Plantago lanceolata growth and Cr uptake after mycorrhizal inoculation in a Cr amended substrate

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Arbuscular mycorrhizal fungi from two chromium contaminated sites, one with 275 mg kg^{-1} of Cr (zone A) and the other with 550 mg kg^{-1} Cr (zone B), were multiplied and tentatively identified. The effect of both fungal consortia on *Plantago lanceolata* plant growth in a substrate amended with 200 mg kg^{-1} of Cr and with 400 mg kg^{-1} Cr was assessed and compared with the growth of plants inoculated with *Glomus intraradices* BEG72. Only the plants inoculated with *G. intraradices* BEG72 and with the fungal consortia obtained from the area with a high Cr contamination (zone B) grew in the soil with 400 mg kg^{-1} of Cr. The consortia of fungi from zone B, decreased the plant's uptake/translocation of the heavy metal compared with *G. intraradices* BEG72. These results underscore the differential effect of AM fungi in conferring bioprotection in Cr contaminated soils.

Key words: arbuscular mycorrhizal fungus, heavy metals, chromium contamination

Introduction

Chromium is a common element of rocks, especially of those of basic and ultramafic origin, and thus soils resulting from them are correspondingly rich in this element. However in other soils, elevated concentrations of chromium are most likely caused by contamination. Chromium has been used for a wide range of industrial applications, including steel, chrome plating, metal finishing, wood preservatives, dyes, leather tanning, textiles and other chemical manufactures (Bewley et al. 2000). Chromium may exist in nine oxidation states (Barnhart 1997) but in natural soil systems the most stable and common forms are chromium (III) and chromium (VI). These two main oxidation states of chromium significantly differ in their biological, geochemical and toxicological properties (Sule and Ingle 1996). Whereas Cr (III), over a narrow concentration range, is considered to be less toxic, Cr (VI) salts have severe toxic effects on humans (Kornhauser et al. 2002). Given the difference in toxicity between the two oxidation forms, the overall objective of most treatments of Cr (VI) contaminated wastes has been that of chemical reduction to Cr (III). The sludge obtained from Cr (III) contaminating industries, like tanneries, or originating from the chemical treatment of Cr (VI) has a high concentration of Cr (III). Before the implementation of international regulations on heavy metal contamination, the sludge was collected and used to be disposed on land, where a subsequent soil treatment system needed to be implemented to reduce the impact of the contamination. Besides the processes involving soil treatments and land filling, the final restoration step

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involves the re-vegetation of the polluted site. Also high levels of chromium can be found in agricultural soils that have been fertilised with composted urban wastes, or in areas that have been exposed to the pollution through airborne emissions of incineration facilities and power stations. Toxic Cr (III) levels in most agronomic plants range from 5 to 100 mg kg^{-1} of available Cr (III) in soil (Ghosh and Singh 2005). The bioavailability of the heavy metal in the soil is of great concern because plants may take up excessive amounts and pass it into the food chain.

The effect of arbuscular mycorrhiza in plant growth and nutrition has been well documented (Smith and Read 2008), however, the effect of the symbiosis in the uptake of heavy metals can vary depending on the fungal isolate, the plant and the metal concerned (Orlowska et al. 2005). With the aim of studying the use of arbuscular mycorrhizal fungi (AMF) for improving plant growth and/or the plant's establishment on polluted soils, in the present work we compared the effect of *Glomus intraradices* (BEG72) with the effect of two consortia of native endophytes obtained from contaminated sites, on the growth and chromium uptake of *Plantago lanceolata* L. grown on soil amended with Cr (III). *Plantago lanceolata*. was selected as a model plant because it is strongly mycorrhizal, common to a wide range of habitats and has been suggested as a potential indicator of soil toxicity (Djingova et al. 2003).

Materials and methods

Isolation of AM fungi from chromium contaminated soils

The study site was in an industrial zone located in the right margin of the Llobregat river (Barcelona, Spain), at 4 km from the river's mouth. The alluvial soil of the zone presented yellow patches in the surface, and was next to an old abandoned industrial factory producing products that contained chromites used for the manufacturing of dyes, chrome plating products and leather tanning and textile products.

