in the remaining soils was extracted using deionised water, as explained above.

### 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), TAAB (epoxy resin 812) and Sigma (Trypan blue stain).

## Results

### Soil properties

Properties of the soil collected at the banks of Esteiro de Estarreja (L) and of the spiked soil (L + Zn) are given in Table 1. The soils used in this study were slightly acidic and presented Zn levels of about 433 mg Zn $kg^{-1}$ dry soil, for the local soil, and 964 mg Zn $kg^{-1}$ dry soil, after spiking

Table 1
Soil properties

| | Local | Local spiked with extra Zn |
|---|---|---|
| pH | 5.02–5.12 | 4.71 – 4.75 |
| Water content (%) | 3.0 ± 0.7 | 2.49 ± 0.08 |
| Organic content (%) | 8.41 ± 0.08 | 8.30 ± 0.07 |
| N (mg $kg^{-1}$) | 3962 ± 42 | 3791 ± 336 |
| P (mg $kg^{-1}$ | 259 ± 3 | 221 ± 11 |
| Total Zn (mg $kg^{-1}$) | 433 ± 13 | 964 ± 42 |
| EDTA extractable Zn (mg $kg^{-1}$) | 179 ± 20 | 699 ± 45 |
| $NH_4$-Ac extractable Zn (mg $kg^{-1}$) | 69 ± 42 | 391 ± 12 |
| $H_2O$ extractable Zn (mg $kg^{-1}$) | 30 ± 7 | 266 ± 14 |

pH is expressed as a range. Other results are expressed as means ± SD ($n = 3$). Further properties of the local soil have been described in Marques et al. (2007a) and Oliveira et al. (2001).

with Zn. The Zn contents extractable by EDTA, $NH_4$-Ac and $H_2O$ represent 41, 16 and 7% of the total for local (L) soil and 73, 41 and 28% of the total for the extra-Zn spiked soil (L + Zn), respectively.

### Mycorrhizal colonisation

No AMF colonisation was found in the non-inoculated control treatments. Addition of either EDDS or EDTA affected the mycorrhizal status of the plant, for both Zn levels in the matrix (L and L + Zn). In general, the application of both chelating agents, especially EDTA, induced a significant decrease on plant colonisation by both AMF (Fig. 1).

The increase in the Zn level in the soil also had a significant negative effect in the percentage colonisation by both *G. claroideum* and *G. intraradices* (Fig. 1).

### Plant Biomass

Roots of *S. nigrum* plants grown with the highest Zn level (L + Zn) presented lower biomass yields than plants grown in the local soil (L) (Table 2). Generally, inoculation with the AMF isolates affected significantly the biomass of all of the sections of *S. nigrum* when growth was made in any of the contaminated soils, either by promoting an increase or a decrease in the biomass of the plant, but with no evident trend in these effects (Table 2).

Before the treatment with EDTA or EDDS, all the plants showed no visual toxicity signs. The application of both EDTA and EDDS to the contaminated soils had no significant effect in the biomass of *S. nigrum* (Table 2) but signs of toxicity appeared, especially in the EDTA treated plants – visual chlorosis and necrosis of leaves.

### EDTA and EDDS effect on Zn water-extractable

The concentrations of water-soluble Zn in the soils were examined to assess the efficiency of EDTA and EDDS in enhancing metal solubilisation. The Zn concentrations in water-extracts of the soils collected at the time of harvest were significantly increased by the addition of EDTA or EDDS to either of the contaminated soils, by up to 4.0- and 3.1-folds, respectively. In some cases, the addition of EDTA has shown to be more efficient than EDDS increasing significantly ($P < 0.05$) the soluble Zn (Fig. 2).

The effect of the inoculation with AMF was also investigated. An increase in the levels of Zn in the water extracts of the extra-Zn spiked soil (L + Zn) when EDTA or EDDS were applied was only observed when *S. nigrum* had been inoculated with *G. intraradices*.

### Zn concentration in the plant

Tissues of plants grown in the soil with higher Zn levels (L + Zn) always presented higher Zn levels, showing an influence of the Zn levels in the matrix in the plant uptake and accumulation. *S. nigrum* plants grown in the local soil (L) accumulated up to 3409 mg $kg^{-1}$ of Zn in the roots, 3747 mg $kg^{-1}$ in the stems and 1191 mg $kg^{-1}$ in the leaves; plants grown in the extra-Zn spiked local soil (L + Zn) accumulated up to 7948, 8267 and 4735 mg Zn $kg^{-1}$ in the roots, stems and leaves, respectively, with the higher uptakes being registered for chelating agents treated plants (Table 3).

The inoculation with AMF had a positive effect on the accumulation of Zn in *S. nigrum* plants grown in the local soil, with both *G. claroideum* and *G. intraradices* inducing significantly higher Zn accumulations in all the plant sections when comparing with non-inoculated plants (Table 3). When the local soil (L) was treated with EDDS or EDTA, the effect of AMF on Zn accumulation in the roots, stems and leaves of *S. nigrum* was always significant, with inoculated plants presenting higher accumulations in these sections. An exception was observed for the accumulations registered for the stems and roots of *S. nigrum* grown in EDDS treated soil, in which no effect of the AMF inoculation was seen. For *S. nigrum* plants growing in extra-Zn spiked soil (L + Zn), the inoculation with *G. intraradices* and *G. claroideum* led to significantly higher Zn accumulation in the leaves when no chelating agent was added, and to significantly ($P < 0.05$) lower or similar Zn accumulations in plants growing in the EDTA or EDDS treated soils (Table 3).

