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Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity

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Abstract

In this study we investigated the interactions among plant, rhizosphere microorganisms and Zn pollution. We tested the influence of two bacterial strains isolated from a Zn-polluted soil on plant growth and on the symbiotic efficiency of native arbuscular mycorrhizal fungi (AMF) under Zn toxicity. The two bacterial strains exhibited Zn tolerance when cultivated under increasing Zn levels in the medium. However, strain B-I showed a higher Zn tolerance than strain B-II at the two highest Zn levels in the medium (75 and 100 mg l^{-1} Zn). Molecular identification placed the strain B-I within the genus *Brevibacillus*. Our results showed that bacterial strain B-I consistently enhanced plant growth, N and P accumulation, as well as nodule number and mycorrhizal infection which demonstrated its plant-growth promoting (PGP) activity. This strain B-I has been shown to produce IAA (3.95 μg ml) and to accumulate 5.6% of Zn from the growing medium. The enhanced growth and nutrition of plants dually inoculated with the AMF and bacterium B-I was observed at three Zn levels assayed. This effect can be related to the stimulation of symbiotic structures (nodules and AMF colonization) and a decreased Zn concentration in plant tissues. The amount of Zn acquired per root weight unit was reduced by each one of these bacterial strains or AMF and particularly by the mixed bacterium-AMF inocula. These mechanisms explain the alleviation of Zn toxicity by selected microorganisms and indicate that metal-adapted bacteria and AMF play a key role enhancing plant growth under soil Zn contamination.

Keywords: Arbuscular mycorrhizal symbiosis; Zn-polluted soil; Plant-growth-promoting bacteria

1. Introduction

Zinc is an essential metal for normal plant growth and development since it is a constituent of many enzymes and proteins. However, excessive concentrations of this metal are well known to be toxic to most living

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organisms. Elevated concentrations of Zn exist in many agricultural soils from management practices including application of sewage sludge or animal manure and from mining activities, and this may represent a risk to environmental quality and sustainable food production (Li and Christie, 2001). Many evidences suggest that microorganisms are far more sensitive to heavy metal stress than animals or plants growing on the same soils (Giller et al., 1998; Crowley and Dungan, 2002). In recent years several studies have shown the harmful effects of metals in high concentrations on microbial diversity and their activity in the soil (Brooks et al., 1986; Chaudri et al., 1992, 1993; McGrath et al., 1995; del Val et al., 1999b).

Zinc only occurs as the divalent cation Zn^{2+}, which does not undergo redox changes under biological conditions. Zinc is a component in a number of enzymes and DNA-binding proteins, for example zinc-finger proteins, which exist in bacteria. In humans, zinc toxicity may be based on zinc-induced copper deficiency; however, zinc is apparently less toxic than copper. In *Escherichia coli*, the toxicity of zinc is similar to that of copper, nickel, and cobalt (Crowley and Dungan, 2002).

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutual symbioses with the majority of the roots of higher plants, providing a direct physical link between soil and plant roots (Smith and Read, 1997). They occur in almost all habitats and climates including disturbed soils such as those derived from mine activities, but soil disestablished usually produce changes in the diversity and abundance of AMF population (del Val et al., 1999a; Jeffries and Barea, 2001). Thus, changes in AMF population diversity produced by the presence of high amounts of metals are expected to interfere with the possible beneficial effects of this symbiotic association.

Mycorrhizal symbiosis generally occurs in the presence of many microorganisms, and there is abundant literature to support the hypothesis that some of these microbes interact in rather specific ways to influence the mycorrhizal relationship and its effects on plant growth. Thus, the associated microorganisms may well complement mycorrhizal activity (Linderman, 1988, 1992; Azcón, 1989; Garbaye, 1994). One of these bacterial groups, the so-called plant-growth-promoting rhizobacteria (PGPR), has been reported by several authors to interact with AMF (Azcón, 1987, 1993; Barea et al., 1997, 2002a,b). The final effect of soil microorganisms, including AMF, on plant development is the result of the interactions among the different soil microbial components involved (Meyer and Linderman, 1986; Puppi et al., 1994; Requena et al., 1997). In contrast, only few studies have been carried out involving interactions between AMF and PGPR and heavy metals as source of soil disturbance (Haselwandter et al., 1994).

PGPR can significantly increase the growth of plants in the presence of heavy metals including nickel, lead and zinc (Burd et al., 1998, 2000; Grichko et al., 2000; Nies et al., 2002). However, the manipulation of beneficial combinations of microorganisms depends on a proper understanding of the ecosystem in order to apply a suitable selection of microbes (Puppi et al., 1994; Díaz et al., 1996).

