Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity

A. Vivas, R. Azcón, B. Biró, J.M. Barea, and J.M. Ruiz-Lozano

Abstract: We isolated two bacterial strains from an experimentally lead (Pb)-polluted soil in Hungary, 10 years after soil contamination. These strains represented the two most abundant cultivable bacterial groups in such soil, and we tested their influence on *Trifolium pratense* L. growth and on the functioning of native mycorrhizal fungi under Pb toxicity in a second Pb-spiked soil. Our results showed that bacterial strain A enhanced plant growth, nitrogen and phosphorus accumulations, nodule formation, and mycorrhizal infection, demonstrating its plant-growth-promoting activity. In addition, strain A decreased the amount of Pb absorbed by plants, when expressed on a root weight basis, because of increased root biomass due to the production of indoleacetic acid. The positive effect of strain A was not only evident after a single inoculation but also in dual inoculation with arbuscular mycorrhizal fungi. Strain A also exhibited higher tolerance than strain B when cultivated under increasing Pb levels in the spiked soil. Molecular identification unambiguously placed strain A within the genus *Brevibacillus*. We showed that it is important to select the most tolerant and efficient bacterial strain for co-inoculation with arbuscular mycorrhizal fungi to promote effective symbiosis and thus stimulate plant growth under adverse environmental conditions, such as heavy-metal contamination.

Key words: arbuscular mycorrhizal symbiosis, Pb-polluted soil, plant-growth-promoting bacteria.

Résumé : Nous avons isolé deux souches de bactéries d'un sol pollué expérimentalement au plomb (Pb) en Hongries, 10 ans après la contamination du sol. Ces souches étaient représentatives des deux groupes de bactéries cultivables les plus abondants dans un tel sol; nous avons analysé leur impact sur la croissance de *Trifolium pratense* L. et sur le fonctionnement de champignons mycorhiziens indigènes soumis à une toxicité au Pb dans une second sol contaminé au Pb. Nos résultats démontrent que la souche bactérienne A a stimulé la croissance végétale et l'accumulation de l'azote et de le phosphore, de même que la formation de nodules et l'infection des mycorhizes, ce qui démontre son activité favorisant la croissance des plantes. De plus, la souche A a diminué la quantité de Pb absorbé par les plantes lorsque exprimé sur la base du poids des racines, grâce à une augmentation de la biomasse racinaire due à la production d'acide indol acétique. L'effet positif de la souche A n'a pas été manifeste seulement après une inoculation simple, mais aussi suite à une inoculation double avec des champignons arbusculaires mycorhiziens. La souche A a aussi démontré une résistance supérieure à la souche B lorsque cultivée dans des taux croissants de Pb dans le sol contaminé. Une identification moléculaire a placé sans ambiguïté la souche A dans le genre *Brevibacillus*. Nous montrons qu'il est important de sélectionner la souche bactérienne la plus tolérante et la plus efficiente pour une co-inoculation avec des champignons arbusculaires mycorhiziens afin de favoriser une symbiose efficace et ainsi stimuler la croissance végétale dans des conditions environnementales défavorables, tel qu'une contamination aux métaux lourds.

Mots clés : symbiose arbusculaires mycorhizienne, sols pollués au Pb, bactéries favorisant la croissance des plantes.

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Introduction

The pollution of soils with toxic metals, such as lead (Pb), due to man-made activities poses a major environmental and human health problem (Leyval et al. 1997). The sources of Pb in the soil are diverse, including the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge, pigments, and spent batteries (Darbon et al. 1992; Mercier et al. 2002). Pb is the principal contaminant in soil and waste deposits (Royer et al. 1992). Various plant species, including trees, vegetable crops, grasses, and weeds, are known to accumulate Pb (Kabata-Pendias and Pendias 1992). There are public health implications if plant foods accumulate high concen-

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trations of Pb; furthermore, leaf fall and dispersal may pose a hazard (Pichtel et al. 2000). In the United States, a detailed analysis of 12 epidemiological studies (Lanphear et al. 1998) showed that 5.9% of American children had more than 10 mg of Pb/dL in their blood. This value is the threshold above which neurotoxicity from lead occurs (Lanphear et al. 1998).

