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Comparative effects of native filamentous and arbuscular mycorrhizal fungi in the establishment of an autochthonous, leguminous shrub growing in a metal-contaminated soil

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ABSTRACT

The aim of this study was to assess the effectiveness of inoculation with a native arbuscular mycorrhizal (AM) fungus, *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, or a filamentous fungus, *Penicillium aurantiogriseum* Dierckx 1901, on the establishment of *Coronilla juncea* L. seedlings grown in a polluted, semiarid soil. For that, root and shoot biomass, nutrient uptake, mycorrhizal colonisation and nitrate reductase (NR) and phosphatase activities were analysed. Six months after planting, the shoot biomass of *C. juncea* was increased only by the inoculation with *G. mosseae* (by about 62% compared with non-mycorrhizal plants). The shoot NR and root acid phosphatase activities were increased more by inoculation with *G. mosseae* than with *P. aurantiogriseum* inoculation. The root NR activity and foliar nutrient contents were increased only by the inoculation with the AM fungus. The root Zn and Cu decreased with the AM fungus. In conclusion, the autochthonous AM fungus was an effective inoculant with regard to stimulating growth and alleviating heavy metal toxicity for plants growing on a soil contaminated by multiple heavy metals. Inoculation with an autochthonous, filamentous fungus does not seem to be a good strategy for phytoremediation of such problematic sites.

1. Introduction

The establishment of an autochthonous plant cover is a commonly-used approach for the remediation of mine tailings (Mendez and Maier, 2008; Carrasco et al., 2009). The canopy serves to reduce eolian dispersion while plant roots help prevent water erosion and leaching. However, in Mediterranean, semiarid areas the low productivity of the soil and the water deficit seriously limit plant growth. There is evidence that arbuscular mycorrhizal (AM) fungi can benefit plant nutrition and enhance plant tolerance of heavy metal pollution, in part by immobilization of metals within or near the root and reducing their translocation to the shoot (Göhre and Paszkowski, 2006). In this context, the use of these fungi, as plant inoculants, is being investigated for phytoremediation of heavy metal-polluted soils (González-Guerrero et al., 2009). As is well-known, these fungi form mycorrhizal symbioses with most plant species and occur in almost all habitats and climates, including disturbed soils such as those derived from mine activities (Del Val et al., 1999). The role of AM fungi in plant acquisition of N may be ascertained by measuring the activity of nitrate reductase (NR). This is the first enzyme in the nitrate assimilation pathway and represents the rate-limiting step in this process (Campbell, 1988). NR is considered to be a limiting factor for the growth, development and protein production of plants, and has been demonstrated to be highly sensitive to metal toxicity (Singh et al., 1997). An increase in NR activity due to the AM symbiosis has been observed (Caravaca et al., 2003), as has analogous enzymatic activity in AM fungi (Ho and Trappe, 1980).

Toxic metals can also affect adversely the number, diversity and activity of soil organisms, resulting in the selection of the most metal-resistant microorganisms. Therefore, autochthonous microorganisms isolated from polluted environments are likely to play an important role in the growth and metal tolerance exhibited by plants growing in metal-contaminated soils. Fungi are known to tolerate and detoxify metals by several mechanisms, including extra- and intracellular precipitation, biosorption to the cell wall and sequestration (Zafar et al., 2007). Additionally, fungi can alleviate heavy metal stress by exuding enzymes, such as acid phosphatase — that catalyses the hydrolysis of various phosphate esters in an acidic environment. Thus, this resistance may be the key to plant survival on contaminated soils (Colpaert and Vandenkoornhuyse, 2001). To the best of our knowledge, there are no previous studies indicating whether the inoculation of fungal strains isolated from heavy metal-contaminated soils may enable plants to thrive under polluted, semiarid conditions.