In this study we have tested on *Trifolium repens* the effect of inoculation with two indigenous bacterial isolates and AMF on Zn tolerance in terms of plant growth, nutrient uptake, Zn acquisition and symbiotic development. The microbial strains used were isolated from a long-term Zn contaminated area from a Hungarian (Nagyhörcsök) experimental field (Kádar, 1995). Microorganisms were assayed in single or in dual coinoculation in soil artificially contaminated with a range of Zn levels. Bacterial indole acetic acid (IAA) production, Zn biosorption ability and number of viable bacterial cells at increasing Zn levels were also determined.

2. Materials and methods

2.1. Experimental design and statistical analysis

The experiment consisted of a two-factor randomized complete block design of: (1) microbial treatments including two rhizobacterial species (B-I or B-II) with or without microbial indigenous mycorrhizal inoculum, including an uninoculated control treatment; and (2) three levels of Zn added to the soil (30, 90 or 270 mg Zn kg^{-1}). Five replicates were made for each treatment, totaling 90 pots.

For each Zn level data were subjected to an analysis of variance with bacterial treatment, AMF treatment, and bacterial-treatment/AMF-treatment interaction as sources of variation (Duncan, 1955). Percentage values were arcsin transformed before statistical analysis.

2.2. Soil and biological materials

A loamy soil from Granada (Spain) was selected for this study on the basis of its high similarity (pH, texture and nutrient contents) to the original soil from Hungary. The soil was sieved (2 mm), diluted with quartz-sand (<1 mm) (4:1 soil:sand v/v) and sterilized by steaming (100 °C for 1 h on three consecutive days). The undiluted soil had a pH of 7.2 (water); 1.6% organic matter, nutrient concentrations (mg kg^{-1}): N (total), 2.1 (Kjeldahl); P, 1.7 Olsen; K, 0.8. The soil texture was made up of 57.8% sand, 19% clay and 23.2% silt.

After sterilization, the soil was supplemented with 30, 90 or 270 mg Zn kg^{-1} by adding adequate amounts of

an aqueous solution of $ZnSO_4$. The soil was left in a greenhouse for a 2 weeks period (for metal stabilization) and then the amount of Zn remaining was determined according to Lakane and Erviö (1971) methodology. After 2 weeks incubation, the amount of available Zn remaining in the soil was 24, 68 and 215 mg Zn kg^{-1}, respectively.

Trifolium repens seeds were sterilized in a 15% sodium hypoclorite solution for 15 min, then washed several times with sterile water to remove any trace of chemical that might interfere in seed germination and placed in plastic pots containing 100 g of sterilized soil/sand mixture (4:1 v/v), previously polluted with Zn. A suspension (1 ml^{-1}) of the diazotrophic bacterium *Rhizobium leguminosarum* bv. *trifolii* (10^8 cell ml^{-1}) was sprinkled over the seeds of all treatments at the time of planting.

Two bacterial strains exhibiting different colony morphology and referred to as strain B-I or strain B-II were isolated from Zn-contaminated soil at Nagyhörcsök Experimental Station (Hungary), (Kádar, 1995). The isolation was carried out following serial dilutions of the soil. For that, 1 g of homogenized 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 two bacterial strains were the most abundant cultivable types in such soil. For inoculation, appropriate pots were sprinkled with 1 ml (10^8 cell ml^{-1}) of each bacterial strain grown in nutrient broth medium for 24–48 h at 28 °C of temperature (Vivas et al., 2003a).

The autochthonous mycorrhizal inoculum (a mixture of fungal *Glomus* species morphologically determined) being a *Glomus mosseae* strain the most abundant AMF spore in this soil also coming from the Zn-contaminated soil at Nagyhörcsök (Hungary). It was bulked in an open-pot culture of red clover and consisted of soil, spores, mycelia and infected root fragments. Ten grams of inoculum were added to appropriate pots at sowing time just below the clover seeds.

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

2.3. Growth conditions

Plants were grown for three months in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15 °C, and a photoperiod of 16 h at a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1} (Licor, Lincoln, NE, USA, model LI-188B).

Each week throughout the experiment, the plants received 10 ml of Hewitt's nutrient solution lacking N and P (Hewitt, 1952).

2.4. Parameters measured

2.4.1. Biomass production and nutrients and metals concentrations

At harvest (three 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 two days. Shoot concentrations of N (micro-Kjeldahl) and P (Olsen and Dean, 1965), as well as of Zn, Cd, Ni and Pb were also determined after wet digestion of the air-dried plant samples with $HNO_3 + H_2O_2$ by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Takács et al. (2001).

2.4.2. 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 lactophenol (v/v), according to Phillips and Hayman (1970). Quantification was performed using the grid-line intersect method (Giovannetti and Mosse, 1980). Nodule number was estimated by direct observation using a binocular microscope. Five replicates were made per treatment.

