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Antioxidant activities and metal acquisition in mycorrhizal plants growing in a heavy-metal multicontaminated soil amended with treated lignocellulosic agrowaste

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ARTICLE INFO

Article history:
Received 11 June 2008
Received in revised form
9 October 2008
Accepted 13 October 2008

Keywords:
Amendment
Antioxidants
Metals contamination
Arbuscular mycorrhizal symbiosis
Bacillus cereus

ABSTRACT

The plant growth, nutrient acquisition, metal translocation and antioxidant activities [ascorbate peroxidase (APX), glutatione reductase (GR), superoxide dismutase (SOD) and catalase (CAT)] were measured in plants growing in a heavy-metal (HM) multicontaminated soil inoculated with selected autochthonous microorganisms [arbuscular mycorrhizal (AM) fungus and/or plant growth promoting bacteria (PGPB)] and/or amended with an *Aspergillus niger*-treated agrowaste. The treated agrowaste on its own increased root growth by 296% and shoot growth by 504% compared with non-treated control plants. Both chemical and biological treatments, particularly when combined, enhanced plant shoot and root development. The stimulation effect on plant biomass was concomitant with increased AM colonization, P and K assimilation, and reduced metal translocation from soil to plant shoot. The treated residue, particularly through interactions with AM inoculation, produced the expected bioremediation effect, leading to enhanced plant development and successful rehabilitation of contaminated soil. The enhancement of CAT, APX and GR activities caused by AM inoculation suggests that AM colonization helped plants to limit oxidative damage to biomolecules in response to metal stress. The response of the plant's antioxidant activities to the amendment appears to be related to enhanced plant biomass production. The application of amendments and/or microbial inoculations to enhance plant growth and reduce metal translocation in multicontaminated soil could be a promising strategy for remediating HM pollution.

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1. Introduction

The exploitation of natural resources has contributed to the pollution of many areas on Earth and has created adverse conditions for plant growth due to nutrient imbalance and soil microbial depletion. Phytoremediation uses plants to extract or sequester pollutants from soil and can be regarded as an inexpensive, effective and sustainable biotechnology (Vivas

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0929-1393/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2008.10.008

et al., 2003a). However, the use of plants for bioremediation purposes depends on their capacity to tolerate HMs. Colonization by AM fungi could contribute to HM tolerance by improving plant nutrition and by providing a HM barrier that prevents metal uptake by the plant (Leyval et al., 1997; Vivas et al., 2003a,b,c,d). The AM-colonized plants grown on contaminated sites use survival strategies such as HM chelation (as organic complexes), sequestration and accumulation in order to keep metal concentrations in the shoots below critical levels (Kaldorf et al., 1994, 1999; Vivas et al., 2006). AM fungi have been observed to interact with plants in HM contaminated soils, and this symbiosis is partly responsible for plant survival in these polluted sites. Plants growing under HM stress conditions may require indigenous microorganisms (AM fungi and PGPR bacteria) in order to survive.

Three different molecular mechanisms of HM toxicity can be outlined according to their distinct chemical and physical properties: (a) production of reactive oxygen species by autoxidation and Fenton-reaction (b) blocking of essential functional groups in biomolecules and (c) displacement of essential metal ions from biomolecules (Schutzendubel and Polle, 2002). Thus, HM affect the plant's metabolic as well as antioxidant enzyme activities. When present in excessive amounts, HM actually cause uncontrolled redox reaction in cells, resulting in the formation of reactive oxygen species (Hall, 2002; Schutzendubel and Polle, 2002). However, plant cells contain an array of protective and repair systems that minimize the occurrence of oxidative damage. Smirnoff (1993) has divided these systems into two categories: those that interact with active forms of oxygen and keep them at low levels [superoxide dismutases (SODs), catalases (CATs), and ascorbate peroxidases (APX)], and those that regenerate oxidized antioxidants [glutathiones (GSHs), glutathione reductases (GRs), ascorbate and mono- and dehydroascorbate reductases]. The first group of enzymes are involved in the detoxification of $O_2^{\bullet-}$ radicals and H_2O_2, thereby preventing the formation of $^{\bullet}OH^{-}$ radicals. GR and GSH are important components of the ascorbate–glutathione pathway responsible for the removal of H_2O_2 in different cellular compartments (Dalton, 1995; Jiménez et al., 1997).

AM-colonized root cells accumulate reactive oxygen species in response to stress (Hause and Fester, 2005). Lanfranco et al. (2001) have described several genes in AM fungi with putative roles in oxidative stress alleviation. Thus, a major function of AM fungi could be to protect plants against HM-induced oxidative stress (Schutzendubel and Polle, 2002).