Furthermore, the chelating agents addition to the soil also increased significantly Zn accumulation in the roots, stems and leaves of *S. nigrum*. The highest Zn accumulations were registered for plants that were grown in substrates that received EDDS or EDTA. An exception was observed for the roots of *S. nigrum* plants grown in extra-Zn spiked soil (L + Zn), for which the application of the chelating agents had no effect on the metal accumulation in this plant section, as shown by the results of two-way ANOVA (Table 3). *S. nigrum* uptake of Zn was thus generally increased by the addition of EDTA or EDDS,

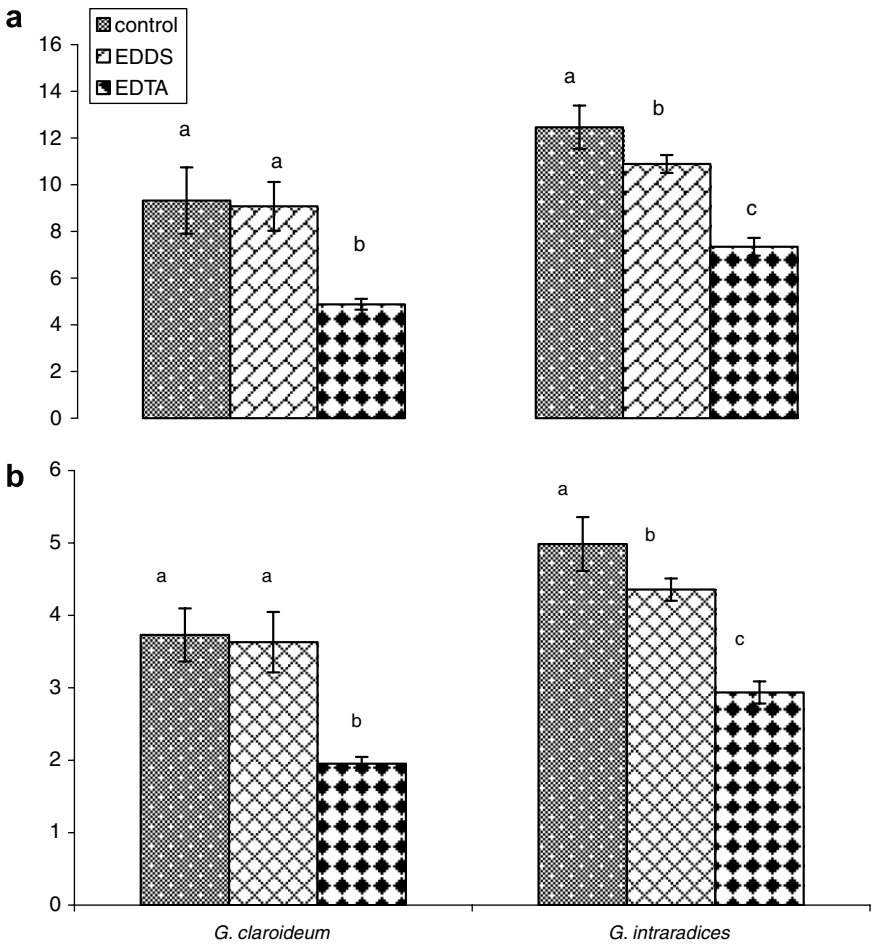


Fig. 1. Percentage of mycorrhizal colonisation for *G. claroideum* and *G. intraradices* in *S. nigrum* grown in local soil (a) and extra-Zn spiked soil (b). Results are expressed as percentual means. The error bars represent the SD. Means with different letters for the same AMF species within each soil are significantly different from each other ($P < 0.05$) according to the Tukey test.

with no significant differences between both chelating agents (Table 3).

### Root to aboveground parts translocation

The translocation factors (TFs) were determined for the different treatments (Fig. 3). Higher Zn levels in the soil induced higher translocation rates in *S. nigrum*. These values were determined using the ratio $\{([\ Zn]_{stems} * A + [Zn]_{leaves} * B)/([Zn]_{roots} * C)\}$ where A, B and C are biomass of stems, leaves and roots, respectively (Marques et al., 2006).

The analysis of the TFs of *S. nigrum* inoculated with each of the AMF species showed that the addition of chelating agents only had a significant effect when higher levels of Zn contamination were present in the soil (L + Zn soil), and in non-inoculated control plants. With these exceptions, neither EDTA nor EDDS had an effect on the root to shoot Zn translocation in *S. nigrum* under these conditions. In fact, when assessing the global effect of the chelating agent treatments and of the inoculation with AMF in the TFs, it was observed that none of these factors had a significant ($P < 0.05$) impact on the translocation rates.

### Percentage of Zn uptake to the plant shoots from the soil

The percentage of Zn taken up by *S. nigrum* shoots relatively to the total Zn initially present in the pots is presented in Fig. 4. For the local soil (L), the treatment that better enhanced the percentage of uptake of Zn to the aboveground tissues of *S. nigrum* was the combination of the inoculation with *G. claroideum* with the addition of EDTA; for the extra-Zn spiked soil, the treatments that led to higher phytoextraction capabilities of the plant were the combination of the inoculation with *G. intraradices* with the addition of EDTA, and also the application of EDDS alone.

### Zn localization on stems of S.nigrum

Zinc localization was determined on stem samples (control, EDTA and EDDS treated soils) by autometalography and no differences were found between treatments. In all cases, dark staining resulting from the metal presence were visible in all the tissues with predominance to the cortex collenchyma cells (Fig. 5A and Fig. 6A). Dark granules indicative of Zn were mainly found in the protoplasm