Among the beneficial fungi in the rhizosphere are the phosphate-solubilising, filamentous hyphomycetes, such as *Penicillium*

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aurantiogriseum (Illmer and Schinner, 1995a). Their effects may be of great interest in soils with scarce assimilable P, such as those in polluted, semiarid ecosystems. The microbiologically-solubilised phosphate could, however, be taken up by a mycorrhizal mycelium, thereby developing a synergistic microbial interaction (Barea et al., 1997). The combined inoculation of selected rhizosphere microorganisms has been recommended for maximising plant growth and nutrition (Probanza et al., 2001). The study of the antagonic or synergic effects of the different microbial inoculants when co-inoculated is a crucial step in the development of effective host-microorganism combinations. In previous studies, it was shown that the presence of *Aspergillus niger* stimulated the growth of ectomycorrhizal shrub species (Caravaca et al., 2005). It has been reported also that dual inoculation with *Glomus intraradices* and *Bacillus subtilis* promoted the establishment of the introduced AM fungus and increased plant biomass and tissue P accumulation (Toro et al., 1997). However, reports on the co-inoculation of AM fungi with phosphate-solubilising fungi (*Penicillium*) in polluted environments are uncommon.

We hypothesised that the inoculation with native AM and filamentous fungi can help autochthonous seedlings (*Coronilla juncea*) to establish in a semiarid soil contaminated by multiple heavy metals and that their effects can be estimated by determining the enzymatic activities of NR and phosphatase.

2. Materials and methods

2.1. Study sites

The study area was located in the La Unión mine district (southeast Spain). The terrain is low lying (<400 m), but with steep slopes (20–30%) because of its proximity to the coast. The climate is semiarid Mediterranean with an annual rainfall around 250–300 mm and a mean annual temperature of 17.5 °C; the potential evapo-transpiration reaches 1000 mm y^{-1}. This zone constituted an important mining nucleus for more than 2500 years. The ore deposits of this zone have iron, lead and zinc as the main metal components. Iron is present in oxides, hydroxides, sulfides, sulfates, carbonates and silicates; lead and zinc occur in galena, sphalerite, carbonates, sulfates, and lead- or zinc-bearing (manganese, iron) oxides (Oen and Fernández, 1975). In this area a neutral mine tailing called "Gorguel" (U.T.M. X687480 Y4162800 Z135, Length: 200–300 m, Width: 95 m, Height: 25 m, Volume: 750,000 m^3, IGME, 1999) was selected (Carrasco et al., 2009). Three soil samples were taken from the tailing. Each soil sample consisted of a mixture of 6 sub-samples randomly taken from the top 20 cm depth of soil. The analytical characteristics of the mine tailing are shown in Table 1.

Table 1
Physico-chemical characteristics of the soil used in the experiment.

pH (H_2O)	7.70
Electrical conductivity (1:5, dS m^{-1})	1.5
$CaCO_3$ (%)	<5
Total Organic Carbon (%)	0.4
Total N (%)	0.02
Clay (%)	5
Silt (%)	24
Sand (%)	71
Total P (g kg^{-1})	6.4
Available P (mg kg^{-1})	7
Total Pb (mg kg^{-1})	5279
Total Zn (mg kg^{-1})	7509
Total Cu (mg kg^{-1})	262

2.2. Materials

The strain of *Penicillium aurantiogriseum* Dierckx 1901 used was isolated from the experimental area by standard serial dilution technique using rose bengal agar. *P. aurantiogriseum* was grown in a medium (nutrient broth, Scharlau Chemie, Spain) composed of meat and yeast extracts, peptone and sodium chloride, for 7 days at 25 °C on a Heidolph Unimax1010 shaker. The fungal culture was centrifuged at 4000 rpm for 5 min at 2 °C and the sediment was re-suspended in sterilised tap water. The suspension obtained had a concentration of 10^6 CFU mL^{-1}.

The mycorrhizal inoculum, originated from the experimental area, was a *Glomus mosseae* (Nicol and Gerd.) Gerd. and Trappe strain being the most abundant AM spore in this soil (Azcón et al., 2009). The AM inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum bicolor*) containing spores, hyphae and mycorrhizal root fragments. The inoculum had a potential infectivity of about 35 infective propagules g^{-1} inoculum.