As the biological properties indicative of soil quality deteriorate in multicontaminated soils partly due to their gradual decline in organic matter content, the use of an organic amendment is recommended in these soils. After treatment with *Aspegillus niger* added to a rock-phosphate (RP) medium, sugar beet (SB) waste, an inexpensive lignocellulosic residue, can be used as an effective amendment for improving soil characteristics (Medina et al., 2006; Vassilev et al., 1996). This SB residue is transformed by *A. niger* into more simple sugar compounds that can be used by rhizosphere microorganisms for metabolic activities and growth (Bowen and Rovira, 1999).

Recently, information has become available on the positive interactive effect of this amendment and AM inoculum on plant development in artificial Zn and Cd contaminated soil (Medina et al., 2005, 2006). Immobilization and/or biosorption of HMs in soil after SB amendment have been attributed to the presence of carboxyl and strong complexes in its constituents (Reddad et al., 2002).

Previous studies have proposed the use of AM fungi and *A. niger*-treated SB amendments as alternative strategies for alleviating the plant's Zn and Cd toxicity as well as Zn and Cd phytoextraction (Medina et al., 2006). In light of these studies, we have investigated the positive effects of microbial treatments (AM fungus and/or bacterium) and *A. niger*-treated amendments on plant growth, nutrition and metals translocation to *Trifolium* plants grown in a natural multicontaminated soil.

Metals affect the plant's metabolic activities by adversely affecting cell integrity and functioning. This phenomenon also involves biochemical adaptations such as changes in the cell's enzymatic activities. These enzymatic activities play an important role in protecting the plant against HM stress. The effect of composted SB and/or microbial inoculants on antioxidant plant defence is not known, and this study represents the first attempt to determine the usefulness of these enzymatic values in detecting changes in the HM tolerance of plants growing in multicontaminated soil.

The main objective of this work was to evaluate metal assimilation in AM and bacteria inoculated (singly or together) plants growing in a multicontaminated soil with or without *A. niger*-treated SB agrowaste. In addition, we tried to determine whether the treatments applied affected the physiological and biochemical basis of plant tolerance to HM through changes in antioxidant enzyme activities (GR, APX, SOD and CAT).

2. Materials and methods

2.1. Fermentation process

The NB2 strain of *A. niger* was used in this study. It had previously been selected as it produces citric acid on complex substrates (Vassilev et al., 1986).

SB waste, a lignocellulosic material, was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10% with 50 ml of Czapek's solution (described in Fluka Chemica, catalogue no. 70185) containing (g/l of distilled water): $FeSO_4$, 0.01; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $NaNO_3$, 3.0; sucrose, 30; K_2HPO_4, 1.0, and with a final pH of 7.3 ± 0.2 for static fermentation in 250-ml Erlenmeyer flasks. The ground SB waste was inoculated with 3 ml of *A. niger* spore suspension (1.2×10^6 spores). RP at a concentration of 1.5 g kg^{-1} was added when appropriate. Static fermentation was performed at 28 °C for 20 days.

2.2. Soil-plant experiment

The experiment consisted of a factorial design involving five treatments: (1) single mycorrhizal inoculation with autochthonous AM inoculum, (2) single bacterial inoculation with an autochthonous bacterial isolate from multicontaminated soil, (3) dual AM/bacterial inoculation, (4) PO_4^{3-} fertilization and (5) untreated control. These treatments were applied to

Table 1 – Chemical, biochemical, microbiological and physical characteristics of the test soil.

pH (H_2O)	7.7	Total N (g kg^{-1})	0.22
EC (1:5, dS m^{-1})	2.5	Avail. P (μg g^{-1})	1.00
Aggregate stability (%)	48.7	Water soluble C (μg g^{-1})	41.00
Dehydrogenase activity (μg INTF g^{-1})	6.9	Water soluble carbohydrates (μg g^{-1})	10.00
	Total		Soluble
Fe (mg kg^{-1})	139,045	Fe (mg kg^{-1})	0.540
Mn (mg kg^{-1})	8,300	Mn (mg kg^{-1})	0.190
Al (mg kg^{-1})	19,385	Al (mg kg^{-1})	0.410
Zn (mg kg^{-1})	47,695	Zn (mg kg^{-1})	1.710
Pb (mg kg^{-1})	8,555	Pb (mg kg^{-1})	0.170
Cu (mg kg^{-1})	168	Cu (mg kg^{-1})	0.114
Cd (mg kg^{-1})	52	Cd (mg kg^{-1})	0.028
Ni (mg kg^{-1})	34	Ni (mg kg^{-1})	0.036
As (mg kg^{-1})	475	As (mg kg^{-1})	0.019
Cr (mg kg^{-1})	31	Cr (mg kg^{-1})	0.015

unamended control soil and soil amended with *A. niger*-treated SB agrowaste residue. Treatments were replicated fives times giving a total of 50 pots.