Table 2
Effects of the addition of chelating agents and AMF species on *S. nigrum* biomass

| | AMF species | Leaves | Biomass (g dry tissue) stems | Roots |
|---|---|---|---|---|
| Local soil | | | | |
| Control | No fungi | $0.8329 \pm 0.0885^{a}$ | $0.9496 \pm 0.0983^{a}$ | $1.0709 \pm 0{,}2894^{a}$ |
| | *G. claroideum* | $0.7815 \pm 0.2676^{a}$ | $0.8703 \pm 0.1668^{a}$ | $0.8583 \pm 0.1100^{a}$ |
| | *G. intraradices* | $0.8996 \pm 0.2183^{a}$ | $1.0302 \pm 0.3181^{a}$ | $0.9909 \pm 0.2906^{a}$ |
| EDDS | No fungi | $0.7921 \pm 0.2584^{a}$ | $0.8215 \pm 0.4148^{a}$ | $1.1586 \pm 0.4936^{a}$ |
| | *G. claroideum* | $0.6444 \pm 0.3862^{a}$ | $0.7385 \pm 0.2050^{a}$ | $0.8630 \pm 0.3651^{a}$ |
| | *G. intraradices* | $0.6072 \pm 0.1989^{a}$ | $0.5811 \pm 0.1070^{a}$ | $0.6080 \pm 0.1095^{a}$ |
| EDTA | No fungi | $1.2274 \pm 0.4138^{a}$ | $1.1126 \pm 0.2367^{a}$ | $1.3390 \pm 0.1821^{a}$ |
| | *G. claroideum* | $0.6134 \pm 0.2913^{b}$ | $0.9122 \pm 0.3436^{ab}$ | $0.9395 \pm 0.1216^{b}$ |
| | *G. intraradices* | $0.4849 \pm 0.1769^{b}$ | $0.5456 \pm 0.1478^{b}$ | $0.5396 \pm 0.0885^{c}$ |
| Amendment (A) | | NS ($F_{2,31} = 1.269$) | $^{*}(F_{2,33} = 3.782)$ | NS ($F_{2,27} = 0.734$) |
| AMF species inoculated (B) | | $^{*}(F_{2,31} = 3.992)$ | $^{*}(F_{2,33} = 3.886)$ | $^{***}(F_{2,27} = 11.121)$ |
| A × B | | NS ($F_{4,31} = 2.286$) | NS ($F_{4,33} = 2.440$) | $^{*}(F_{4,27} = 2.730)$ |
| Local + Zn soil | | | | |
| Control | No fungi | $0.9058 \pm 0.2544^{a}$ | $0.9635 \pm 0.1901^{b}$ | $0.6518 \pm 0.1886^{c}$ |
| | *G. claroideum* | $0.3674 \pm 0.0470^{b}$ | $0.8259 \pm 0.2278^{b}$ | $0.2781 \pm 0.1249^{b}$ |
| | *G. intraradices* | $1.1544 \pm 0.3704^{a}$ | $1.5759 \pm 0.1983^{a}$ | $1.2760 \pm 0.3097^{a}$ |
| EDDS | No fungi | $1.0689 \pm 0.2649^{a}$ | $1.0807 \pm 0.2194^{ab}$ | $0.5057 \pm 0.1717^{a}$ |
| | *G. claroideum* | $0.5034 \pm 0.1591^{b}$ | $0.9072 \pm 0.1072^{b}$ | $0.5440 \pm 0.1447^{a}$ |
| | *G. intraradices* | $0.6638 \pm 0.0408^{b}$ | $1.2475 \pm 0.1879^{a}$ | $0.9081 \pm 0.5510^{a}$ |
| EDTA | No fungi | $0.6988 \pm 0.4351^{a}$ | $0.8833 \pm 0.3966^{b}$ | $0.3176 \pm 0.1958^{b}$ |
| | *G. claroideum* | $0.5011 \pm 0.1640^{a}$ | $0.7411 \pm 0.1793^{b}$ | $0.7507 \pm 0.7216^{ab}$ |
| | *G. intraradices* | $1.1730 \pm 0.5455^{a}$ | $1.7196 \pm 0.4699^{a}$ | $1.4860 \pm 0.5953^{a}$ |
| Amendment (A) | | NS ($F_{2,33} = 0.106$) | NS ($F_{2,36} = 0.060$) | NS ($F_{2,25} = 0.413$) |
| AMF species inoculated (B) | | $^{***}(F_{2,33} = 14.191)$ | $^{***}(F_{2,36} = 29.901)$ | $^{***}(F_{2,257} = 12.359)$ |
| A × B | | $^{*}(F_{4,33} = 2.973)$ | NS ($F_{4,36} = 2.416$) | NS ($F_{4,25} = 2.264$) |

One-way ANOVA was performed for each chelating treatment. Means in the same treatment 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 chelators addition to each soil. The test results are shown with the test statistic and as: NS – Non significant at the level $P < 0.05$; $^{*}$, $^{***}$ – Significant at the level $P < 0.05$ and $P < 0.001$, respectively.

(Fig. 5A – arrows). In Fig. 5B, showing a stoma and air chamber, Zn was found inside all the cells; outside the cells Zn was only found in the air chamber of the stoma. The starch sheath, typical of this type of stem structure, is one of the main Zn accumulation sites (Fig. 5C and D, arrows). As shown in Fig. 5E and F the medullar parenchyma is not a Zn accumulation site. On the other hand, the inner phloem and associated parenchyma as well as the parenchyma of the primary xylem (proto and metaxylem) show a high density of dark particles indicating intense accumulation of Zn in these structures (Fig. 5G, H and Fig. 6A).

## Discussion

The soils used in the current study presented Zn levels similar or higher than those established as toxic to plants. According to Kabata-Pendias and Pendias (1984) – a total fraction of 400 mg $kg^{-1}$ Zn in the soil would already be considered as phytotoxic. In addition, the Zn contents extractable by EDTA, potentially available for plant uptake, represent a significant portion of the total Zn present in the soils. Therefore, the levels of Zn would normally impose toxic effects on plant growth, especially in the Zn spiked soil (L + Zn), inhibiting root vitality and plant growth, and preventing the absorption of inorganic nutrients (Borregard and Rydin, 1989). Although no signs of Zn excess were visible in the plants grown in the present study, when no chelating agents were added to the soils, the Zn level in the soil affected the behaviour of the plant-AMF associations. The increase in the Zn level in the soil (from L to L + Zn) promoted a reduction in the colonisation by AMF. A similar trend has been reported by Marques et al. (2006), who observed a reduced colonisation of *S. nigrum* by different AMF (*G.* sp. BEG140, *G. claroideum*, *G. mosseae*, and *G. intraradices*), when grown in sand spiked with high Zn levels. Citterio et al. (2005) have also reported this negative effect on mycorrhizal colonisation of *Cannabis sativa* as a consequence of the addition of heavy metals to the growing matrix. In addition, plants growing in the highly contaminated soil (L + Zn) accumulated higher levels of Zn in their roots, stem and leaves, and presented a reduction in their biomass. The Zn levels for all the sections of *S. nigrum* registered in this study are above those considered as phytotoxic for plants according to Chaney (1989) – 500 to 1500 mg $kg^{-1}$. The possible increase in biomass formation promoted by the inoculation with AMF could be a means to compensate the depletion of biomass production due to the stress caused by Zn presence in the matrix. However, this was not observed in this study. Only in some cases the biomass of *S. nigrum* was improved when AMF were present, while in other cases no enhancement, or even a decrease, in the