High metal concentrations in the soil are toxic to the bacteria and fungi living in it, as well as to plants. In recent years, several studies have shown the harmful effects of metals on microbial diversity and their activity in the soil (Brooks et al. 1986; Chaudri et al. 1993; McGrath et al. 1995; del Val et al. 1999*a*). Ecosystem functioning is largely governed by soil microbial activity because the biogeochemical cycles of major plant nutrients are carried out by microorganisms (Jeffries and Barea 1994; Kennedy and Smith 1995). Low-input sustainable agriculture requires a more profound understanding of the interactions of agricultural plants with soil microorganisms, mainly those microorganisms that may have a direct effect on plant productivity (Gryndler et al. 2000). Among these microorganisms, arbuscular mycorrhizal (AM) fungi are ubiquitous in soils throughout the world and form symbiotic relationships with the roots of most terrestrial plants. AM fungi play a crucial role not only in improving plant growth and nutrition but also in protecting the host plant against biotic and abiotic stresses (Smith and Read 1997). A number of studies have demonstrated the importance of AM symbiosis for plant survival and development in heavy-metal-polluted environments (Díaz et al. 1996; Leyval et al. 1997; Kaldorf et al. 1999).

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; Garbaye 1994). One of these bacterial groups, the so-called plant-growth-promoting rhizobacteria (PGPR), has been reported by several authors to interact with AM fungi (Meyer and Linderman 1988, 1992; Barea et al. 2002*a*, 2002*b*). The definitive effect of soil microorganisms, including AM fungi, on plant development is the result of the interactions among the different soil microbial components involved (Requena et al. 1997). During the last decade the role of PGPR as modifiers of soil fertility and as facilitators of plant establishment and development has been considered (Linderman 1992; Glick 1995; Requena et al. 1997). However, the manipulation of beneficial combinations of microorganisms depends on a proper understanding of the ecosystem to apply a suitable selection of microbes (Puppi et al. 1994).

Both AM fungi and soil bacteria can adapt to specific environmental conditions and develop tolerance to stressful environments (Brundrett 1991; Ruiz-Lozano and Azcón 2000). Heavy-metal tolerance in soil microorganisms has been studied not only in the context of removing metals from polluted soils but also in providing a biological understanding of the adaptation of living organisms to extreme environments. In the interaction between AM and heavy metals, two important aspects must be considered: (*i*) the effect of the metal on the AM fungus and its tolerance to the metal and (*ii*) the effect of the fungus on the availability and transfer of the metal to the host plant (Leyval et al. 1997). A higher tolerance to heavy metals of indigenous mycorrhizal fungi from sludge-polluted sites in comparison with those of reference isolates from unpolluted soils has been described previously (Gildon and Tinker 1983; Díaz et al. 1996; del Val et al. 1999*b*). Indigenous bacterial populations may have adapted in a similar way to metal toxicity and evolved capacities that enable the bacteria to survive in polluted soils. Under natural conditions, however, soil becomes contaminated with more than one metal, making it difficult to determine which metals are responsible for the toxic effects observed (Chaudri et al. 1992). Therefore, only long-term experiments with soils supplemented with a single metal salt can give the opportunity to study the real toxic effects of each heavy metal on the beneficial microbes for any length of time (Biró et al. 1998). An agricultural soil from Nagyhörcsök Experimental Station (Hungary) was contaminated in 1991 with suspensions of 13 microelement salts applied separately. Each salt was applied at four levels (0, 30, 90, and 270 mg kg^{-1}), as described by Biró et al. (1998). In this study, we have isolated two indigenous bacterial strains from the Pb-polluted soil 10 years after contamination and tested their influence on both plant growth and on the functioning of native mycorrhizal fungi in the face of Pb toxicity. The study was carried out in a local soil previously polluted with Pb to test whether or not such microorganisms maintain their properties against Pb when growing in a different soil from that where they were isolated.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a three-factor randomized complete block design of (*i*) bacterial treatment, including assays with two rhizobacterial species and one uninoculated control treatment; (*ii*) inoculation with an indigenous mycorrhizal inoculum, including an uninoculated control treatment; and (*iii*) three levels of Pb added to the soil (30, 90, or 270 mg Pb kg^{-1}). Five replicates were made for each treatment, totalling 90 pots.

For each Pb level, data were subjected to an analysis of variance (ANOVA) with bacterial treatment, AM treatment, and bacterial treatment plus AM treatment interaction as sources of variation, followed by Duncan's multiple range test (Duncan 1955). Percentage values were arcsin transformed before statistical analysis.