2.4.3. Bacterial growth under increasing Zn levels in the medium

Both bacterial isolates (B-I or B-II) were cultivated for 24–48 h at 28 °C in nutrient broth supplemented with 0, 25, 50, 75 or 100 mg l^{-1} Zn as $ZnSO_4$. The number of viable cells was estimated as the number of cfu ml^{-1} at 1 h intervals from 0 to 16 h as described by (Vivas et al., 2003a). Four replicates were used in each determination.

2.4.4. Production of indole-3-acetic acid (IAA)

The production of IAA by the bacteria was measured by the method of Wöhler (1997). The bacteria were grown overnight on nutrient broth and then collected by centrifugation at 7000*g* for 5 min. The bacterial pellet was then incubated at 37 °C for 24 h with 3 ml of phosphate buffer (pH 7.5) with glucose (1%) and 2 ml of L-tryptophan (1%). After incubation, 2 ml of 5% trichloroacetic acid and 1 ml of 0.5 M CaCl were added. The solution was filtered (Whatman No. 2 of pore size). Three milliliter of the filtrate were put in a test tube, and to this 2 ml of salper solution (2 ml 0.5 M $FeCl_3$ and 98 ml 35% perchloric acid) were added. This mixture was incubated for 30 min at 25 °C in the dark. Then the absorbance of the resulting solution was measured at 535 nm with a Shimadzu UV-1603 spectrophotometer.

The calibration curve was made using indole acetic acid in the range of 0–20 mg l^{-1}.

2.4.5. Molecular identification of the most effective bacterial strain

Total DNA from bacterial isolate B-I was obtained as described by Giovannetti et al. (1990) and characterized by sequence analysis of the small ribosomal subunit (16S ribosomal DNA). Polymerase chain reaction amplification was carried out as described previously (Vivas et al., 2003a).

2.4.6. Bacterial capability for Zn biosorption

The biosorption study was carried out as described by Kanazawa and Mori (1996) with some modifications. Bacteria were grown in 250 ml of nutrient broth until reaching one unit of optical density (600 nm). Then the cells were harvested by centrifugation at 7000*g* for 30 min and the bacterial pellet washed twice with Ringer's solution (NaCl 0.85%, $CaCl_2$ 0.03%, KCl 0.025%, $NaHCO_3$ 0.02%). The harvested biomass was incubated for 1 h at 28 °C with a solution containing 267.4 μg Zn ml^{-1} as $ZnSO_4 \cdot 7H_2O$.

The suspension was then centrifuged at 7000 rpm and filtered through a 0.45 μm Millipore membrane to separate the biomass from the filtrate. The biomass was dried, weighed, and heavy metals were extracted with nitric acid (24 h). The Zn contents were determinated on both microbial biomass and supernatants by atomic absorption spectrometry.

3. Results

Results on shoot and root growth showed the effectiveness of B-I and AMF for plant growth in Zn-contaminated medium. The two inoculated bacteria showed different effectiveness on shoot and root biomass production when they were singly or coinoculated with AMF inoculum mainly at the lowest Zn level assayed (Fig. 1). The effectiveness of microbial inocula to promote shoot growth was higher under 68 μg g^{-1} of Zn in the growing medium and no differences on shoot growth were observed between single or dual AMF treatments. Root growth was the highest in dual AMF + B-I treated plants irrespective of Zn amount in soil (Fig. 1).

Nitrogen accumulation in plants decreased as Zn in the medium increased. Nevertheless, microbial treatments were effective in enhancing N uptake mainly at the lowest Zn level in the medium. Under 24 μg Zn g^{-1} single or dually inoculated mycorrhizal plants highly enhanced N content. At the three Zn levels, the maximum N content was observed in mycorrhizal plants coinoculated with the bacterium B-I (Fig. 2A).

A similar picture was evidenced in relation to P content. Single bacterial inoculation was only effective at the lowest Zn contaminated medium. Nevertheless, under the two highest Zn concentrations B-I was highly effective in increasing P content in AMF-colonized plants (Fig. 2B).

A negative effect of Zn application to the growing medium on nodule formation of non-mycorrhizal plants was found (Fig. 2C). The effectiveness of B-I to enhance this symbiotic value was more relevant than that of B-II both in single and in dual inoculated plants. The bacterium B-I increased nodule numbers at whatever Zn levels in non-mycorrhizal and mycorrhizal plants. In soil supplied with 215 μg Zn g^{-1} no nodules were formed in non-inoculated control plants (Fig. 2C).