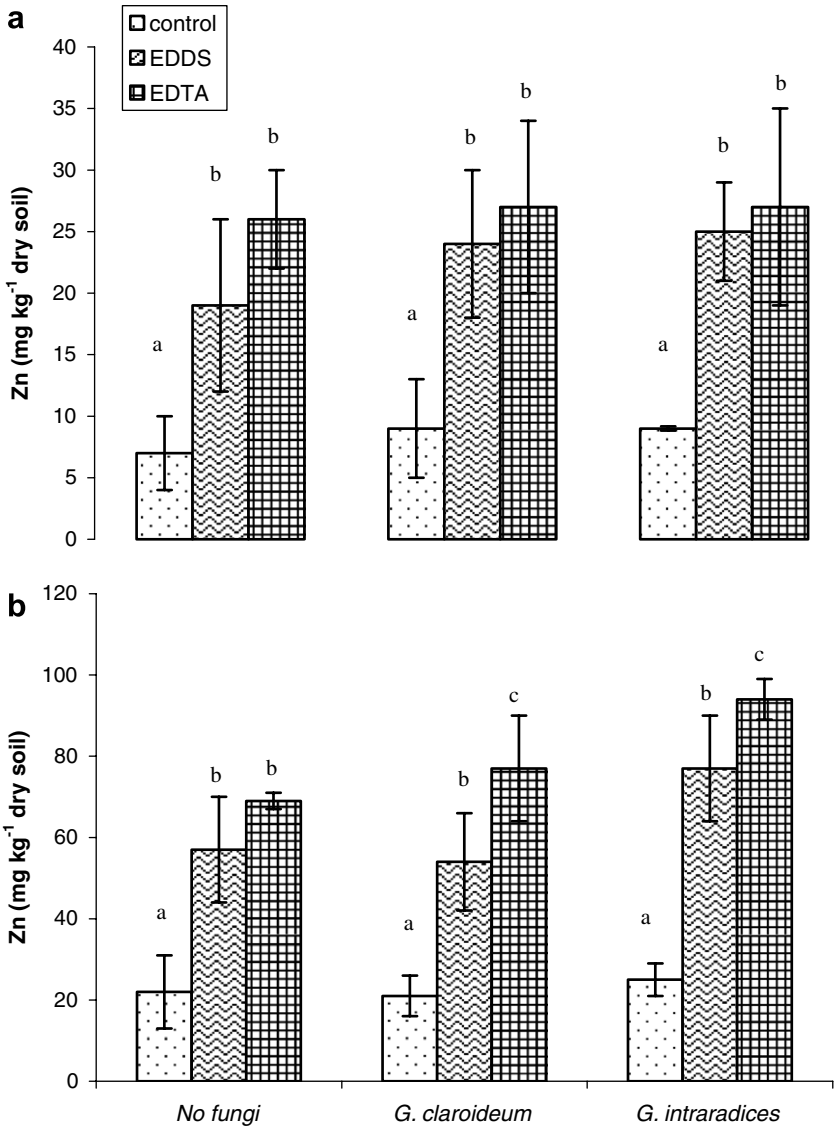


Fig. 2. Effects of the addition of chelating agents on the Zn availability in local soil (a) and in extra-Zn spiked soil (b). Results are expressed as means. The error bars represent the SD. Means with different letters for the same AMF treatments within each soil are significantly different from each other ($P < 0.05$) according to the Tukey test.

biomass of the different plant sections occurred. In fact, although the relationships between plants and AMF are generally reported as mutualistic (Smith and Read, 1997), with most studies reporting enhanced plant growth with mycorrhizal colonisation (Chen et al., 2005), negative (Johnson et al., 1997) or even neutral (Marques et al., 2006) plant responses have been observed, under certain environmental conditions. Nevertheless, it seems that *S. nigrum* inoculated with these AMF has the potential to grow and accumulate Zn in Zn contaminated sites. The growth of this species occurred even in soils with Zn levels as those registered for the extra-Zn spiked soil (L + Zn). It seems that the AMF protected the plant of excessive Zn, which is translated in a decrease in metal accumulation in AMF inoculated plants (Göhre and Paszkowski, 2006). On the other hand, there was an increase in the metal accumulation when Zn levels in the growing matrix were lower suggesting that this plant-AMF association can enhance the accumulation abilities demonstrated by the plant alone. Similar tendencies were reported by Diaz et al. (1996) for the uptake of Zn and Pb by *Lygeum spartum* and *Anthylis cytisoides* inoculated with *G. mosseae* in soils with different levels of theses metals: at low doses, mycorrhizal plants had equal or higher Zn or Pb concentrations than non-inoculated controls; at higher doses, however, metal concentrations in the plants inoculated with *G. mosseae* were lower than those found in the corresponding controls.