The selected study area was in the outer part of the yellow patch, and there, two sampling sites were chosen. The first one was located next to the heavily chromium contaminated zone where few plants were present (zone B). The second one was 100 m apart (zone A). In the sampling sites the vegetation was scarce but *P. lanceolata* was growing in both locations.

The soil sampled from the study area was a sandy loam heavily carbonated, with an alkaline pH (Table 1). There was no soil structure, in accordance with the probable origin of the soil as a mixture of refuse from building yards and added external contamination with chrome.

Table 1. Physico-chemical properties of the soil. Data are the average value of six combined samples.

Parameter	Value
Texture	Sandy loam
Organic matter (%)	0.84
pH (H_2O)	8.73
pH (KCl)	8.35
E.C. (dS/m)	2.87
Carbonates (%$CaCO_3$)	29.4
Ca (mg/kg)	18197
Mg (mg/kg)	490
Na (mg/kg)	4477
K (mg/kg)	240
CEC (meq/100g)	69.6
N (Kjeldahl) (%)	0.1
P (Olsen) (mg/kg)	7
Total Cr (extr. aqua regia) (mg/kg)	275 (zone A) 550 (zone B)

Five composite rhizosphere soil samples were taken from both zones at 20 cm deep from *P. lanceolata*, *Taraxacum* sp., *Medicago* sp. plants and from plants belonging to an undetermined genus of the poaceae family. Roots of the sampled plants were stained to determine AM root colonization (Pitet et al. 2009). Three replicates were used per each sampled plant. To recover the AM fungi that might be present but not sporulating, native *P. lanceolata* plants, growing in both locations, were transplanted in 1 l containers with sterile sand, taking care to preserve the root system, and leek seedlings were transplanted in the same pot.

The remaining soil samples were combined to yield one sample per location. The resulting soils were divided in two portions. One portion was dried at 35ºC and used for soil physicochemical analysis. The other portion was used to isolate the native arbuscular mycorrhizal fungi that might be present. As a first step, 100 g of soil were processed (Gerdemann and Nicolson 1963) to extract AM spores from the samples. Different morphotypes of AMF spores recovered were identified on the basis of spore size, colour, wall structure, and hyphal attachments.

Multiplication of AM native fungi

After 8 weeks growth, leeks growing in the pots with native *P. lanceolata* were uprooted and transplanted to pots filled with sterile sand to produce inoculum for the growth experiment. Plants were harvested after 4 months growth.

Inoculum production

The production of inoculum from the isolate *G. intraradices* BEG 72 (Camprubí and Calvet 1996) was done using colonised leek roots that were used to inoculate leek plantlets. The BEG 72 isolate of *G. intraradices* is a native arbuscular fungus isolated from a citrus nursery of the mediterranean area (Camprubí and Calvet 1996). It was and has proved effective in many agricultural and landscape restoration situations (Calvet et al. 2001, Estaún et al. 2007).

The production of the inocula from the AM fungi consortia recovered from the contaminated site and of *G. intraradices* BEG 72 was done in parallel.

Plant growth experiment

The experiment had a factorial design with two main factors: Inoculation and Chrome Contamination. The Inoculation factor had 4 levels: non-inoculated, inoculated with the isolate *G. intraradices* BEG 72, inoculated with the consortia from zone A, and inoculated with the consortia from zone B. The Chrome Contamination factor had three levels equivalent to the added chrome to the potting mix: 0 mg kg^{-1}, 200 mg kg^{-1} and 400 mg kg^{-1}. The experiment thus had 12 different treatment combinations that were replicated 7 times.

Plantago lanceolata seeds were germinated in 10 cm deep seed trays filled with autoclaved sand where a layer of each respective inoculum was distributed at 5 cm depth. One liter pots were filled with a pasteurized (at 90 ºC during 60 minutes, repeated three times) mixture of sandy soil, quartz sand and sphagnum peat substrate (3:2:1,v/v). The substrate had low P content (8 mg kg^{-1}) and a pH of 7.8 and it was spiked with chromium chloride ($CrCl_3$), added as a water solution to each pot to achieve a soil concentration 200 and 400 mg kg^{-1} of chromium respectively. Chromium (III) was chosen as the contaminant because being less toxic than Cr (VI), many remediation practices end at the reduction of Cr (VI) to Cr (III) by physicochemical processes. Plants were kept in a greenhouse (25–18°C day/night) and watered when needed.