The multicontaminated test soil used in the greenhouse experiment is described in Table 1. The soil was air-dried, sieved to less than 2 mm and mixed with quartz sand (<1 mm) at a soil/sand (v/v) ratio of 1:1. The test soil came from Gorguel in the province of Murcia (Spain).

The amendment was mixed at a rate of 5% with half of the soil/sand mixture and left for equilibration for 3 weeks at room temperature. Four *Trifolium repens* seeds were sown in each pot (300 ml capacity). The seedlings were thinned to two plants per pot after emergence.

2.3. Selection of metal-tolerant microbes

The bacterial strain was isolated from the above-mentioned soil following serial soil dilutions, 1 g of homogenised soil was suspended in 100 ml of sterile water (dilution 10^2) and this suspension was further diluted to reach dilution 10^4 to 10^7. The suspension was sown on agar plates (Gryndler et al., 2000). The most abundant cultured bacterial strain was selected. For inoculation purposes, appropriate pots were sprinkled with 1 ml (10^8 cell ml^{-1}) of this bacterial strain grown in nutrient broth medium for 24–48 h at 28 °C (Vivas et al., 2003e).

The autochthonous mycorrhizal inoculum, also from the multicontaminated soil, was a mixture of a morphologically distinct fungal *Glomus* species, the *Glomus mosseae* strain being the most abundant AM fungal spore in this soil. The inoculum was bulked in an open-pot culture of red clover and consisted of soil, spores, mycelia and infected root fragments. Five grams of inoculum were added to appropriate pots at sowing time.

The non-mycorrhizal treatments received the same amount of autoclaved inoculum together with a 2-ml aliquot of a filtrate (<20 μm) of the AM inoculum to provide a general microbial population free of AM propagules.

2.4. Growth conditions

The plants were grown in a greenhouse under the following conditions: 16–8 h day/night cycle, 21–15 °C, and 50% relative humidity. The photosynthetic photon flux density was 500 μmol m^{-2} s^{-1} as measured with a light meter (LICOR, model LI-188B). Water lost was replaced daily by top watering with tap water.

2.5. Parameters measured

2.5.1. Biomass production, water content and nutrient and metal concentrations

At harvest (3 months after planting), the root system was separated from the shoot, and dry weights were measured after drying in a forced-draught oven at 70 °C for 2 days. Shoot concentrations of K were determined by flame photometry and P by the method described by Olsen and Dean (1965). Al, Fe, Cu, Mn, Cd, Cr, Ni and Zn were measured after wet digestion of the air-dried plant samples with $HNO_3 + H_2O_2$ by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Takács et al., 2001).

Water content (WC) was calculated on the basis of loss of weight on drying as a % of fresh weight.

2.5.2. Symbiotic development

The percentage of mycorrhizal root length infected was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v) (Phillips and Hayman, 1970). Quantification was carried out using the grid-line intersect method (Giovannetti and Mosse, 1980). The total root length colonized was calculated from total root fresh weight based on the average length (in cm) of four 20 mg fresh root samples per plant.

2.5.3. Molecular identification of the bacterial strain

Total DNA from the bacterial isolate selected in this study was obtained (Giovannetti et al., 1990). Bacterial identification was carried out by 16S rDNA cloning and sequencing (Vivas et al., 2003e). Database searches for 16S rDNA sequence similarity unambiguously identified the HM-tolerant bacterium as *Bacillus cereus* (Accession AY795568).