For both Zn contaminated soils, the addition of EDTA promoted a decrease in the root colonisation of *S. nigrum* by both *G. claroideum* and *G. intraradices*, reducing the colonisation to significantly lower levels than the ones registered for plants growing in the EDDS or in the non-treated

Table 3
Effects of the addition of chelating agents and AMF species on *S. nigrum* Zn accumulation

| | AMF species | Leaves | Zn (mg kg$^{-1}$ dry tissue) stems | Roots |
|---|---|---|---|---|
| Local soil | | | | |
| Control | No fungi | $531 \pm 21^{c}$ | $1431 \pm 57^{c}$ | $1356 \pm 359^{c}$ |
| | *G. claroideum* | $656 \pm 35^{b}$ | $1938 \pm 134^{b}$ | $1798 \pm 148^{b}$ |
| | *G. intraradices* | $851 \pm 69^{a}$ | $2482 \pm 40^{a}$ | $2687 \pm 49^{a}$ |
| EDDS | No fungi | $764 \pm 55^{b}$ | $3068 \pm 615^{a}$ | $2769 \pm 541^{a}$ |
| | *G. claroideum* | $1016 \pm 20^{a}$ | $2855 \pm 343^{a}$ | $3409 \pm 561^{a}$ |
| | *G. intraradices* | $1020 \pm 52^{a}$ | $2899 \pm 205^{a}$ | $3095 \pm 40^{a}$ |
| EDTA | No fungi | $506 \pm 55^{b}$ | $2190 \pm 293^{b}$ | $2461 \pm 343^{b}$ |
| | *G. claroideum* | $1191 \pm 214^{a}$ | $3747 \pm 912^{a}$ | $3069 \pm 58^{a}$ |
| | *G. intraradices* | $978 \pm 108^{a}$ | $2809 \pm 183^{ab}$ | $3150 \pm 277^{a}$ |
| Amendment (A) | | $^{***}(F_{2,31} = 29.964)$ | $^{***}(F_{2,34} = 25.835)$ | $^{***}(F_{2,25} = 43.999)$ |
| AMF species inoculated (B) | | $^{***}(F_{2,31} = 62.139)$ | $^{***}(F_{2,34}$v8.710) | $^{***}(F_{2,25} = 17.887)$ |
| A × B | | $^{***}(F_{4,31} = 12.895)$ | $^{***}(F_{4,34} = 7.668)$ | $^{**}(F_{4,25} = 4.442)$ |
| Local + Zn soil | | | | |
| Control | No fungi | $1434 \pm 161^{b}$ | $3690 \pm 441^{b}$ | $5628 \pm 388^{a}$ |
| | *G. claroideum* | $2542 \pm 525^{a}$ | $5446 \pm 490^{a}$ | $7465 \pm 1662^{a}$ |
| | *G. intraradices* | $2082 \pm 180^{a}$ | $4423 \pm 464^{b}$ | $6399 \pm 429^{a}$ |
| EDDS | No fungi | $3435 \pm 455^{a}$ | $8267 \pm 1250^{a}$ | $6463 \pm 83^{a}$ |
| | *G. claroideum* | $3627 \pm 228^{a}$ | $7317 \pm 439^{a}$ | $7363 \pm 150^{a}$ |
| | *G. intraradices* | $3221 \pm 84^{a}$ | $4787 \pm 501^{b}$ | $7140 \pm 997^{a}$ |
| EDTA | No fungi | $4735 \pm 480^{a}$ | $7028 \pm 414^{a}$ | $7948 \pm 514^{a}$ |
| | *G. claroideum* | $3444 \pm 430^{b}$ | $6236 \pm 415^{b}$ | $6953 \pm 53^{b}$ |
| | *G. intraradices* | $3070 \pm 248^{b}$ | $6474 \pm 215^{ab}$ | $6816 \pm 359^{b}$ |
| Amendment (A) | | $^{***}(F_{2,3}1 = 102.573)$ | $^{***}(F_{2,35} = 68.144)$ | NS $(F_{2,24} = 2.166)$ |
| AMF species inoculated (B) | | $^{**}(F_{2,31} = 6.354)$ | $^{***}(F_{2,35} = 17.481)$ | NS $(F_{2,24} = 1.396)$ |
| A × B | | $^{***}(F_{4,31} = 18.298)$ | $^{***}(F_{4,35} = 21.173)$ | $^{*}(F_{4,24} = 3.004)$ |

One-way ANOVA was performed for each chelating treatment. Means in the same treatment 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 chelators addition to each soil. The test results are shown with the test statistic and as: NS – Non significant at the level $P < 0.05$; *; **; *** – Significant at the level $P < 0.05$, $P < 0.01$and $P < 0.001$, respectively.

soils. Other reports have shown similar results: Grčman et al. (2003) reported that EDTA addition to a metal contaminated soil induced fungi stress; Chen et al. (2004) indicated lower AMF colonisation of *Z. mays* when grown in EDTA treated soil. On the other hand, the addition of EDTA had no effect on the biomass formation of *S. nigrum* in the present study, probably due to the late addition of the chelating agents, not allowing the effect of this addition to be translated in significant differences in the growth of treated and not treated plants. Other studies reported the same effect demonstrating that the late addition of the chelating agents avoids loss of potential plant development. Wu et al. (2004) reported no influence of EDTA application to the soil at a rate of 3 mmol kg$^{-1}$ in the biomass yield of *B. juncea* and Meers et al. (2005) reported that the addition of up to 4 mmol kg$^{-1}$ EDTA had no influence on the biomass production of *Helianthus annuus*.

The soil treatment with EDTA led to an increase in the water extractable fraction of Zn, as has also been described in other reports (Lai and Chen, 2004; Luo et al., 2004). The concentration of metal extracted using deionised water can be considered equal to the water soluble form in the contaminated soil that is easily leached into the groundwater along with possible rainfall (Lai and Chen, 2004) and easily taken up by plants. This was also observed in the present study, as Zn accumulation by *S. nigrum* increased when EDTA was added to the growing matrix.

The addition of EDDS to the contaminated soil promoted a reduction in the root colonisation of *S. nigrum* by both *G. claroideum* and *G. intraradices*. Sudová et al. (2007) also reported a reduced AMF colonisation of *Nicotiana tabacum*, another plant from the Solanaceae family, when growing in EDDS treated soil. However, EDDS induced significantly lower reductions in the colonisation by both AMF than EDTA. Grčman et al. (2003) reported that EDDS addition to a metal contaminated soil induced earlier fungi stress; however, EDDS toxicity was revealed much later than EDTA-induced stress. EDDS is a naturally occurring aminopolycarboxylic acid, produced by a number of microorganisms (Goodfellow et al., 1997) and this is the likely reason for its lower toxicity.