After 6 weeks growth in the seed tray, once AM colonization was established, *P. lanceolata* plants were transplanted to the pots where the contaminant had already been added. Each experimental unit was a pot with three *P. lanceolata* plants. Survival was recorded and after another 8 weeks growth plants were harvested. Each plant was treated separately when determining shoot weight and heavy metal concentration; however, the root system of each pot was considered a whole unit when measuring AM colonisation. The dried shoots were milled and mineralised in concentrated HNO_3 at 155 ºC during 2 hours with vapor control. The heavy metal content was determined by atomic absorption spectrometry. Bioconcentration factors (BCF) were calculated by dividing the total heavy metal concentration in the soil by the heavy metal concentration in the above-ground plant tissue (McKone and Maddalena 2007). Root samples were stained with 0.05 % trypan blue in lactic acid (Phillips and Hayman 1970, Koske and Gemma 1989). The percentage of root colonisation was determined using the grid-line intersect method (Giovannetti and Mosse 1980).

Statistical analysis

Data from the plant growth experiment were analyzed by a one way ANOVA for each Cr (III) treatment followed by a Tukey posthoc analysis. The bifactorial statistical analysis was not performed, as plants from two treatments did not survive at the end of the experiment. The statistical program used was SAS System for Windows V.8.0.

Results

Isolation of AMF from contaminated soils

Morphological spore identification suggested that there was a low AMF species diversity in chromium contaminated soils. Only two morphotypes were isolated in each contaminated soil sample, one with a bigger spore size, and the other one with smaller spore size. All of them were identified as *G. intraradices*.

When roots from the leek plants planted next to the native *P. lanceolata* plants were observed under a dissecting binocular, typical *G. intraradices* structures were observed. Thus, the spore morphological identification present in the chromium contaminated soils was confirmed.

Effect of AMF inoculation on plant growth in a substrate amended with Cr

In the highest Cr (III) concentration treatment, 400 mg kg^{-1}, *P. lanceolata* plants non-inoculated with AMF and *P. lanceolata* plants inoculated with the AMF consortia from zone A did not survive until the end of the experiment. Considering plant growth, when the substrate was not amended with Cr (III), plants inoculated with the fungal consortia from zone A were the only ones presenting significantly higher shoot dry weight (Fig. 1A). However, at increasing concentrations, the highest growth was observed in the plants inoculated with *G. intraradices* BEG 72 and with the fungal consortia isolated from the area with the highest concentration of chromium in the soil (zone B). Mycorrhizal root colonization ranged from 30% to 50% in the AMF-inoculated treatments with a high variability, and no significant differences were found between the different fungi assayed (Fig. 1B). The heavy metal concentration in leaves of plants growing in the substrate amended with 200 mg kg^{-1} Cr (III) was the highest in the non-inoculated treatment, and was the lowest in plants inoculated with both AMF consortia from zone A and zone B. In the substrate amended with 400 mg kg^{-1} of Cr (III) *P. lanceolada* plants inoculated with *G. intraradices* accumulated more Cr (III) in the leaves compared to the plants inoculated with the AMF consortia from zone B (Fig. 1C). Data from BCF followed the same pattern (Fig. 1D).