2.5.4. Antioxidant enzymatic activities

Total SOD activity (EC 1.15.1.1) (Beyer and Fridovich, 1987) was measured on the basis of SOD's ability to inhibit the reduction

Table 2 – Shoot and root fresh weights (mg) and plant water content (%) of *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Shoot fresh W		Root fresh W		Plant water content (%)	
	No SB	SB	No SB	SB	No SB	SB
Control	304d	1838b	500e	1980c	64.3d	73.0b
PO_4^{3-}	1102c	1884b	660e	2260cb	74.8b	75.5a
B	458d	2768a	1280d	3260a	70.6c	73.8b
AM	630d	2983a	880e	3140a	70.0c	73.8b
AM + B	970c	2074b	1180d	2540b	71.2c	73.4b

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.11.1.6) was measured (Aebi, 1984). Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 100 μl of enzyme extract in a 2 ml volume. APX activity (EC 1.11.1.11) was measured in a 1-ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). GR activity (EC 1.20.4.2) was estimated by measuring the decrease of absorbance at 340 nm and 25 °C due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 ml) contained 0.1 M HEPES–NaOH 100 mM (pH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, 150 μl enzyme extract, and 0.2 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in μmol NADPH oxidized g^{-1} PF min^{-1}, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}).

The metal translocation factor was calculated as the ratio of the metal present in plant shoot and the total metal extractable from the soil, using the formula supplied by Stoltz and Greger (2002).

2.6. Statistical analyses

Five replicates were carried out per treatment. Data were subjected to analysis of variance using microbial inoculation treatment and SB amendment treatment as sources of variation. Post hoc comparisons with Tukey's LSD test were used to compare treatment means. Percentage values were arcsin transformed before statistical analysis.

3. Results

3.1. Plant growth

Although in this HM multicontaminated soil plant growth was limited, all the biological and chemical treatments applied increased shoot and particularly root biomass (Table 2). The application of treated agrowaste to the multicontaminated soil positively affected plant growth, particularly in the case of plants inoculated with AM alone. The increase was 373% for shoot and 257% for root biomass when the AM with SB treatment is compared with the AM without SB treatment (Table 2). AM fungal inoculation in non-amended soil increased root biomass (by 76% and 136% for single and dual AM + B coinoculation, respectively) compared with the non-inoculated control plants (Table 2). The treated agrowaste itself increased roots by 296% and shoots by 504% (Table 2). Although the single inoculation with bacteria significantly increased shoot growth, this only occurred in non-amended soil (Table 2).

3.2. Mycorrhizal colonization

The total length of AM-colonized roots was significantly greater for plants growing in amended soil than for those growing in non-amended soil (Table 3). Microbial inoculation, particularly with dual AM fungal/bacterial inoculation, greatly enhanced AM colonization in the non-amended natural multicontaminated soil. In this soil, values for the total length of AM-colonized roots ranged from 20 cm (control) up to 861 cm (dual AM + B inoculated plants) (Table 3). In amended soil, the single AM inoculation produced a mycorrhizal root measuring 1758 cm (Table 3).

3.3. Plant nutrition

The average P concentration values for shoots were higher in plants grown in amended soils, particularly those fertilized with PO_4^{3-} (Table 4). In non-amended soil, PO_4^{3-} fertilization and dual AM fungal/bacterial inoculation were the most effective treatments for increasing the plant's P and K concentrations (Table 4). The most effective microbial treatment therefore turned out to be AM fungal/bacterial inoculation.

P content in shoots showed trends similar to those described for plant growth, with P content being enhanced by the amendment, particularly when associated with single AM-inoculation, which matched the P content of PO_4^{3-} fertilized plants (Table 4). The largest increases in P content were observed for plants in non-amended soils, where microbial treatments were effective in enhancing P content though to a lesser extent than for P-fertilized plants (Table 4).

Table 3 – Percentage and total length (cm) of AM-colonized roots of *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Percentage of AM-colonized roots		Total length of AM-colonized roots (cm)	
	No SB	SB	No SB	SB
Control	4e	8de	20e	158d
PO_4^{3-}	1e	12d	13e	271d
B	14d	7de	92d	228d
AM	56b	58b	493c	1758a
AM + B	73a	42c	861b	1067b

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

Table 4 – P and K concentrations and contents of *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Conc. (mg g^{-1})		Content (mg)	
	No SB	SB	No SB	SB
P				
Control	0.46e	0.97b	0.14g	1.77b
PO_4^{3-}	1.30a	1.22a	1.44c	3.38a
B	0.61d	0.91b	0.28f	1.72b
AM	0.64cd	1.01b	0.40e	3.01a
AM + B	0.76c	0.96b	0.73d	1.99b
K				
Control	1.90b	1.81b	0.06g	3.33d
PO_4^{3-}	2.54a	1.89b	2.80de	5.25b
B	2.06ab	2.33a	0.09g	4.40c
AM	1.78b	2.21a	1.12f	6.59a
AM + B	2.33a	2.40a	2.26e	4.97b

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

Similarly, the highest K content was found in AM-inoculated plants interacting with the amendment, which increased K content by 98% in relation to amended control plants and by 1040% compared with control plants not treated with agrowaste. In unamended soil, P-fertilized plants showed the highest K content (Table 4).