The bacterium B-I enhanced the value of AMF colonization in plants growing under 68 μg g^{-1} of Zn (Fig. 3A). The two highest Zn amounts applied (68

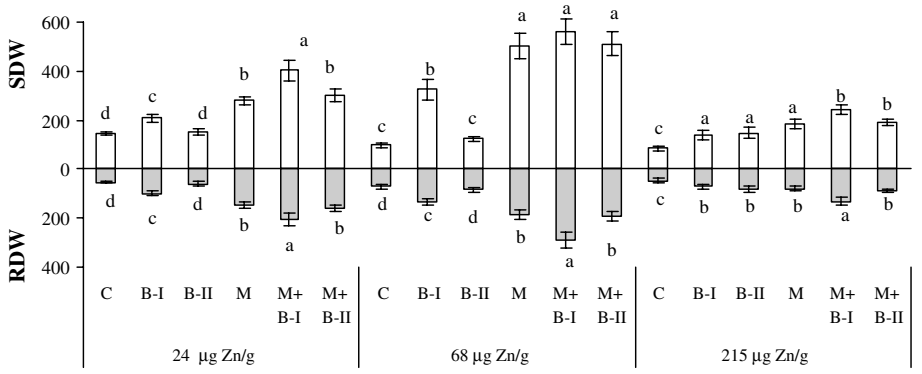

Fig. 1. Shoot and root dry weights (mg $plant^{-1}$) of *Trifolium repens* plants cultivated in soil amended with 24, 68 or 215 μg Zn g^{-1}. Treatments are designed as C (control), B-I (bacterium B-I), B-II (bacterium B-II), M (mycorrhizae), M + B-I (mycorrhizae + bacterium B-I), M + B-II (mycorrhizae + bacterium B-II). Vertical bars represent standard errors. Values followed by the same letter are not significantly different according to Duncan's multiple range test ($n = 5$).

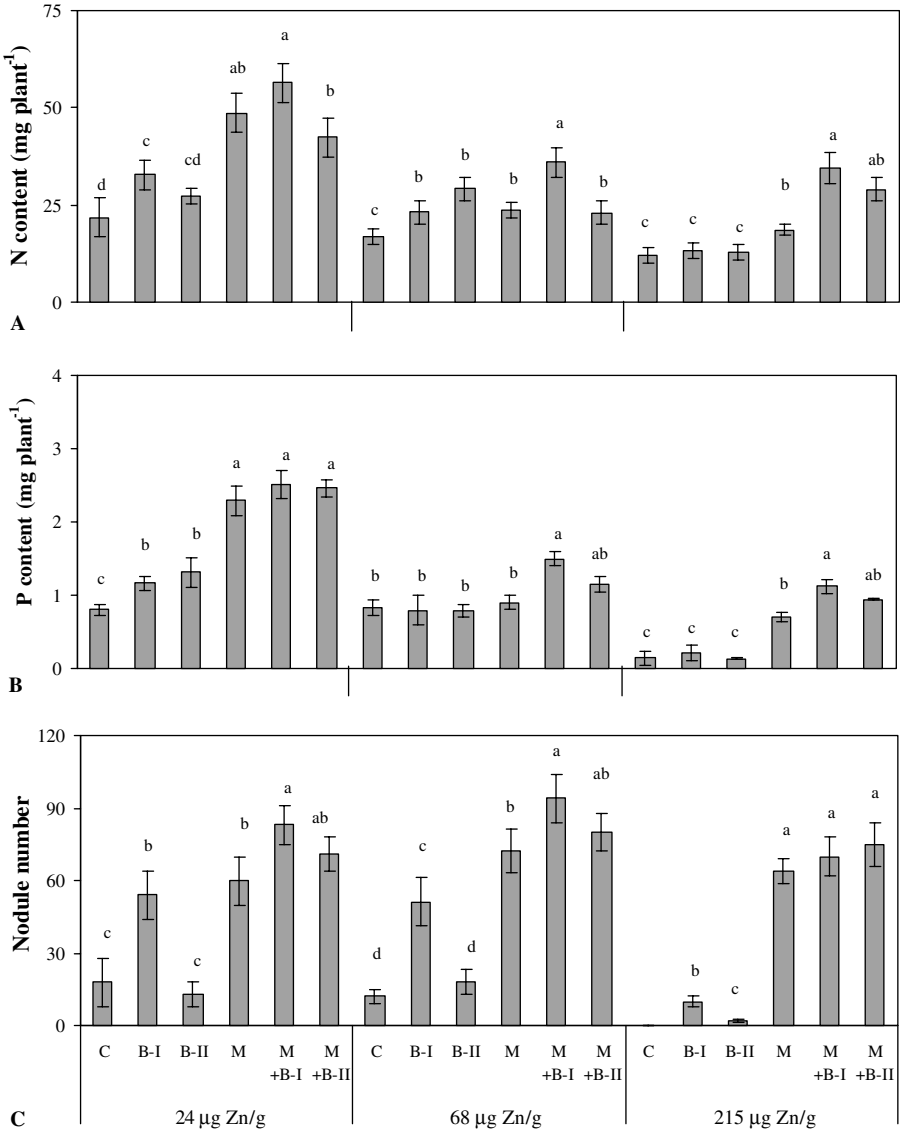

Fig. 2. N content (A), P content (B) and nodule number (C) in *Trifolium repens* plants cultivated in soil amended with 24, 68 or 215 µg Zn g^{-1}. Treatments are designed as in Fig. 1. Vertical bars represent standard errors. Values followed by the same letter are not significantly different according to Duncan's multiple range test ($n = 5$).

and 215 µg Zn g^{-1}) reduced AMF-colonization rate by about 30%.