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 content) 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, and the following nutrient concentrations (mg kg^{-1}): nitrogen (N), 140 (Kjeldahl); phosphorus (P), 17 ($NaHCO_3$-extractable P); and potassium (K), 80. The cadmium (Cd), nickel (Ni), and zinc (Zn) concentrations in the soil were 0.3, 2.6, and 1.3 mg kg^{-1}, respectively. The particle size distribution 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 Pb kg^{-1} by adding adequate amounts of an aqueous solution of $Pb(NO_3)_2$ at 0.145 mol L^{-1}, 0.43 mol L^{-1}, or 1.3 mol L^{-1}, respectively, into three soil subsamples. The soil was left in a greenhouse for a 6-week period and then the amount of available Pb remaining was determined following EDTA extraction. After the 6-week incubation, the amount of Pb remaining in the soil was 29, 79, and 255 mg Pb kg^{-1}, respectively. These three contamination levels were selected to range from a low contamination level (30 mg Pb kg^{-1}), a medium contamination level (90 mg Pb kg^{-1}), and a high contamination level (270 mg Pb kg^{-1}).

Red clover (*Trifolium pratense* L.) seeds were sterilized in a 15% sodium hypochlorite solution for 15 min, 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 three Pb levels. A suspension (1 mL seed) of the diazotrophic bacterium *Rhizobium leguminosarum* bv. *trifolii* (109 cells mL^{-1}) was sprinkled over the seeds of all treatments at sowing time.

Two bacterial strains exhibiting different colony morphology and referred to as strain A or strain B were isolated from Pb-contaminated soil at Nagyhörcsök Experimental Station (Hungary). 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 a dilution of 10^4–10^7. The suspension was cultured on nutrient broth (Panreac, Montcada i Reixac, Spain) agar plates (Gryndler et al. 2000). The two bacterial strains selected for this study were the most abundant cultivable types growing on nutrient broth plates. For inoculation, appropriate pots were sprinkled with 1 mL (109 cells mL^{-1}) of each bacterial strain over the seeds and then covered with a layer of soil/sand mixture.

The autochthonous mycorrhizal inoculum (a mixture of AM fungal species), also isolated from the Pb-contaminated soil at Nagyhörcsök (Hungary), consisted of soil, spores, mycelia, and infected root fragments and was enriched in an open-pot culture of red clover (Brundrett et al. 1996). Ten grams (fresh weight) of inoculum was added to appropriate pots at sowing time just below the clover seeds.

Nonmycorrhizal 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.

Growth conditions

Plants were grown for 3 months in a controlled environmental chamber with 70%–80% relative humidity, light:dark temperatures of 25 °C : 15 °C, and a photoperiod of 16 h at a photosynthetic photon flux density of 460–500 μmol m^{-2} s^{-1} (Licor, Lincoln, Neb., U.S.A., model LI-188B).

Plants were daily watered to avoid any water deficit during the growth period. A characteristic soil moisture curve was previously constructed in our laboratory and used to determine soil water content by gravimetric measurement in the pots (Richards 1954; Ruiz-Lozano et al. 2001). Pots were daily weighed (at the end of the afternoon), and the amount of water lost was added to the pot to maintain the soil water content near field capacity. Each week throughout the experiment, the plants received 10 mL of Hewitt's nutrient solution lacking N and P (Hewitt 1952).

Parameters measured

Biomass production and nutrients and metals 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 N (micro-Kjeldahl) and P (Olsen and Dean 1965) were determined. Pb, Cd, Ni, and Zn concentrations were measured after wet digestion of the air-dried plant samples with HNO_3 + H_2O_2, followed by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Takács et al. (2001).

Symbiotic development

The percentage of infected mycorrhizal root length 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 with the grid – line intersect method (Giovannetti and Mosse 1980). Nodule numbers were estimated by direct observation with a binocular microscope. Five replicates were used per treatment.

Bacterial growth under increasing Pb levels in the medium

Bacterial isolates A and B were cultivated at 28 °C in nutrient broth supplemented with 0, 10, 100, 200, or 300 mg $Pb(NO_3)_2$ L^{-1}. The number of viable cells was estimated as the number of CFU mL^{-1} at 1-h intervals from 0 to 16 h. Four replicates were used in each determination.

Bacterial ability for Pb sorption

A *Brevibacillus* sp. from the Spanish Microbiology Collection at Burjasot, Valencia, was used as a reference bacterium for comparison with strain A. The biosorption study was carried out as described by Kanazawa and Mori (1996). Bacteria were grown in 200 mL of nutrient broth until reaching 1 U of optical density (600 nm). Cells were then harvested by centrifugation at 3000*g* for 15 min and the bacterial pellet washed twice with Ringer's solution. The harvested biomass was incubated for 1 h at 28 °C with a solution containing 200 mg Pb L^{-1}. After the incubation, the cells were centrifuged again and the bacterial biomass was used for Pb determination after digestion with nitric acid. The amount of Pb accumulated by the bacteria was measured on a dry biomass basis (Kanazawa and Mori 1996). The Pb remaining in the supernatant after bacterial incubation for 1 h was also determined.