Concentrations of other elements such as Fe and Cu, significantly decreased in plants growing in amended soil, while the highest Fe and Cu concentrations were observed in AM-inoculated plants growing in non-amended soil (Table 5). The highest Al and Ni concentrations were found in plants singly inoculated with bacteria in non-amended soil (Tables 5 and 6).

In non-amended soil, concentrations of Mn, Cd and Zn were greatest in control plants (Tables 5 and 6), while concentrations of Cr increased more with PO_4^{3-} fertilization (Table 6). Overall, amendments reduced metal concentrations in plants (Tables 5 and 6).

The translocation of metals from soil to plant shoots was also greatly affected by the treatments applied. AM fungal colonization translocated more Fe (+203%), Al (+41%), Cu (+63%), Mn (+22%) and Zn (+33%) than PO_4^{3-} fertilized plants growing in non-amended soil (Tables 7 and 8). However, when the AM fungi were coinoculated with the bacterium, AM symbiosis reduced these increases in Fe, Al and Mn translocation (Table 7). The highest translocation rate for Cr was found in PO_4^{3-} fertilized plants grown in soil without amendments (Table 8). Amendments significantly reduced translocation for all metals (Tables 7 and 8). Important differences were observed between more and less active treatments in the translocation of metals from polluted soil to plant shoot (Tables 7 and 8).

3.4. *Plant antioxidant enzyme activities*

APX activity was enhanced by PO_4^{3-} fertilization, inoculation with AM fungi and amendments. The highest level of APX activity was found in PO_4^{3-} fertilized plants growing in amended soil. Curiously, the dual AM fungal/bacterial inoculation did not significantly affect APX activity as compared to the control treatment (Fig. 1).

GR activity was enhanced by amendments and AM fungal inoculation. By contrast, inoculation with bacteria alone or in combination with AM fungi did not significantly affect this enzyme activity (Fig. 2).

Table 5 – Fe, Al, Cu and Mn concentrations in *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Fe (μg g^{-1})		Al (μg g^{-1})		Cu (μg g^{-1})		Mn (μg g^{-1})	
	No SB	SB	No SB	SB	No SB	SB	No SB	SB
Control	716.3b	31.5f	63.6b	3.2e	15.8c	10.4d	260a	127d
PO_4^{3-}	342.0d	54.0e	28.4d	5.5e	12.7d	10.9d	151c	158c
B	540.9c	244.6d	121.8a	37.1c	17.2b	15.2c	174c	130d
AM	1074.6a	75.4e	40.0c	8.2e	20.7a	11.0d	184b	106e
AM + B	503.3c	83.5e	26.6d	6.6e	19.2a	12.1d	160c	132d

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

Table 6 – Cd, Cr, Ni and Zn concentrations in *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Cd ($\mu g\,g^{-1}$)		Cr ($\mu g\,g^{-1}$)		Ni ($\mu g\,g^{-1}$)		Zn ($\mu g\,g^{-1}$)	
	No SB	SB	No SB	SB	No SB	SB	No SB	SB
Control	0.19a	0.09c	1.95c	1.21e	1.6b	0.31e	39.0a	15.3c
PO_4^{3-}	0.13b	0.10c	6.26a	1.04e	1.2c	0.51d	26.5b	17.1c
B	0.16b	0.11c	3.94b	1.71dc	2.1a	0.69d	34.3a	22.7b
AM	0.15b	0.08c	4.05b	1.55d	1.1c	0.52d	35.3a	16.2c
AM + B	0.13b	–	3.30b	3.19b	1.0c	0.57d	35.4a	13.1c

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

Table 7 – Translocation factor (ratio of the metal present in plant shoots to the total metal extractable from the soil) for Fe, Al, Cu and Mn in *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Fe		Al		Cu		Mn	
	No SB	SB	No SB	SB	No SB	SB	No SB	SB
Control	1026.5b	58.3f	144.2b	7.8e	138.6c	91.2d	1368.4a	668.4d
PO_4^{3-}	633.3d	100.0e	69.3d	13.4e	111.4d	95.6d	794.7c	831.6c
B	1001.7c	453.0d	297.1a	90.5c	150.8b	133.3c	915.8c	684.2d
AM	1919.0a	139.6e	97.6c	20.1e	181.6a	96.5d	968.4b	557.8e
AM + B	932.0c	154.6e	64.9d	16.1e	168.4a	106.0d	842.1c	694.7d

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

Table 8 – Translocation factor (ratio of the metal present in plant shoots to the total metal extractable from the soil) for Cd, Cr, Ni and Zn in *Trifolium* plants growing in a natural multicontaminated soil supplied or not with treated agrowaste residue (SB). Plants were either inoculated with autochthonous AM fungus and/or bacteria (B) or remained uninoculated (control). An uninoculated PO_4^{3-} fertilized control was also included.