Mature seeds of *Coronilla juncea* L. were collected near the mine tailing. The seeds were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) for 5 min and subsequently rinsed thoroughly with sterilized water prior to a wetting treatment with sterilized water for two hours. Seeds were placed in darkness on double layered Whatman No.1 filter paper moistened with distilled water in sterilized Petri dishes until germination.

2.3. Experimental design and layout

The experiment was conducted as a completely randomised factorial design with two factors. The first factor had two levels: non-inoculation or inoculation with *G. mosseae*, and the second had two levels: non-inoculation or inoculation with *P. aurantiogriseum*. Six replicates per treatment were carried out, making a total of 24 pots.

Four hundred grams of air-dried soil were placed in each of 600 mL pots. In early February 2009, *C. juncea* seedlings were transplanted to the pots (three seedlings per pot). When appropriate, the soil was inoculated with 10^7 CFU of *P. aurantiogriseum* per pot. The arbuscular mycorrhizal inoculum was applied at a rate of 5% (v/v). The same amount of the autoclaved inoculum was added to control plants, supplemented with a filtrate (Whatman no. 1 paper) of the culture to provide the microbial populations accompanying the mycorrhizal fungi. The experiment was conducted in a greenhouse, located in the SACE service at the Campus of Espinardo (Murcia, Spain). During the experiment, the temperature ranged from 8 °C to 32 °C, and the relative humidity was between 60% and 80%. Midday photosynthetically active radiation (PAR) averaged 260 μE m^{-2} s^{-1}. Plants were watered regularly with decalcified water, without any fertiliser treatment. Six months after planting, plants were harvested.

2.4. Plant analyses

Fresh and dry mass of shoots and roots (105 °C, 5 h) were recorded.

The percentage of root length colonized by arbuscular mycorrhizal fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after staining with trypan blue (Phillips and Hayman, 1970).

Plant tissues were ground before chemical analysis. The foliar contents of phosphorus were determined, after digestion in nitric-perchloric acid (5:3) for 6 h, by colorimetry (Murphy and Riley, 1962) and the plant K was estimated by flame photometry. The foliar N was determined with the Kjeldahl method, consisting of titration after sample digestion and distillation (Bremner and Mulvarey, 1982). Sub-samples were oven-dried at 480 °C, then the ashes were dissolved in 0.6 M nitric acid and were filtered through an Albert® 145 ashless filter paper. All metals were quantified using an ICP-MS (Agilent

7500A). The precision and accuracy of this method were tested by analysing (five replicates) the CTA-VTL-2 (Dybczyński et al., 1997) and CRM027-050 (Resource Technology Corporation, USA) certified materials, corresponding to Virginia Tobacco leaves and a soil, respectively.

Nitrate reductase activity was assayed *in vivo* by measuring NO_2^- production in tissue that has been vacuum infiltrated with buffered NO_3^- solutions (Downs et al., 1993). The roots and shoots from the seedlings were collected in the morning between 8:30 and 11:00 solar time. Roots and shoots of *C. juncea* were cut into 5-mm sections. Approximately 300 mg of tissue was placed into tubes containing 2 mL of an incubation medium consisting of 0.05 M tris–HCl pH 7.8 and 0.25 M KNO_3. The tubes were sealed and kept in the dark at 30 °C for 1 h. The nitrite released into the medium was determined after incubation by treating 1 mL of the aliquots with 1 mL of 1% sulphanilamide in 1 M HCl and 1 mL of 0.01% N-1-napthyl-ethylene-diamine hydrochloride (NNEDA). After 15 min, the optical density was measured at 540 nm with Beckman spectrophotometer (Keeney and Nelson, 1982).

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two mL of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid and 0.5 mL of substrate were added to 100 mg of fresh root tissue and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

2.5. Statistical analysis

Percentage colonization was arcsin-transformed, and the other parameters were log-transformed to compensate for heterogeneity of variance, before analysis of variance. Both fungal inoculations and their interactions effects on measured variables were tested by a two-way analysis of variance. Statistical procedures were carried out with the software package SPSS 17.0 for Windows.