The Zn concentration in plants increased as did Zn availability in the growth medium (Fig. 3B). Substantial differences among mycorrhizal and non-mycorrhizal plants were found at whatever Zn level since AMF colonization strongly decreased Zn concentration in shoot plants. The inoculation of bacteria B-I or B-II also reduced Zn concentration in mycorrhizal and non-mycorrhizal plants. In single inoculation, B-I was more effective than B-II in decreasing plant Zn accumulation. Dual inoculation with AMF and bacterium B-I or B-II highly decreased Zn shoot concentration regardless of the Zn level in soil (Fig. 3B).

Colonization by AMF strongly reduced Zn transport to shoot per unit of root (Table 1). The mycorrhizal effect decreasing Zn transport was enhanced by bacteria, particularly by B-I at the two highest Zn levels (68 and 215 µg Zn g^{-1}).

Comparing the effect of coinoculation with AMF plus B-I at the different Zn levels we can see that Zn transports per unit of root were 17.5 (24 µg Zn g^{-1}), 17.3 (68 µg Zn g^{-1}) and 81.5 (215 µg Zn g^{-1}) fold lower than those in non-inoculated control plants. An

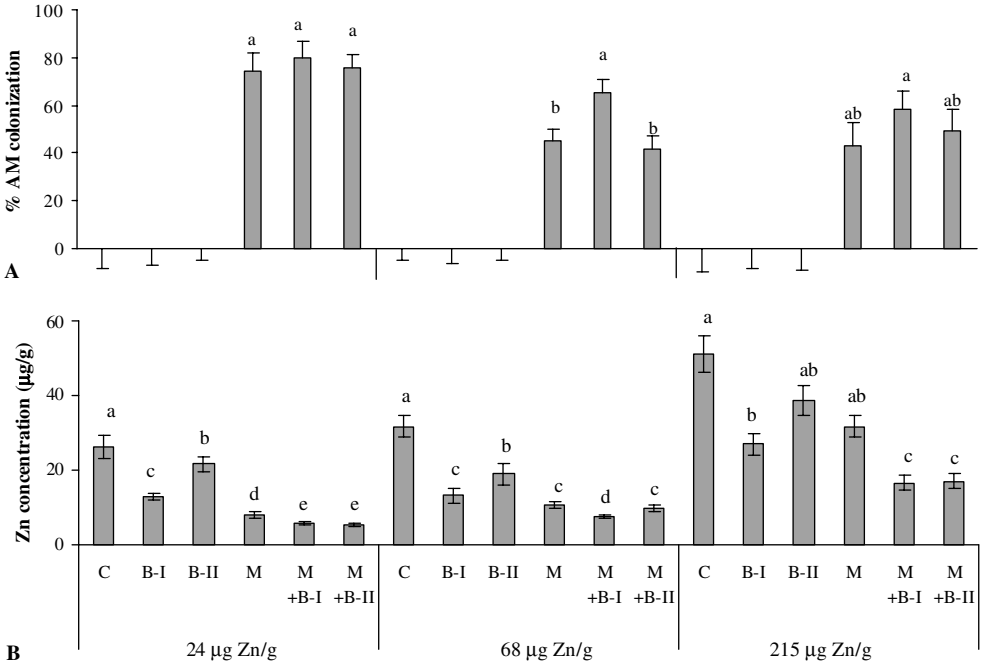

Fig. 3. Percentage of mycorrhizal root length (A) and Zn concentration (B) in *Trifolium repens* roots cultivated in soil amended with 24, 68 or 215 μg Zn g^{-1}. Vertical bars represent standard errors. Values followed by the same letter are not significant different according to Duncan's multiple range test ($n = 5$).

Table 1
Ratio Zn concentration to root weight unit (Zn g^{-1} dw root) in *Trifolium repens* plants cultivated in soil amended with 24, 68 or 215 μg Zn g^{-1}

Treatment	24 μg g^{-1}	68 μg g^{-1}	215 μg g^{-1}
C	0.49 a	0.45 a	1.06 a
B-I	0.13 c	0.10 c	0.37 c
B-II	0.35 b	0.23 b	0.47 b
M	0.05 d	0.05 d	0.40 b
M + B-I	0.03 e	0.02 e	0.13 c
M + B-II	0.03 e	0.05 d	0.19 d

Treatments are designed as C (uninoculated control), B-I (bacterium B-I), B-II (bacterium B-II), M (mycorrhizae), M + B-I (mycorrhizae + bacterium B-I), M + B-II (mycorrhizae + bacterium B-II). Values followed by the same letter are not significantly different according to Duncan's multiple range test ($n = 5$).

interesting result is that the ratio of Zn concentration in shoot per unit of root in control plants growing in soil supplied with the lowest Zn level matched that of all single inoculated treatments growing in soil supplied with the highest Zn level. For dually inoculated plants this value was even more reduced (Table 1). These results indicate that in inoculated plants the Zn transfer per unit of root to the plant shoot was highly reduced particularly at the highest Zn level assayed.