The application of EDDS resulted in lower Zn availability in the water extracts, suggesting that the addition of EDDS appears as a less dangerous solution than applying EDTA, which can increase the danger of further metal leaching and groundwater contamination. Enhanced phytoextraction is more feasible if fewer disturbances are applied to the contaminated site, and fewer risks of possible transference from soil to other environmental compartments are taken. EDTA and its complexes are known for being poorly photo-, chemo- and biodegradable in soil environments (Nörtemann, 1999), with a minimum observed half-life of ca. 36 d (Meers et al., 2005): its longevity will cause higher metal mobility, even long after harvest,

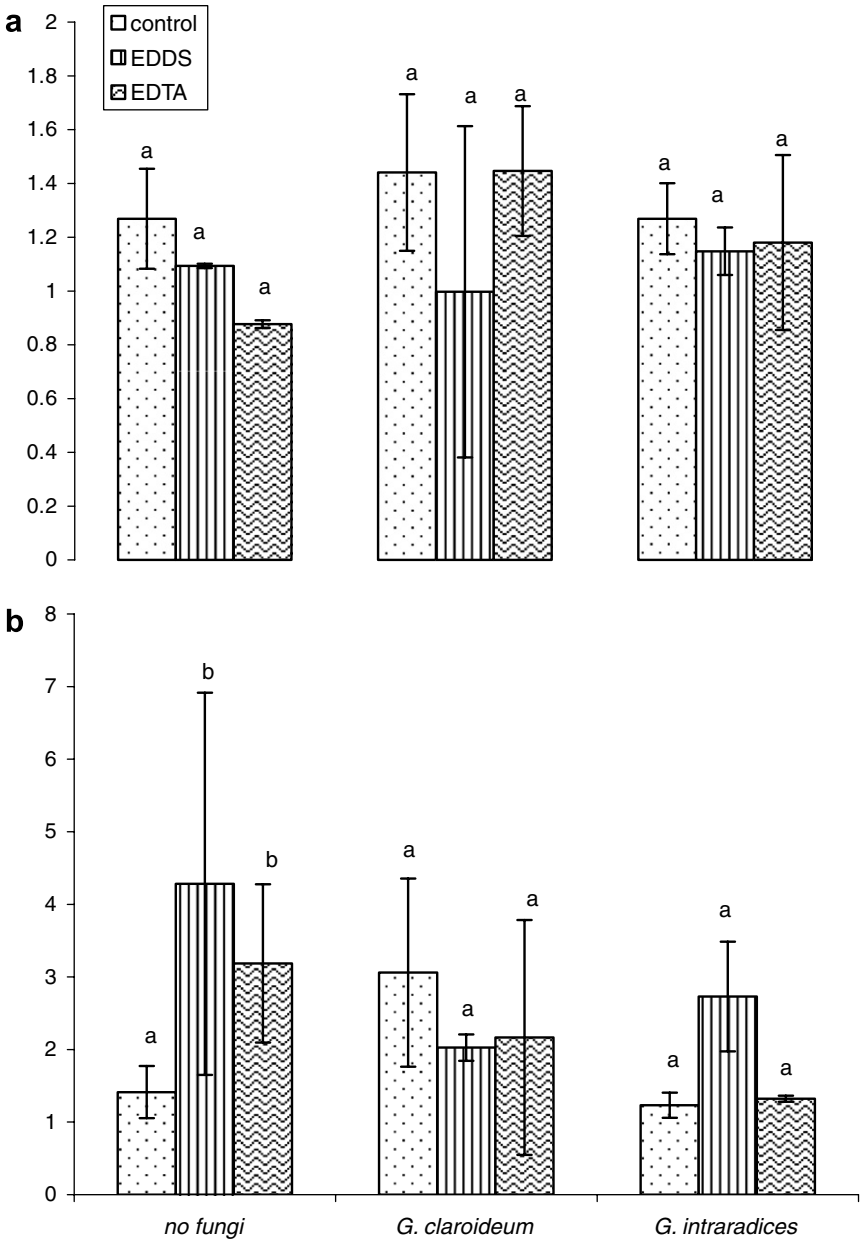


Fig. 3. Translocation factor of *S. nigrum* for different AMF treatments in local soil (a) and in extra-Zn spiked soil (b). Results are expressed as means. The error bars represent the SD. Means with different letters for the same AMF treatments within each soil are significantly different from each other ($P < 0.05$) according to the Tukey test.

implying leaching of metals and an increased risk of contamination of the groundwater. On the other hand, EDDS half-life is reported as ca. 2.5 d in soil, achieving complete mineralisation in 28 d (Schowanek et al., 1997). Nevertheless, the addition of EDDS enhanced Zn accumulation in a similar way to EDTA. Other studies have reported a neutral effect of EDDS addition in the growth of *B. rapa* and *H. annuus*, respectively (Grčman et al., 2003; Meers et al., 2005). These reports also indicate that the rates of metal uptake enhancement by EDDS addition were similar to the enhancement of those obtained when EDTA was added to the growing matrix.