3. Results

3.1. Plant growth parameters and symbiotic development

The shoot biomass of the *C. juncea* plants was increased only by the inoculation with *G. mosseae* (Tables 2 and 3), producing increases of about 62% with respect to non-mycorrhizal plants. The combined inoculation produced a greater increase in root biomass than each fungal inoculation alone. The mycorrhizal inoculation produced a significant increase in the percentage of root colonisation (Table 3), but had no effect when combined with the fungus *P. aurantiogriseum*. Nodules in the plants of *C. juncea* were clearly present, ranging between 10–14 nodules per gram dry root, but with no significant differences among the treatments (data not shown).

3.2. Nutrient acquisition

Mycorrhizal inoculation increased the contents of nutrients in shoots of *C. juncea* (Table 2), particularly foliar P (76% higher than in the non-mycorrhizal plants). However, inoculation with the filamentous fungus had no significant effect on the foliar nutrient levels. Mycorrhizal inoculation increased the root nutrient contents to a significantly-greater extent than fungal inoculation (Tables 2 and 3).

Table 2
Shoot and root biomass, colonisation and nutrients of *C. juncea* seedlings in response to filamentous and AM fungi inoculations six months after planting (N = 6).

	Control	*G. mosseae* (M)	*P. aurantiogriseum* (P)	M × P
Shoot biomass (g dw plant^{-1})	0.17 ± 0.01b	0.45 ± 0.05a	0.17 ± 0.02b	0.51 ± 0.04a
Root biomass (g dw plant^{-1})	0.09 ± 0.01bc	0.18 ± 0.02b	0.13 ± 0.02b	0.38 ± 0.01a
Colonisation (%)	29.0 ± 0.1b	54.2 ± 2.7a	28.0 ± 0.7b	35.2 ± 1.5b
Foliar N (mg plant^{-1})	2.1 ± 0.2b	3.7 ± 0.1a	1.8 ± 0.1b	4.6 ± 0.3a
Foliar P (mg plant^{-1})	0.14 ± 0.03b	0.30 ± 0.02a	0.13 ± 0.01b	0.29 ± 0.02a
Foliar K (mg plant^{-1})	2.26 ± 0.33b	2.75 ± 0.32ab	1.90 ± 0.22b	4.66 ± 0.67a
Root N (mg plant^{-1})	1.07 ± 0.06c	2.22 ± 0.09b	1.63 ± 0.08b	4.78 ± 0.19a
Root P (mg plant^{-1})	0.06 ± 0.02a	0.08 ± 0.02a	0.12 ± 0.03a	0.14 ± 0.03a
Root K (mg plant^{-1})	887 ± 22c	2155 ± 30a	1426 ± 21b	2929 ± 33a

Mean ± standard error.
Values in rows, followed by the same letter, do not differ significantly (P<0.05) as determined by the Tukey test.

3.3. Nitrate reductase and phosphatase activities

Inoculation with *G. mosseae* was the most-effective treatment with regard to increasing the NR activity in the shoots of *C. juncea*, followed by inoculation with *P. aurantiogriseum* (Table 4). The NR activity in the roots of *C. juncea* seedlings was lower than in shoots and was only increased significantly by the inoculation with the AM fungus.

The acid phosphatase activity in the roots was increased by both mycorrhizal and fungal inoculation (Table 4), without significant differences between treatments.

3.4. Heavy metal concentrations

The foliar and root concentrations of Pb were decreased in the plants inoculated with the AM fungus and/or the filamentous fungus (Table 5). The fungal inoculations had no significant effect on the concentrations of shoot Zn and Cu (Table 3). The *P. aurantiogriseum*- and *G. mosseae*-inoculated plants showed the lowest concentrations of shoot Zn, as well as the highest concentrations of shoot Cu. The mycorrhizal inoculation, alone or in combination with the

Table 3
Two factor ANOVA (filamentous and AM fungi inoculations) F probabilities.