Treatments	Cd		Cr		Ni		Zn	
	No SB	SB	No SB	SB	No SB	SB	No SB	SB
Control	68a	32c	130.0c	80.7d	44.4b	8.6e	22.8a	8.9c
PO_4^{3-}	46b	39c	417.3a	69.3d	33.3c	14.1d	15.5b	10.0c
B	57a	36b	262.6b	114.0c	58.3a	19.2d	20.1a	13.3b
AM	54b	29c	270.0b	103.3c	30.6c	14.4d	20.6a	9.5c
AM + B	46b	36c	220.0b	212.7b	27.8c	15.8d	20.7a	7.7c

For each variable, results having a common letter are not significantly different ($P < 0.05$) as determined by the Tukey's test ($n = 5$).

SOD activity was reduced by AM inoculation, though only in non-amended soil. On the other hand, SOD activity reached maximum levels following coinoculation with AM fungi and bacteria (Fig. 3).

CAT activity was significantly reduced by amendments, particularly in AM and bacterial inoculated plants. In the absence of amendments, CAT activity was enhanced by AM and bacterial inoculation but not by coinoculation (Fig. 4).

4. Discussion

The presence of HMs in plants and therefore their toxicity depends on complex rhizospheric reactions involving interactions between soil and plants as well as microbial activities. In this respect, mycorrhizal fungi appear to play a central modulating role (Schutzendubel and Polle, 2002). Several studies have reported a possible alleviation of metal toxicity through mycorrhization (Hartley et al., 1997; Jentschke and Godbold, 2000; Leyval et al., 1997). However, it remains unclear whether the observed alleviation is a consequence of improved nutrition, the fungal effect on the plant's physiological stress reactions or simply hindered access of HMs to the root surface caused by the latter's fungal sheath (Jentschke and Godbold, 2000).

In this study, in terms of shoot and root growth of plants growing in natural multicontaminated soil, autochthonous microorganisms (AM fungi and/or bacteria) and PO_4^{3-} fertilization were both equally effective. The addition of a treated SB amendment improved plant growth, particularly when combined with inoculation with autochthonous microbial strains. This was related to improved root development,

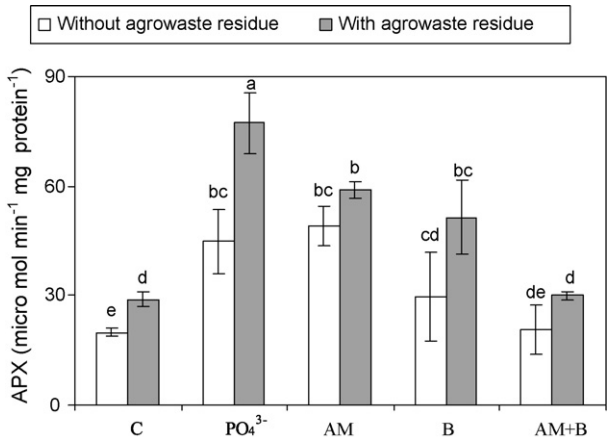

Fig. 1 – Effects of treated agrowaste residue, single or dual autochthonous AM fungus (AM) and/or bacterial (B) inoculation or PO_4^{-3} fertilization on ascorbate peroxidase activity (APX) in *Trifolium* plants growing in multicontaminated soil. Bars represent means plus the standard error ($n = 5$). Means with the same letter are not significantly different ($P < 0.05$) as determined by the Tukey's test.