Regarding shoot uptake of metals such as Cd, Ni and Pb (Table 2) results show that at the lowest Zn level in the soil the Cd concentration increased significantly only in plants singly inoculated with AMF. At the medium Zn level, Cd increased significantly in plants singly inoculated with bacterium B-I and in plants dually inoculated with AMF plus either bacterium. In contrast the mycorrhiza alone did not affect this value. At the highest Zn level, bacterium B-II, mycorrhization and dual inoculation with AMF and bacterium B-I enhanced Cd content (Table 2).

The Ni content was clearly enhanced by mycorrhizal colonization in combination with either bacterial strain at the three Zn levels in the soil. Inoculation with bacterium B-I alone also enhanced Ni content at the two lowest Zn levels assayed (Table 2).

Regarding plant Pb content, at the two lowest Zn levels there was a clear enhancement of Pb by single AMF-colonization. Under 68 μg Zn g^{-1} the maximum Pb content was observed in dual combination with bacterium B-II. However at the highest Zn level this effect disappeared and only single inoculation with bacterium B-II enhanced the plant Pb content (Table 2).

3.1. Molecular identification

The most efficient bacterial strain (B-I) was selected for molecular identification and we obtained its 26S rDNA sequence. FASTA (Pearson and Lipman, 1988) and BLAST (Basic local alignment search tool) analyses unambiguously identified the strain B-I as a *Brevibacil-*

Table 2
Ni, Cd and Pb contents (μg plant^{-1}) in *Trifolium repens* plants cultivated in soil amended with 24, 68 or 215 μg Zn g^{-1}

Treatment	24 μg g^{-1}			68 μg g^{-1}			215 μg g^{-1}		
	Cd	Ni	Pb	Cd	Ni	Pb	Cd	Ni	Pb
C	0.26 b	0.18 d	0.14 c	0.14 b	0.12 d	0.24 c	0.20 c	0.11 c	0.15 c
B-I	0.28 b	0.38 c	0.40 b	0.34 a	0.53 c	0.63 b	0.40 b	0.26 b	0.20 b
B-II	0.27 b	0.07 e	0.16 c	0.17 b	0.52 c	0.18 c	1.00 a	0.08 d	0.34 a
M	0.84 a	0.21 d	0.71 a	0.15 b	0.46 c	0.56 b	0.60 ab	0.14 c	0.20 b
M + B-I	0.21 b	0.63 b	0.55 ab	0.20 ab	0.89 b	0.83 b	1.00 a	0.38 b	0.21 b
M + B-II	0.10 c	1.21 a	0.56 ab	0.22 ab	1.72 a	1.50 a	0.30 ab	0.76 a	0.26 ab

Treatments are designed as C (control), B-I (bacterium B-I), B-II (bacterium B), M (mycorrhizae), M + B-I (mycorrhizae + bacterium B-I), M + B-II (mycorrhizae + bacterium B-II). Values followed by the same letter are not significantly different according to Duncan's multiple range test ($n = 5$).

lus sp., with *Brevibacillus brevis* (Accession AB039334) as its closest relative.

3.2. Bacterial growth in the presence of Zn

Both bacterial isolates were grown in nutrient broth at increasing Zn concentrations ranging from 0 to 100 mg Zn l^{-1} (Fig. 4). The growth of both bacterial strains decreased with an increase of Zn in the medium. However, strain B-I exhibited a higher tolerance to Zn than strain B-II. At low Zn in the medium (25 and 50 mg Zn l^{-1}), strain B-I reached almost 10^8 cfu, while strain B-II never exceeded 10^5 cfu. In addition, at the highest Zn levels in the growth medium (75 and 100 mg Zn l^{-1}), strain B-I produced 10^8 cfu after 10 h of incubation, while strain B-II only reached 10^2 cfu.

3.3. Production of indole-3-acetic acid (IAA)

The production of IAA by bacterial strain B-I was tested against two PGPR-characterized bacteria such as *Bacillus pumillus* (isolate B.3) and *Bacillus licheniformis* (isolate B 21) (Probanza et al., 1996; Gutierrez Mañero et al., 1996). Strain B-I showed a higher production of IAA (3.95 mg l^{-1}) than both reference PGPRs (average of 1.4 mg l^{-1}).

3.4. Bacterial capability for Zn biosorption

The Zn adsorbing capability of B-I was 5.6% of the biomass dry weight.