	G. mosseae (M)	*P. aurantiogriseum* (P)	M × P
Shoot dry weight	0.001	NS	NS
Root dry weight	<0.001	0.002	0.031
Colonization	0.014	NS	NS
Foliar N	<0.001	NS	NS
Foliar P	0.001	NS	NS
Foliar K	0.046	NS	0.001
Root N	0.001	0.033	0.026
Root P	NS	NS	NS
Root K	0.001	0.045	NS
Shoot Nitrate reductase	<0.001	<0.001	NS
Root Nitrate reductase	<0.001	NS	NS
Root Acid phosphatase	<0.001	<0.001	NS
Shoot Pb	0.001	0.001	NS
Shoot Zn	NS	NS	<0.001
Shoot Cu	NS	NS	<0.001
Root Pb	0.001	NS	NS
Root Zn	0.001	NS	NS
Root Cu	0.005	0.023	0.041

NS: not significant.

Table 4
Nitrate reductase (NR) and acid phosphatase activities in root and leaf of *C. juncea* seedlings as affected by fungal and mycorrhizal inoculations six months after planting (N = 6).

	Control	*G. mosseae* (M)	*P. aurantiogriseum* (P)	M X P
NR activity (leaf) (nmol NO_2^- g FW^{-1} h^{-1})	0.014 ± 0.000b	0.035 ± 0.002a	0.026 ± 0.001ab	0.038 ± 0.001a
NR activity (root) (nmol NO_2^- g FW^{-1} h^{-1})	0.07 ± 0.01b	0.17 ± 0.03a	0.07 ± 0.00b	0.06 ± 0.01b
Acid phosphatase (root) (μmol PNP g^{-1} h^{-1})	10.8 ± 0.6b	13.7 ± 0.5a	12.7 ± 0.4a	12.2 ± 0.3a

Mean ± standard error.
Values in rows, followed by the same letter, do not differ significantly (P<0.05) as determined by the Tukey test.

filamentous fungus, was effective at decreasing the root concentrations of Zn and Cu.

4. Discussion

In this study, the inoculation with *G. mosseae* was the most-effective treatment with respect to stimulating the production of shoot biomass for *C. juncea* plants grown in a polluted soil. In contrast, *P. aurantiogriseum* was not able to promote their growth. This type of filamentous fungus is able to excrete H^+ into the soil and solubilise "insoluble" phosphate, making it available to plants (Illmer and Schinner, 1995a). However, there were no differences in the foliar phosphorus content between inoculated and control plants. The scarcity of nutrients in this soil might be the reason for the lack of effect of *P. aurantiogriseum* on the plant growth. The findings of this study agree with those of previous ones which also showed that plant-available phosphorus was only increased when *P. aurantiogriseum* disposed of a sufficient nutrient supply (Illmer and Schinner, 1995b). Total plant nutrient contents can be taken as a representative indicator of mycorrhizal effectiveness, because they take into account the well-balanced effects of nutrient acquisition and biomass production (Jeffries et al., 2003). The mycorrhizal inoculation treatments appeared effective on improving nutrient contents. The fact that the foliar N, P and K contents of plants inoculated with the AM fungus were higher than those of non-inoculated plants reaffirms the key role of mycorrhizae in sustaining the plant cover in nutrient-deficient soils. The greater effectiveness of *G. mosseae* was related to the extent of mycorrhizal infection, because the local AMF community showed little capacity for colonisation of shrub roots and was much-less effective than the added, native Glomus inoculum at stimulating host plant growth for *C. juncea*.