Production of indole-3-acetic acid

The production of indole-3-acetic acid (IAA) by the bacteria was measured by the method of Wöhler (1997). The bacteria was grown overnight on nutrient broth and then collected by centrifugation at 1000*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 mol L^{-1} $CaCl_2$ were added. The solution was filtered through a Whatman filter

paper No. 2. Three millilitres of the filtrate was transferred in a test tube, and to this 2 mL of salper solution (2 mL 0.5 mol L^{-1} $FeCl_3$ and 98 mL 35% perchloric acid) was 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 ranged from 0.5 to 10 mg IAA L^{-1}.

Molecular identification of the most effective bacterial strain

Total DNA from bacterial isolate A was obtained as described by Giovannetti et al. (1990) and characterized by sequence analysis of the small ribosomal subunit (16S ribosomal DNA). PCR was carried out to amplify nearly the entire gene with the eubacterial primers 27f and 1495r (Lane 1991) located at the 5′ and 3′ ends of the ribosomal rDNA sequence, respectively. The amplification reactions were performed in a 20-µL volume containing 0.5 µmol L^{-1} concentrations of each primer, 100 µmol L^{-1} deoxynucleoside triphosphates, 1× PCR buffer (Sigma, St. Louis, Mo., U.S.A.), 2.5 mmol L^{-1} $MgCl_2$, 10 ng of genomic DNA, and 0.25 U *Taq* DNA polymerase (Sigma). A PerkingElmer/Cetus DNA Thermal Cycler (Norwalk, Conn.) was used with the following parameters: initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. The amplified DNA was purified following electrophoresis through a 1.2% agarose gel with the QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany) and cloned into plasmid pGME (Promega, Madison, Wis.) for sequencing (Sanger et al. 1977).

Results

Shoot dry weight

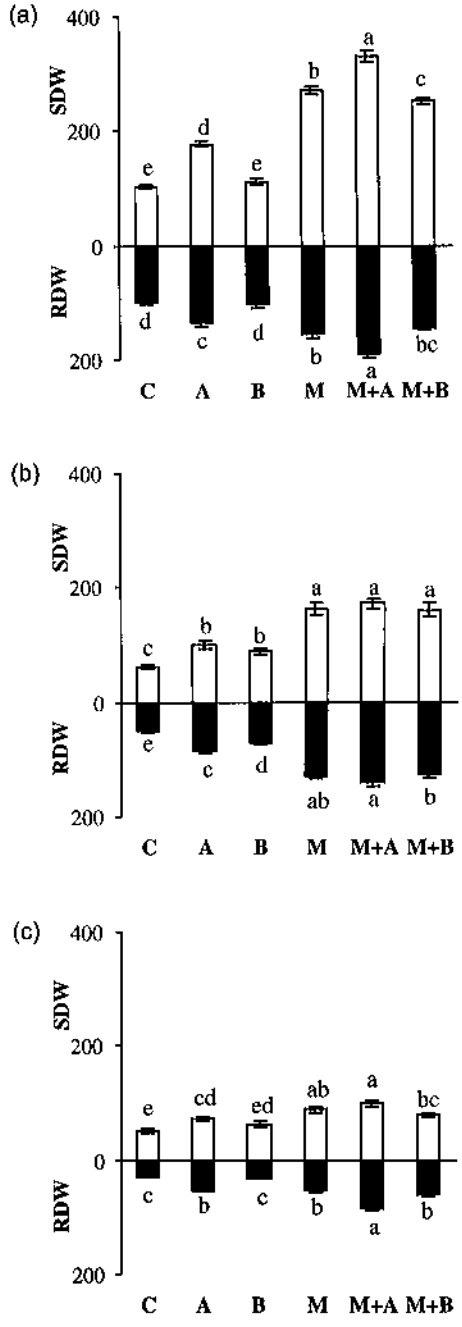
Under the lowest Pb level (30 mg kg^{-1}), nonmycorrhizal plants inoculated with strain A had higher shoot dry weight than control plants (Fig. 1). Mycorrhizal colonization by autochthonous AM fungi increased shoot dry weight by 165% compared with control plants. This increase jumped to 223% when mycorrhizal plants were also co-inoculated with strain A. On the other hand, at the mid Pb level (90 mg kg^{-1}), both bacterial treatments slightly increased shoot dry weight in nonmycorrhizal plants. Mycorrhizal colonization enhanced shoot biomass production by over 160%, both with single inoculation and after co-inoculation with strain A or B, compared with nonmycorrhizal control plants.