4. Discussion

Microorganisms play important roles in the environmental fate of toxic metals with physicochemical mechanisms affecting transformations between soluble and insoluble phases. Such mechanisms are important components of natural biogeochemical cycles for metals and associated elements, e.g., sulfur and phosphorus, with some processes being of potential application to the treatment of contaminated materials (Gadd, 2000).

In this study, we have tested that toxic concentrations of Zn initially inhibited the growth rate of two bacterial strains isolated from Zn-polluted soil and representing the two most abundant cultivable bacterial groups in such soil. However after few hours, *Brevibacillus* sp. (strain B-I) recovered its ability to grow in a Zn-polluted medium. The development of resistance against heavy metal ions is a generally observed phenomenon (Leyval et al., 1997).

Plant nodule formation decreased as available Zn in the soil increased and the beneficial effect of this symbiosis (on N acquisition) was reduced in highly Zn polluted soil. In the case of AMF infection similar trends were observed. Nevertheless, under the more stressed conditions (215 μg Zn g^{-1}) mycorrhizal colonization improved in a greater extent nodule formation. Nodulation by *Rhizobium* resulted more sensitive to Zn contamination than AM symbiosis. Evidences suggest that both these symbiotic microorganisms can be constitutively or adaptatively resistant to increasing Zn concentrations and specific strategies can be developed by adapted strain to resist high metal concentrations (Kanazawa and Mori, 1996).

Regarding results of bacterial growth in culture medium, autochthonous bacteria showed different Zn tolerance in terms of number of viable cells. In microcosm experiment, the most tolerant bacterial strain (B-I) also affected biomass production by plants growing in Zn polluted soil. Such effectiveness seems to be related to a reduced Zn uptake and/or transport per root unit.

Chaudri et al. (1992) suggested the use of biotechnological procedure in remediation strategies. Results here presented provide evidence to support this proposition.

The potential of saprophyte (bacteria) and symbiotic (AMF and *Rhizobium*) Zn-adapted microbial groups to alleviate Zn toxicity is evident. The effectiveness of these microorganisms on plant growth and nutrition must be

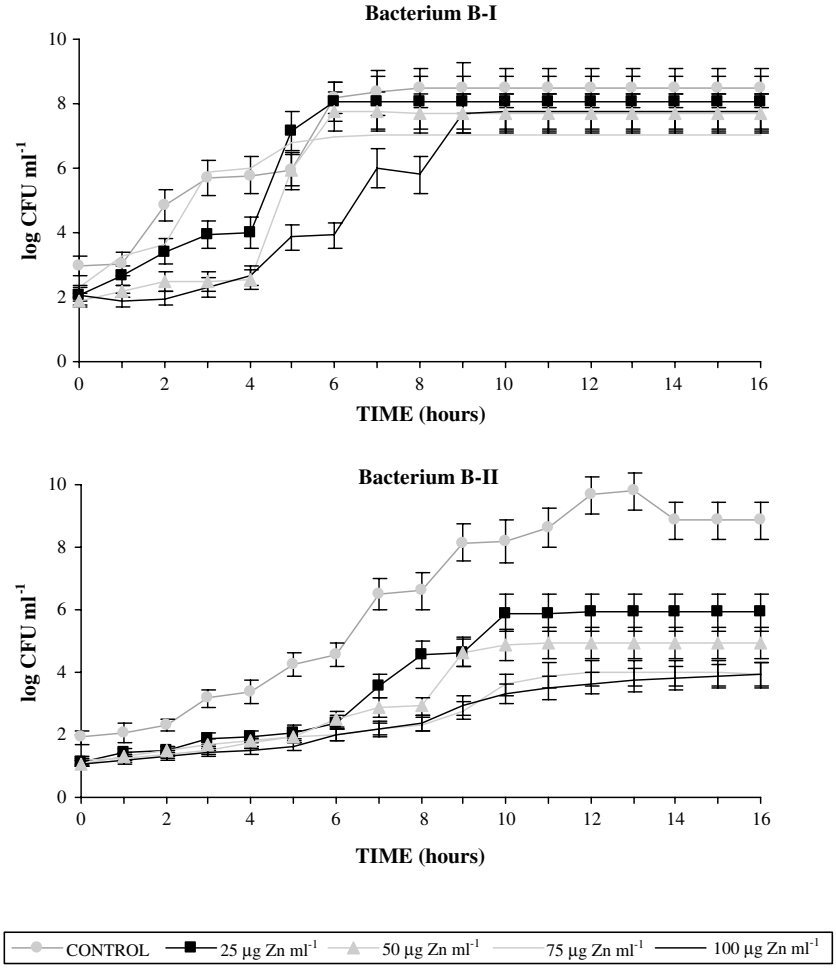

Fig. 4. Number of viable cells (log cfu ml^{-1}) at different time intervals of bacterium B-I and bacterium B-II grown in nutrient broth supplemented with 0 (control), 10, 25, 50 or 100 μg Zn ml^{-1}. Data correspond to the average value plus the standard error ($n = 4$).

considered for their activities in metal tolerance (Díaz et al., 1996; Biró et al., 1998; Vivas et al., 2003a,b, 2005). In fact, Zn content in plant depended on Zn accumulation in soil and on the microbial activities in the rhizosphere. Microbially treated plants reduced shoot Zn concentration since the proportion of soil/plant Zn transfer was decreased by the inoculants, particularly in association.