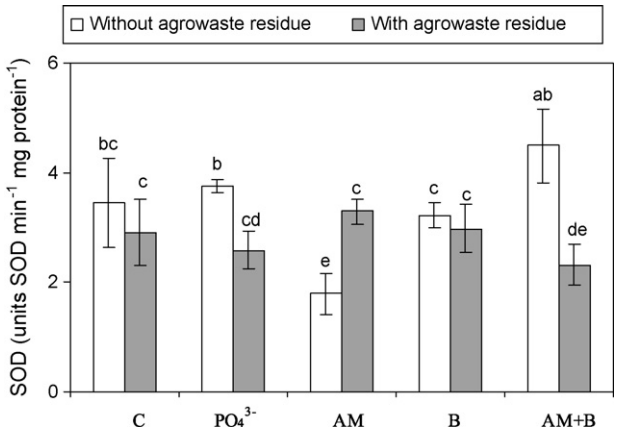

Fig. 3 – Effects of treated agrowaste residue, single or dual autochthonous AM fungus (AM) and/or bacteria (B) inoculation or PO_4^{-3} fertilization on superoxide dismutase activity (SOD) in *Trifolium* plants growing in multicontaminated soil. Bars represent means plus the standard error ($n = 5$). Means with the same letter are not significantly different ($P < 0.05$) as determined by the Tukey's test.

higher concentrations of P in shoot tissue and lower metal concentrations (with some exceptions) in these plants compared with unamended and uninoculated plants.

An important effect of microbial inoculation is the stimulation of root biomass and the macronutrient uptake by the inoculated roots. These microbial effects on nutrient acquisition and metal depletion are an indication of plant metal tolerance. In previous studies involving an artificial single metal contaminated soil, the microbial inoculations with autochthonous *G. mosseae* and *Brevibacillus brevis* proved

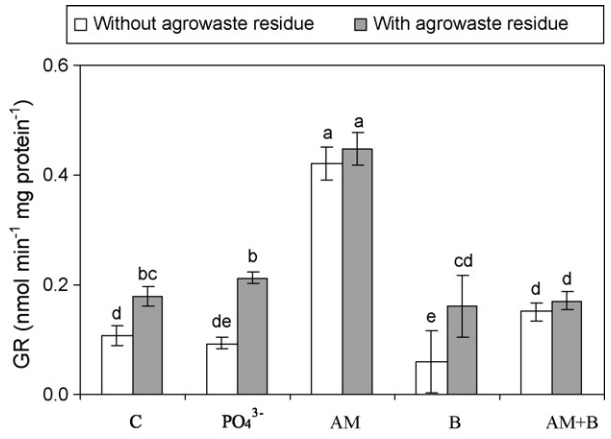

Fig. 2 – Effects of treated agrowaste residue, single or dual autochthonous AM fungus (AM) and/or bacteria (B) inoculation or PO_4^{-3} fertilization compared to control (T) on glutathione reductase (GR) activity in *Trifolium* plants growing in multicontaminated soil. Bars represent means plus the standard error ($n = 5$). Means with the same letter are not significantly different ($P < 0.05$) as determined by the Tukey's test.

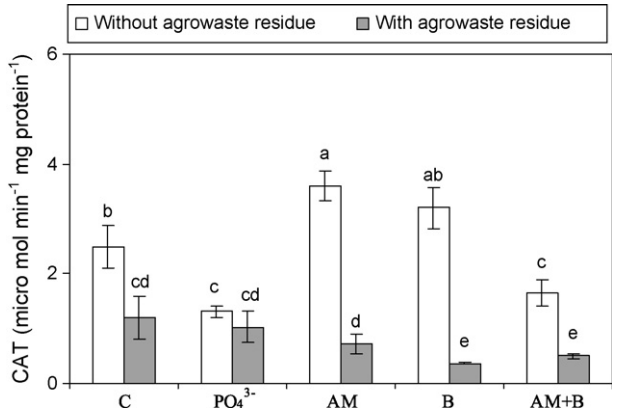

Fig. 4 – Effects of treated agrowaste residue, single or dual autochthonous AM fungus (AM) and/or bacteria (B) inoculation or PO_4^{-3} fertilization on catalase activity (CAT) in *Trifolium* plants growing in multicontaminated soil. Bars represent means plus the standard error ($n = 5$). Means with the same letter are not significantly different ($P < 0.05$) as determined by the Tukey's test.

to be highly effective in increasing biomass production and symbiotic structures (nodules and AM colonization) formation and activity (Vivas et al., 2005).

A. niger-treated SB, a lignocellulosic agrowaste, provides an organic amendment rich in polysaccharide compounds and available P through the RP applied during the fermentation process. This amendment significantly increased nutrients and reduced metal concentrations in plant shoot tissue. Thus, the amendment, particularly combined with AM inoculation, had the expected bioremediation effect of reducing metal concentrations in most cases. The amendment also increased macro- and micronutrients which enhanced plant growth

and, consequently, enabled metal to be effectively phytoextracted from the contaminated soil. The use of this amendment and AM colonization for contaminated soil recovery is therefore a successful and promising biotechnological procedure.