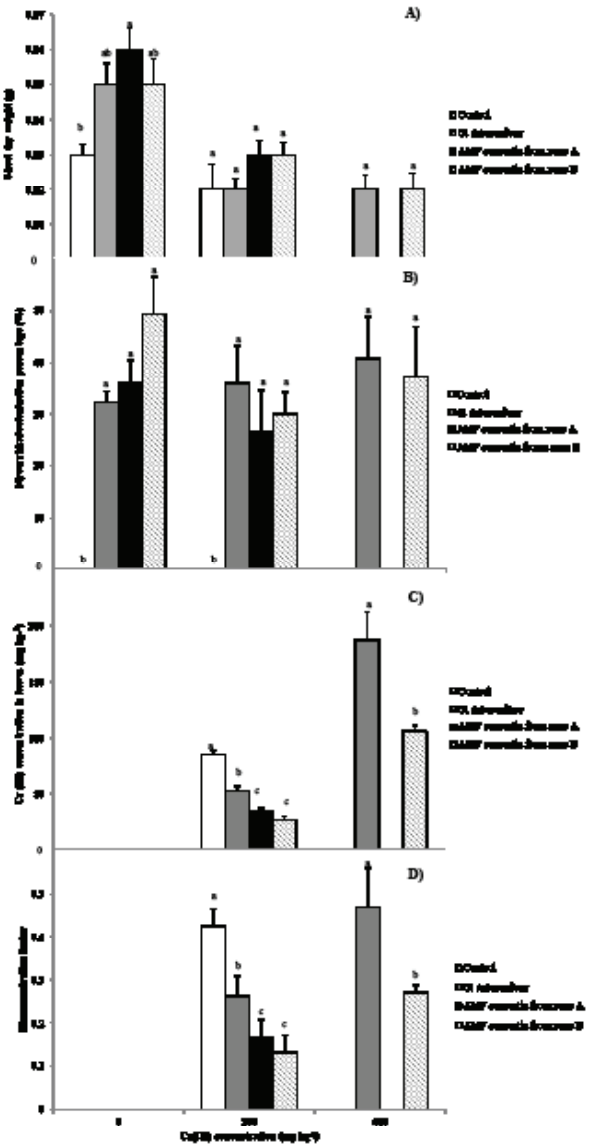

Fig. 1. Shoot biomass (A), mycorrhizal colonization percentage (B), Chromium (III) concentration in leaves (C) and Chromium (III) Bioconcentration Factor (D) of *Plantago lanceolata* plants inoculated with *Glomus intraradices* BEG 72, with two AMF consortia isolated from two chromium contaminated zones or non-inoculated plants. Plantago lanceolata plants were grown 8 weeks in the presence of 0, 200 and 400 mg kg-1 of Cr (III). Data were compared by one way ANOVA for each chromium treatment followed by a Tukey post hoc test. In (B) data were transformed to arcsine prior to the ANOVA analysis.

Discussion

The inoculation with AMF can be a strategy to accelerate the revegetation process in areas polluted by heavy metals (Karimi et al. 2011). Few studies (e.g. Jordao et al. 1997, Farmer et al. 2006, Estaún et al. 2010) have dealt with chromium toxicity although it is a common occurrence among many industrial activities, such as tanneries and metal plating with chromates. Although the contaminated sites are not widespread, they are abundant worldwide. *Plantago lanceolata* is a well-studied species, tolerant

to many environmental stresses, strongly mycorrhizal and has been considered useful to measure soil toxicity. In non-contaminated soils *P. lanceolata* can survive and grow without the symbiosis, however, when Cr is added to the soil, the survival of *P. lanceolata* decreases rapidly and most of non-inoculated plants do not survive at concentrations higher than 100 mg kg^{-1} of Cr (III) (Estaún et al. 2010). In our case, few non-mycorrhizal plants survived in the presence of Cr (III) concentrations of 200 mg kg^{-1} and those that survived grew badly and accumulated higher concentrations of chromium (Figure 1C). In the highest Cr (III) concentration treatment any of the non-mycorrhizal plants survived. Also in the mycorrhizal treatments, *P. lanceolata* plants inoculated with the AMF fungal consortia from zone A, which had 275 mg kg^{-1} of chromium content in the soil (Table 1), did not survive at 400 mg kg^{-1} Cr (III) concentration. The transplant stress might have contributed to the low survival of the non-inoculated plants and plants inoculated with the consortia from the zone A in the presence of the highest Cr (III) concentration, suggesting that not only *P. lanceolata* plants are dependent on the mycorrhization to survive after transplant in those substrates, but also the AMF isolate or isolates which are associated to the roots are important to ensure their survival.