Most commonly the mechanism of resistance in prokariotes is an efflux of the toxic metals by the action of P-type ATPases or secondary efflux systems (Nies and Silver, 1995; Paulsen and Saier, 1997). An important mechanism on this respect is the synthesis of extracellular polymeric substances, a mixture of polysaccharides, mucopolysaccarides and proteins which can bind significant amounts of potentially toxic metals and entrap precipitated metal sulfides and oxides. In bacteria, peptidoglycan carboxyl groups are main cationic binding sites in Gram-positive species. Chitin, phenolic polymers, and melanins are important structural components of fungal walls and these are also effective biosorbents for metals and radionuclides.

The *Brevibacillus* strain (B-I) posses cellular mechanisms (biosorption and bioaccumulation) involved in the detoxification of Zn in the growth medium. Such metabolic abilities may be related to the Zn tolerance and also to the Zn reduction in the medium (Zhou, 1999). *Brevibacillus* cells accumulated only a 5.6% of Zn from a culture medium supplemented with 267 μg Zn ml^{-1} as $ZnSO_4 \cdot 7H_2O$. However, this biomass sorption did not totally explain the effects here found.

In AMF-colonized plants the expression of genes encoding plasma membrane transporters affecting element accumulation by plants has been reported (Burleigh and Bechmann, 2002). The expression of Zn transporter gene (MtZ1P2) was decreased in root of mycorrhizal plants at a high Zn concentration of

100 mg g^{-1} as described by Burleigh et al. (2003). Thus, concentration of Zn in tissues of AM plants was lower than in non-mycorrhizal plants growing under Zn contamination. Recently, González-Guerrero et al. (2005) suggested the role of *GintZnT1,* encoding a putative Zn transporter in Zn compartmentalization and in the protection of *Glomus intraradices* against Zn stress.

Variations in the chemical behavior of metal species, as well as the composition of microbial cell walls and extracellular materials can result in wide differences in biosorptive capacities (Gadd, 2000). The decreasing ratio of shoot Zn concentration to root weight in bacterial inoculated plants is indicative of a Zn binding mechanism, particularly by strain B-I.

The data from this study suggest that the ability of the bacteria tested to protect plants against the inhibitory effects of high concentrations of zinc is related to its capacity to stimulate plant growth by synthesis of IAA, as has been shown for many PGPRs (Burd et al., 1998, 2000). A low level of IAA produced by rhizobacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Xie et al., 1996). Thus plant growth-promoting bacteria can facilitate plant growth by altering the plant hormonal balance.

On the other hand, some mycorrhizal fungi can protect plants from toxic arsenate and zinc by decreasing their uptake of these metals (Joner et al., 2000; Sharples et al., 2000) as happened in this study for Zn acquisition. Chen et al. (2003) reported that below the critical Zn application (a rate of 50 mg kg^{-1}), the root acquisition of this metal was enhanced in AM plants while above this critical rate of 50 mg kg^{-1} Zn translocation to the shoots decreased. In this study this AMF effect resulting in a decreased Zn translocation to the shoot was observed at the three Zn concentrations (24, 68 and 215 μg g^{-1}) used. Chen et al. (2003) also found that at whatever Zn level, AMF colonization increased the Zn accumulation in the roots and this may help to explain the alleviation of Zn toxicity at high Zn concentrations in the medium.

A number of different mechanisms may be involved in the interactions between mycorrhizal colonization and accumulation of heavy metals, including tissue dilution of the toxic element due to interactions with P nutrition (and increased plant yield), sequestration of the toxic metal by the fungus, decreased Zn solubility in the soil due to changes in rhizosphere pH and development of tolerance by the fungus (Smith and Read, 1997; Li and Christie, 2001).

If AMF colonized plants accumulate Zn in the roots such as Chen et al. (2003) observed and on the other hand, bacteria can also bind some amounts (5.6%) of this metal, apart from benefit on plant growth and nutrition, the additive effects can explain the alleviation of the detrimental effects caused by Zn in dually inoculated plants.

In summary, we have shown a protective effect of the interaction AMF-bacteria against uptake of potentially toxic Zn by *Trifolium* plants growing in moderately polluted soil. The use of these microorganisms can be considered as a biotechnological tool of great economical and ecological relevance.

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