The objectives of phytoextraction include the extraction of metals into bioavailable forms that can be taken up by plants and the enhancement of metal translocation from roots to shoots (Huang et al., 1997). While chelating agents have been proven to be successful at increasing metal bioavailability through enhancing metal solubility, their possible success at overcoming the barrier root to shoot (stems and leaves) translocation is not well documented (Lombi et al., 2001). Therefore, in addition to evaluating a chelating agent's effectiveness at mobilising the metals to the root zone, and understanding its effects on plant biomass and accumulation, the extent of translocation to the shoots is an important factor for the choice of a phytoextraction strategy. Few studies focused on the translocation variations imposed by the addition of chelating agents. Luo et al. (2004) have reported that the application of EDTA

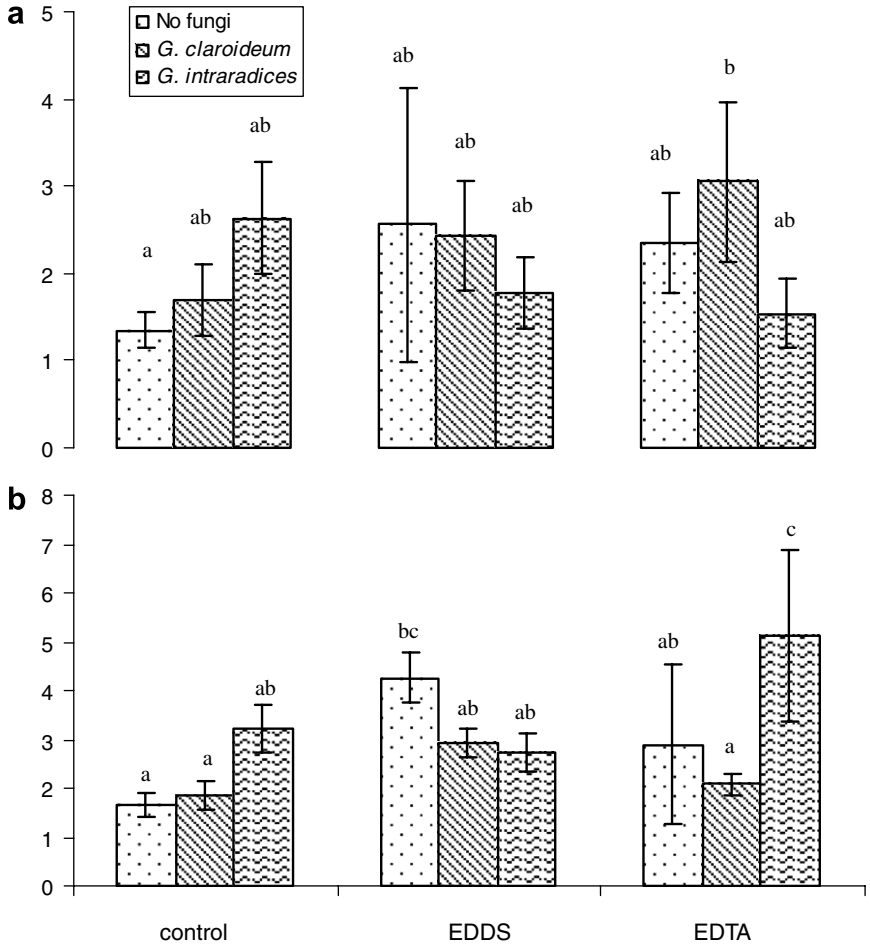


Fig. 4. Percentage of Zn uptake to the plant shoots from the local soil (a) and extra-Zn spiked soil (b). Results are expressed as percentual means. The error bars represent the SD. Means with different letters within each soil are significantly different from each other ($P < 0.05$) according to the Tukey test.

and EDDS increases the root-to-shoot ratios of the metals Cu, Pb, Zn and Cd in *Z. mays* and *Phaseolus vulgaris*, but that was not observed in the present study. Despite the enhancement of Zn accumulation by *S. nigrum* shoots (stem + leaves) in the EDTA and EDDS treatments, no effect of the chelating agents was translated in enhanced translocation of the metals to the aboveground parts of the plants, as the chelate-increased solubilisation of Zn in the soils induced higher accumulations not only in the shoots but also in the roots of *S. nigrum*. Autometallography was performed on stem samples with no AMF inoculation, as it has been reported in previous studies (Marques et al., 2007a) that the inoculation with AMF had not effect on the pattern of localisation of the metal in the plant tissues. In the present study, and for all treatments, Zn was located predominantly in the collenchyma of the cortex, the starch sheath and also internal phloem and xylem parenchyma. As the metal is translocated through the plant, it is unloaded into the vascular parenchyma. Since the stem of *S. nigrum* has stomata, the xylem sap (water and other materials) is transported from the vascular parenchyma to the outer zones of the stem. One barrier in this direction is the starch sheath, another site of Zn accumulation pointed out earlier. Substances may then be transported to the outer cortex and may be carried out into the stoma air chamber. The stems of the plant are not excluding the metal from inside the cells, pattern that was observed for all treatments and thus is not an effect of the addition of chelating agents (Fig. 5 and 6), that in fact are not affecting the localisation of accumulation sites in the plant stem tissues.

When analysing the percentage of Zn stored in the aboveground tissues of *S. nigrum* from the pots in which the plants were growing, additional information can be extracted. The data presented on Fig. 4 can be extrapolated to a field situation – considering equal plant development and similar soils characteristics, contamination nature and depth – in order to estimate the number of crops of *S. nigrum* required to clean-up the soil to a certain level. The background levels for agricultural soils should not be higher than 150–300 mg Zn $kg^{-1}$ dry soil, for soils with pH ranging from 6 to 7 (European Council Directive 86/278/CEE of 12 June 1986). The studied soils are clearly above the established limits and fall within the range of contamination seen as potential for the application of phytoremediation based clean-up strategies, as one of the