Exposure of plants to heavy metals may result in the alteration of nutrient assimilation as a consequence of the inhibition of the activities of enzymes involved in nutrient cycles, such as NR (Xiong et al., 2006) and root acid phosphatase (Cumming and Ning, 2003; Tamás et al., 2008). For example, copper is an essential micronutrient for normal plant metabolism but it has been reported to be among the toxic heavy metals (Xiong et al., 2006). Excessive Cu accumulated in plant tissue can be toxic, affecting several physiological and biochemical processes and growth. Exposure to elevated Cu concentrations results in increased levels of free amino acids and inhibition of NR activity, reducing the total nitrogen concentration (Xiong et al., 2006). In the present study, the Cu concentrations in the roots of the control plants and of those inoculated with the filamentous fungus could have been toxic to the plants (Balsberg Påhlsson, 1989). However, the degree of toxicity is dependent on the nutritional status of the plants. In this sense, phosphorus deficiency may enhance the toxicity of Cu. The significant reduction in root biomass for the control plants could have been due to the toxic effects of Cu. Our results also demonstrate adverse effects of Cu on N metabolism: Cu exposure led to a significant reduction in NR activity in the roots of the control plants and those inoculated with the filamentous fungus. Decreases in NR activity under Cu exposure may involve a direct effect of Cu on nitrate reductase by binding to important sulphydryl groups, to inactivate the enzyme. By contrast, the inoculation with the AM fungus induced a significant increase in the root NR activity, which was accompanied by a lower root concentration of Cu. Some authors have indicated that the increase in the NR activity of mycorrhizal plants with respect to non-mycorrhizal ones could be related to the phosphate requirements of this enzyme (Ruiz-Lozano and Azcón, 1996). In this sense, the mycorrhizal effect could be interpreted as an indirect response to the improved nutrient status, particularly of phosphorus. Thus, the fact that the highest shoot contents of P occurred for plants inoculated with the AM fungus could explain how these plants had the highest values of NR activity. Likewise, this increased enzymatic activity in *G. mosseae*-colonised plants could have been a consequence of fungal NR activity. In fact, Kaldorf et al. (1998) described assimilatory NR activity in mycorrhizal fungi.

The main function of acid phosphatase is to catalyse the hydrolysis of various phosphate esters, increasing the P available to plants and thus enhancing plant P uptake and growth. Phosphatases are inhibited by the final product of the enzymatic reaction, inorganic phosphorus, representing a feed-back inhibition. Moreover, the activity of this enzyme is sensitive to the adverse effects of metals in the soil solution (Juma and Tabatabai, 1977). In addition, it has been observed that the heavy metal inhibition of phosphatase activity depends on the species and concentration (Gabbrielli et al., 1989). Mycorrhizal and fungal inoculation enhanced the acid phosphatase activity in the roots of *C. juncea*. The increased root acid phosphatase activity in plants inoculated with *P. aurantiogriseum* did not stimulate plant growth, suggesting that a high phosphatase activity does not compensate for an inadequate supply of assimilable P to the plant (Azcón and Barea, 1997). In contrast, the phosphatase activity in roots of *C. juncea* was related to the phosphorus status and growth of mycorrhizal plants. Thus, the mycorrhizal inoculum enabled the plants to increase their nutrient uptake, protecting them from heavy metal toxicity. Some authors have suggested that increased acid

Table 5
Metal concentrations in shoot and root of *C. juncea* seedlings in response to fungal and mycorrhizal inoculations six months after planting (N = 6).

	Control	*G. mosseae* (M)	*P. aurantiogriseum* (P)	M X P
Shoot				
Pb (mg kg^{-1})	23 ± 6a	4 ± 2b	5 ± 2b	7 ± 3b
Zn (mg kg^{-1})	137 ± 11a	161 ± 13a	148 ± 10a	95 ± 9b
Cu (mg kg^{-1})	9 ± 2b	9 ± 2b	11 ± 3b	72 ± 8a
Root				
Pb (mg kg^{-1})	387 ± 17a	153 ± 10b	305 ± 11a	113 ± 10b
Zn (mg kg^{-1})	879 ± 21a	342 ± 15b	736 ± 18a	257 ± 13b
Cu (mg kg^{-1})	166 ± 14a	40 ± 9c	93 ± 11b	25 ± 10c

Mean ± standard error.
Values in rows, followed by the same letter, do not differ significantly (P<0.05) as determined by the Tukey test.

phosphatase activity under heavy metal stress is one possible process involved in detoxification and resistance (Zheng et al., 2009). It is worth noting that the existence of this mechanism of heavy metal tolerance in plants inoculated with native AM fungi, involving increased root acid phosphatase activity, has a great adaptive significance as the low phosphate availability in these polluted soils would require both efficient uptake of phosphorus and its recycling within the plant.