Compared with non-amended plants, the amendment reduced metal translocation in control plants. AM inoculation in non-amended soils also decreased the translocation of Al, Mn, Cd and Ni. These results suggest that the quality and productivity of soils can be improved by the application of organic amendments and/or AM inoculation (Caravaca et al., 2004). As well as acting as metal chelators, amendments and/or AM fungal inoculations play a variety of roles in reducing the plant's uptake of HMs, particularly Al and Ni, and have a major impact on plant metal tolerance. These treatments (amendments and/or AM inoculations) could be recommended for alleviating HM toxicity in plants. Physiological immobilization of metals in soils could be achieved through the use of amendments (Bolan and Duraisamy, 2003). Organic matter forms complexes with HMs, and Reddad et al. (2002) have attributed SB pulp's metal-binding capacity to the presence of carboxyl functions in this material.

Many of the degenerative reactions associated with HM stress are mediated by reactive oxygen species (ROS). ROS is a generic term embracing not only free radicals such as superoxide and hydroxyl radicals but also H_2O_2 and singlet oxygen. While it is generally assumed that hydroxyl radicals and singlet oxygen are so reactive that their production must be minimized (Jakob and Heber, 1996), $O_2^{\bullet-}$ and H_2O_2 are synthesized at very high rates even under optimal conditions (Noctor and Foyer, 1998). The most important aspect of $O_2^{\bullet-}$ and H_2O_2 toxicity is thought to be their ability to initiate cascade reactions that result in the production of hydroxyl radicals capable of causing lipid peroxidation, protein denaturation and DNA mutations (Bowler et al., 1992).

The effective destruction of $O_2^{\bullet-}$ and H_2O_2 requires the synchronous action of several antioxidant enzymes. Superoxide is rapidly converted to H_2O_2 by SOD activity (Bowler et al., 1992). CATs convert H_2O_2 to water and molecular oxygen in peroxisomes (Noctor and Foyer, 1998). An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and have a much greater affinity to H_2O_2 than CAT (Jiménez et al., 1997). The enzymes in the ascorbate–glutathione cycle, where H_2O_2 is scavenged, are highly active. In this cycle, APX catalyzes the reduction of H_2O_2 to water by ascorbate, and the resulting dehydroascorbate is reduced back to ascorbate with the help of GR (Iturbe-Ormaetxe et al., 2001).

The ability of plants to increase antioxidative protection in order to combat the negative consequences of HM stress appears to be limited as many studies have shown that exposure to elevated concentrations of redox reactive metals reduces rather than increases antioxidative enzyme activities (Schutzendubel and Polle, 2002). However, in this study, we found that chemical or biological treatments make a major contribution to plant antioxidant activities under HM stress conditions. SOD and CAT activities were reduced by amendments while the opposite effect was observed for APX and GR activities. In fact, the effect of amendments on antioxidant enzyme activities actually differed between the enzymes analyzed. Amendments were also effective in improving plant water content and nutrient uptake in HM polluted soil, which could lead to improved plant establishment in this poor-quality polluted soil.

The enhancement of GR that we found in amended, and particularly in AM-inoculated, plants has been associated with less foliar damage and photoinhibition caused by stress conditions, which suggests that this plays an important role in protecting plants against stress (Aono et al., 1993; Foyer et al., 1995). The results obtained in relation to these antioxidant activities showed that CAT, APX and GR activities recorded a higher rate of increase in single AM-inoculated plants than in non-AM-inoculated plants, suggesting that these activities helped to reduce oxidative damage to biomolecules (Becana et al., 2000). Burritt et al. (2002) have postulated that the ability of plants to produce ROS was due to the increased activity of the enzymes required to regenerate ascorbate and glutathione. In our study, this was seen to occur in the case of APX and GR when plants were AM inoculated in amended soil. However, it has been suggested that fungal cells in the symbiosis may primarily need to resist HM-induced oxidative stress and that the major function of AM proteins in making the plant–AM symbiosis HM tolerant may involve protection against HM-induced oxidative stress and removal of ROS (Ouziad et al., 2006).

Mechanisms and factors affecting the HM tolerance of plants are difficult to determine due to the multiple factors involved. However, the combination of plant growth enhancement and reduced metal translocation caused by amendments and/or microbial inoculations in multicontaminated soil could be regarded as a promising strategy for remediating HM pollution.

Acknowledgements

This study was supported by ''Plan Nacional I+D'' Spain (Project AGL 2006-09453-CO2-02).

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