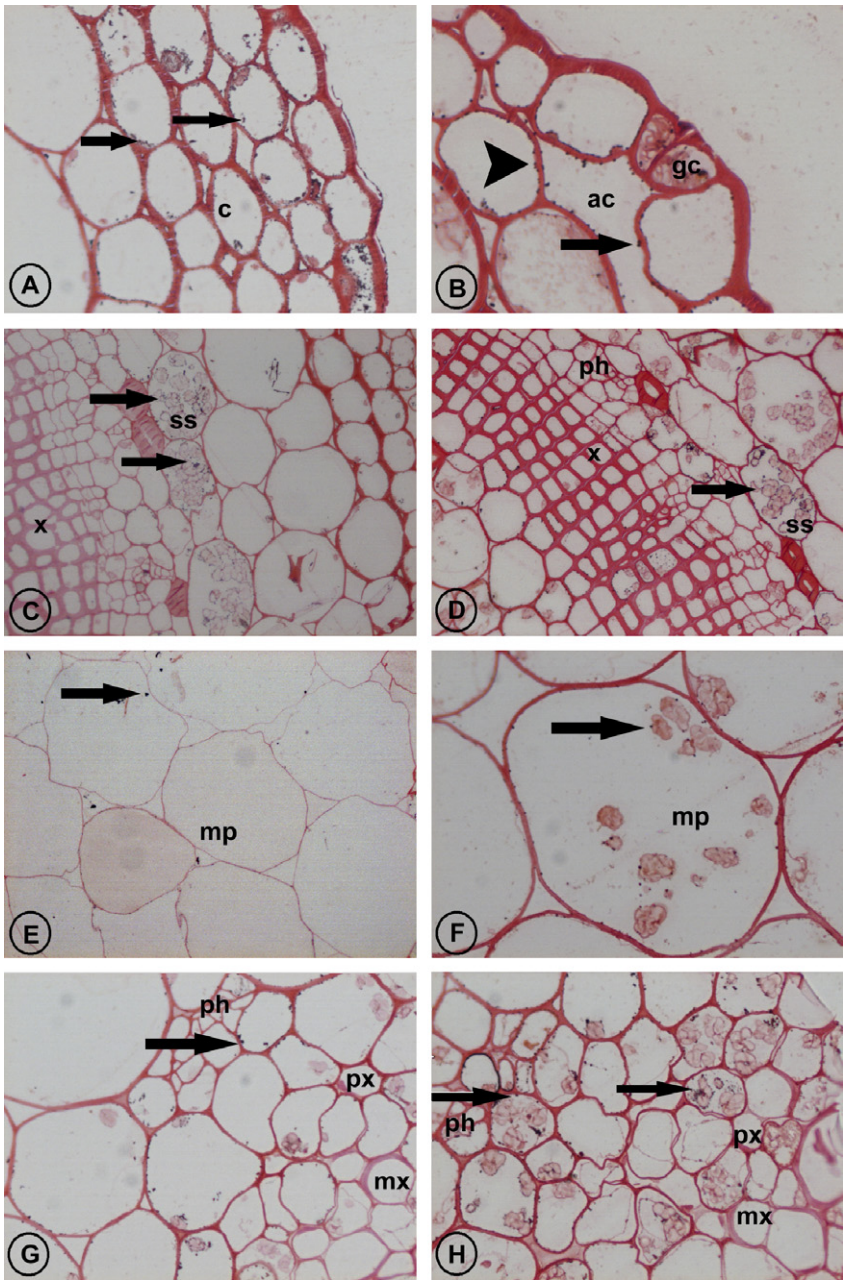


Fig. 5. Light microscopy images of stem cross-sections of plants treated with EDDS (A, C, E, G) or EDTA (B, D, F, H).

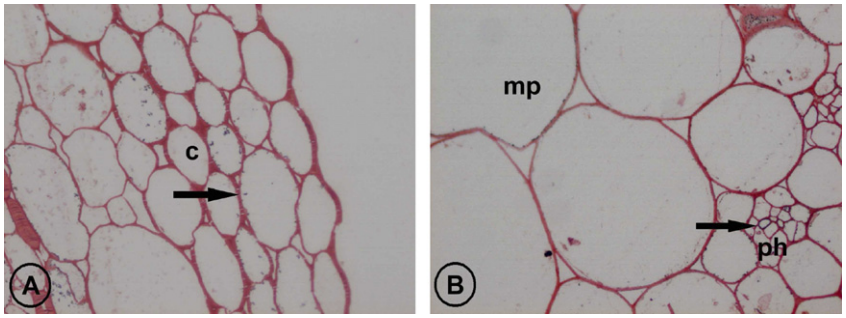


Fig. 6. Light microscopy images of stem cross-sections of plants without without chelating agent treatment.

disadvantages of the technique is the possible toxicity of high levels of metals present in the soil to plants (Glass, 2000). If only *S. nigrum* was applied to the local soil (L), a minimum of 23 crops would be necessary to achieve soil cleanup, according to those standards, while the extra-Zn spiked soil would have to undergo a minimum of 42 crops of the plant. This would correspond to a time span of ca. 12–21 years. In general, all the other treatments applied (AMF and/or chelating agents) increased the soil Zn decontamination abilities of the plant. In some cases this increase was similar or higher with the sole inoculation with AMF than with the application of chelating agents, but the application of EDTA, especially when associated with one of the AMF [*G. intraradices* in the case of the local soil (L) and *G. claroideum* in the case of the extra-Zn spiked (L + Zn) soil] appears as the treatment that is able to enhance to significantly higher levels the Zn taken up from the soil by *S. nigrum*. These offtakes of Zn would imply a minimum sequence of 13 and 14 crops to achieve soil cleanup, for the local (L) and the extra-Zn spiked (L + Zn) soils respectively, which would correspond to a time spam of ca. 7 years. Despite the possible time span reduction brought by a chelate enhanced strategy, phytoextraction using the tested combinations is still a time-consuming remediation strategy and the application of EDTA has some risks at the level of metal percolation.

## Conclusions

This study demonstrated that EDDS and EDTA are equally efficient in increasing the concentrations of Zn in the tissues of *S. nigum* grown in metal contaminated soils. The lower toxicity of EDDS is indicated by a reduced decrease in the AMF colonisation of *S. nigrum* roots, and by less toxicity signs in the plants exposed to that chelating agent, when compared to EDTA. In addition, the level of Zn availability in water after harvest when EDDS was applied to the soil was similar or even lower than that registered for the EDTA-treated soil. However, EDTA in association with one of the tested AMF appeared as the soil treatment that enhanced to higher levels the amount of Zn taken up from the soil to the aboveground tissues of the plant, thus being the treatment that better promoted the phytoextraction abilities of *S. nigrum* and reduced the time-consumption inherent to this technique.

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