In conclusion, the results of this study show that an autochthonous, filamentous fungus was not an effective inoculant with regard to stimulating plant growth and alleviating heavy metal toxicity. Inoculation with an autochthonous, mycorrhizal fungus improved the nutrient status and growth of *C. juncea* plants in a soil contaminated by multiple heavy metals. The enhanced metal tolerance of this plant bestowed by the AM fungus was at least partly due to its influence on the N and P metabolism and nutrient uptake, as well as to reduced uptake of heavy metals by root tissues.

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References

Azcón R, Barea JM. Mycorrhizal dependency of a representative plant species in Mediterranean shrublands (*Lavandula spica* L.) as a key factor to its use for revegetation strategies in desertification-threatened areas. Appl Soil Ecol 1997;7:83–92.

Azcón R, Perálvarez MDC, Biró B, Roldán A, Ruíz-Lozano JM. Antioxidant activities and metal acquisition in mycorrhizal plants growing in a heavy-metal multicontaminated soil amended with treated lignocellulosic agrowaste. Appl Soil Ecol 2009;41:168–77.

Balsberg Påhlsson AM. Mineral nutrients, carbohydrates and phenolic compounds in leaves of beech (*Fagus sylvatica* L.) in southern Sweden as related to environmental factors. Tree Physiol 1989;5:485–95.

Barea JM, Azcón-Aguilar C, Azcón R. Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil–plant systems. In: Gange AC, Brown VK, editors. Multitrophic interactions in terrestrial systems. Cambridge: Blackwell Science; 1997. p. 65–77.

Bremner JM, Mulvarey CS. Nitrogen total. In: Page AL, Miller RH, Keeny DR, editors. Methods of Soil Analysis. Chemical and Microbiological PropertiesMadison: American Society of Agronomy; 1982. p. 595–622.

Campbell WH. Nitrate reductase and its role in nitrate assimilation in plants. Physiol Plant 1988;74:214–9.

Caravaca F, Alguacil MM, Díaz G, Roldán A. Use of nitrate reductase activity for assessing effectiveness of mycorrhizal symbiosis in *Dorycnium pentaphyllum* under induced water deficit. Commun Soil Sci Plant Anal 2003;34:2291–302.

Caravaca F, Alguacil MM, Azcón R, Parladé J, Torres P, Roldán A. Establishment of two ectomycorrhizal shrub species in a semiarid site after *in situ* amendment with sugar beet, rock phosphate, and *Aspergillus niger*. Microb Ecol 2005;49:73–82.

Carrasco L, Caravaca F, Azcón R, Kohler J, Roldán A. Addition of microbially-treated sugar beet residue and a native bacterium increases structural stability in heavy metal-contaminated Mediterranean soils. Sci Total Environ 2009;407:5448–54.

Colpaert JV, Vandenkoornhuyse P. Mycorrhizal fungi. In: Prasad MNV, editor. Metals in the Environment: Analysis by Biodiversity. New York: Dekker; 2001. p. 37–58.

Cumming JR, Ning J. Arbuscular mycorrhizal fungi enhance aluminium resistance of broomsedge (*Andropogon virginicus* L.). J Exp Bot 2003;54:1447–59.

Del Val C, Barea JM, Azcón-Aguilar C. Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environ Microbiol 1999;65:718–23.

Downs MR, Nadelhoffer KJ, Melillo JM, Aber JD. Foliar and fine root nitrate reductase activity in seedlings of four forest tree species in relation to nitrogen availability. Trees (Berl.) 1993;7:233–6.

Dybczyński R, Polkowska-Motrenko H, Samczyński Z, Szopa Z. Preparation and certification of the Polish reference material "Virginia Tobacco Leaves" (CTA-VTL-2) for inorganic trace analysis including microanalysis, Raporty IChTJ, Seria A, Nr 3/97. Warszawa (Poland): Institute of Nuclear Chemistry and Technology; 1997.