In the lowest Cr (III) concentration treatment, 200 mg k^{-1}, differences were found in the plant shoot biomass, non-inoculated plants being those with the lowest growth. Chromium (III) uptake was reduced in all mycorrhizal plants when compared with non-mycorrhizal ones (Fig. 1C), but this effect was dependent on the fungus/consortia of fungi that were forming the symbiosis. In a previous study, Estaún et al. (2010) also found that *P. lanceolata* mycorrhizal plants took-up less Cr than non-mycorrhizal plants, and in those plants the higher concentration of Cr was found in roots. However, the opposite response has been also observed in other plants. *Helianthus annuus*, which has a low tolerance to Cr (Shahandeh and Hossner 2000), had an increased Cr tolerance and uptake through inoculation with *G. intraradices*. Davies et al. (2001), working with a range of both Cr (III) and Cr(VI) concentrations, found that although mycorrhizas reduced *H. annuus* plant stress due to Cr toxicity, the symbiosis enhanced Cr concentration both in shoots and roots. It has been shown that the result of mycorrhizal colonisation in heavy metal uptake depends on the plant–fungus combination and on the heavy metal type, and those are influenced also by soil conditions. This could explain the diversity of results found in the literature.

The effect of the inoculation with *G. intraradices* BEG 72, with the AMF consortia from zone A and with the AMF consortia from zone B was different in *P. lanceolata* plants. In 200 mg kg^{-1} Cr (III) concentration treatment, the highest shoot biomass was observed in plants inoculated with both indigenous consortia of the chromium contaminated soils, and those had also the lowest Cr (III) concentration in the shoots. Several studies have reported that AMF from metal-contaminated sites have developed tolerance against metal toxicity and are well adapted (Weissenhorn et al. 1993, 1994, Leyval and Joner 2001, Toler et al. 2005, Sudova et al. 2007). It has been observed that certain AMF isolates from contaminated soils stimulate plant growth better than non-indigenous isolates, increase host heavy metal tolerance or reduce the heavy metal uptake into plant tissues (reviewed by Gaur and Adholeya 2004). However, there are also contrasting results, e.g Rydlova and Vosatka (2001) who reported low effectivity of the native AMF from mine spoil banks when compared to a non-native *Glomus fistulosum* Skou & Jakobsen isolate.

In the highest Cr (III) concentration, even if no differences were found in shoot biomass between the non-native *G. intraradices* isolate and the consortia from zone B, the Cr (III) concentration in the shoots was higher in the plants inoculated with *G. intraradices* BEG 72. It has been shown that AM fungal isolates differ in their effect on heavy metal uptake by plants. Some reports indicate higher concentrations of heavy metals in plants due to AM, whereas others have found a reduced plant concentration, as in our case (reviewed by Gaur and Adholeya 2004). The effect of AMF in decreasing heavy metal stress has been

assigned to the selective immobilisation of the toxic metal within the root tissues that are colonised by the fungus (Kaldorf et al. 1999) or to the high metal absorption capacity of the extraradical mycelium of the AMF (Joner et al. 2000). Our results with Cr are consistent with a buffering effect of the AMF increasing the protective effect against this heavy metal contamination while decreasing the intake and the translocation of the contaminant to the shoots. This effect was most important in the case of the AMF consortia from the highly contaminated soil. Christie et al. (2004) have also shown an alleviation effect of Zn toxicity by AMF, decreasing the Zn translocation towards the shoot. They suggested a direct effect of the AMF through adsorption and binding of the heavy metal in the mycorrhizosphere and an indirect effect through the improvement of the plant nutrition.

In view of the different response of the plants inoculated with AMF isolated from Cr contaminated zones and non-native AMF isolates, it is important to make a good choice of the AMF isolates that will be used in the phytoremediation strategies, as not all the isolates have the same ability to bind heavy metals into roots, restricting in this way their translocation into shoot tissues.

The use of mycorrhizal plants as a tool in phytoremediation strategies needs further research to understand the mechanisms involved in the plant's protection against metal toxicity. These research efforts will help to integrate this biotechnology in agricultural and environmental engineering processes.

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