Gabbrielli R, Grossi L, Vergnano O. The effects of nickel, calcium and magnesium on the acid phosphatase activity of two *Alyssum* species. New Phytol 1989;111:631–6.

Giovannetti M, Mosse B. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol 1980;84:489–99.

Göhre V, Paszkowski U. Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 2006;223:1115–22.

González-Guerrero M, Benabdellah K, Azcón-Aguilar C, Ferrol N. In: Azcón-Aguilar C, Barea JM, Gianinazzi S, Gianinazzi-Pearson V, editors. Heavy Metal Tolerance in Arbuscular Mycorrhizal Fungi. Mycorrhizas: Functional Processes and Ecological ImpactHeidelberg: Springer; 2009. p. 107–22.

Ho I, Trappe JM. Nitrate reductase activity of nonmycorrhizal Douglas-fir rootlets and of some associated mycorrhizal fungi. Plant Soil 1980;54:395–8.

Illmer P, Schinner F. Solubilization of inorganic calcium phosphates — solubilization mechanisms. Soil Biol Biochem 1995a;27:257–63.

Illmer P, Schinner F. Phosphate solubilizing microorganisms under non-sterile conditions. Bodenkultur 1995b;46:197–204.

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 2003;37:1-16.

Juma NG, Tabatabai MA. Effects of trace elements on phosphatase activity in soils. Soil Sci Soc Am J 1977;41:343–6.

Kaldorf M, Schmelzer E, Bothe H. Expression of maize and fungal nitrate reductase in arbuscular mycorhiza. Mol Plant-Microbe Interact 1998;11:439–48.

Keeney DR, Nelson D.W. Nitrogen-inorganic forms. In: Page AL, editor. Methods of soil analysis, Part 2. Agronomy 9. American Society of Agronomy, Inc, Soil Science Society of America, Inc, Madison, WI, USA, 1982, pp. 643-698.

Mendez MO, Maier RM. Phytoremediation of mine tailings in temperate and arid environments. Rev Environ Sci Biotechnol 2008;7:47–59.

Murphy J, Riley JPA. Modified single solution method for determination of phosphate in natural waters. Anal Chim Acta 1962;27:31–6.

Oen IS, Fernández JC. The lead-zinc and associated ores of La Unión, Sierra de Cartagena, Spain. Econ Geol 1975;70:1259–78.

Phillips JM, Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 1970;55:158–61.

Probanza A, Mateos JL, Lucas JA, Ramos B, de Felipe MR, Gutiérrez-Manero FJ. Effects of inoculation with PGPR Bacillus and *Pisolithus tinctorius* on *Pinus pinea* L. growth, bacterial rhizosphere colonization and mycorrhizal infection. Microb Ecol 2001;41:140–8.

Ruiz-Lozano JM, Azcón R. Mycorrhizal colonization and drought stress as factors affecting nitrate reductase activity in lettuce plants. Agric Ecosyst Environ 1996;60:175–81.

Singh RP, Tripathi RD, Sinha SK, Maheshwari R, Srivastava HS. Response of higher plants to lead contaminated environment. Chemosphere 1997;34:2467–93.

Tabatabai MA, Bremner JM. Use of *p*-nitrophenol phosphate in assay of soil phosphatase activity. Soil Biol Biochem 1969;1:301–7.

Tamás L, Dudíková J, Ďurčeková K, Huttová J, Mistrík I, Zelinová V. The impact of heavy metals on the activity of some enzymes along the barley root. Environ Exp Bot 2008;62:86–91.

Toro M, Azcón R, Barea JM. Improvement of arbuscular mycorrhizal development by inoculation of soil with phosphate solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl Environ Microbiol 1997;63:4408–12.

Xiong Z-T, Liu C, Geng B. Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr. Ecotoxicol Environ Saf 2006;64:273–80.

Zafar S, Aqil F, Ahmad I. Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technol 2007;98:2557–61.

Zheng W, Fei Y, Huang Y. Soluble protein and acid phosphatase exuded by ectomycorrhizal fungi and seedlings in response to excessive Cu and Cd. J Environ Sci 2009;21:1667–72.