At the highest Pb level (270 mg kg^{-1}), inoculation with strain A enhanced shoot dry weight compared with uninoculated control plants. Again, the most beneficial treatment was mycorrhization, which considerably enhanced plant biomass production, mainly in dual combination with bacterial strain A (an increase of 94%).

Root dry weight

At the lowest Pb level, root dry weight of mycorrhizal and nonmycorrhizal plants was slightly enhanced by strain A (Fig. 1). At the mid Pb level, root development followed a similar pattern to the shoot dry weight. In fact, both bacterial strains stimulated root development in nonmycorrhizal plants, but the most important effect was caused by mycorrhization, both alone and in combination with either bacterial strain. A similar effect was observed at the highest Pb level, except for nonmycorrhizal plants inoculated with strain B, which showed the same values of root development as that of the

Fig. 1. Shoot (SDW) and root (RDW) dry weights (mg $plant^{-1}$) of red clover plants cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are designated as C (control), A (bacterium A), B (bacterium B), M (mycorrhizae), M + A (mycorrhizae + bacterium A), and M + B (mycorrhizae + bacterium B). Within each Pb level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 5$).



controls. The toxic effect of Pb decreased root dry weight in all treatments compared with the lower Pb levels.

Nodule number

At the lowest Pb level, inoculation with either bacterial strain considerably enhanced the nodule number of nonmycorrhizal plants compared with control (Fig. 2). Mycorrhization also increased the nodule number by 385% compared with control plants. This increase was even higher after co-inoculation of AM fungi with either bacterial strain, reaching an increase of 519% in the case of strain A. At the mid Pb level, the nodule number was not affected by any bacterial strain in nonmycorrhizal plants. Mycorrhizal colonization increased the nodule number by 383% and even higher (558%) when co-inoculated with strain A. Dual inoculation with AM fungi and strain B increased the nodule number by 275%. An increase to 270 mg Pb kg^{-1} in the growth medium caused a decrease in the number of nodules compared with the lower Pb levels. As a consequence of Pb toxicity, the control plants did not exhibit any nodules in their roots. However, the benefits of bacterial inoculation on this parameter were still evident, mainly in mycorrhizal plants, producing between 31 and 49 nodules per plant.

AM colonization

No mycorrhizal colonization was observed in control plants. At the lowest Pb level, the percentage of mycorrhizal colonization of AM-inoculated plants was similar for all three treatments and no effect of bacterial co-inoculation on mycorrhizal development was observed (Fig. 3). At the mid Pb level, mycorrhization was stimulated by co-inoculation with strain A, and the same was observed at the highest Pb level assayed. The percentage of mycorrhizal infection decreased concomitantly with an increase in the Pb level.

Nutrient concentrations

At the three Pb levels, the N concentration of nonmycorrhizal plants increased when they were inoculated with bacterial strain A, while those inoculated with strain B only showed an increase in N concentration when compared with control plants at the lowest Pb level (Fig. 4). Except at 30 mg Pb kg^{-1}, mycorrhization also increased the N concentration in plants when compared with controls. The increase due to AM symbiosis was not affected by co-inoculation with either bacterial strain. Concerning P concentration (Fig. 5), in nonmycorrhizal plants there was a significant increase in P after inoculation with strain A. Mycorrhizal plants also showed enhanced P concentration at all Pb levels when compared with uninoculated controls. Again, the increase in P concentration was not affected by co-inoculation of AM fungi with either bacterial strain.

Metal concentrations

The Pb concentration in shoots was also evaluated (Fig. 6). Results obtained showed that the Pb concentration in plants increased as Pb in the spiked soil also increased. No significant differences among treatments were found at the lowest Pb level. At the medium Pb level, only mycorrhization alone and co-inoculation of AM fungi plus strain A showed lower Pb concentration compared with the rest of treatments. At the highest Pb level, co-inoculation of AM fungi plus either bacterial strain resulted in enhanced Pb concentration.

We also calculated the ratio of Pb concentration to root weight unit to estimate the amount of Pb absorbed by plants on a root weight unit basis (Table 1). Results showed that at the lowest Pb level, bacterial strain A and mycorrhization (alone or in combination with strain A) decreased this ratio by 36% and 45%, respectively, in comparison with the control treatment. At the medium and high Pb levels, both bac-

Fig. 2. *Rhizobium* sp. nodule numbers in red clover roots cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are as described in Fig. 1. Within each lead (Pb) level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 5$).

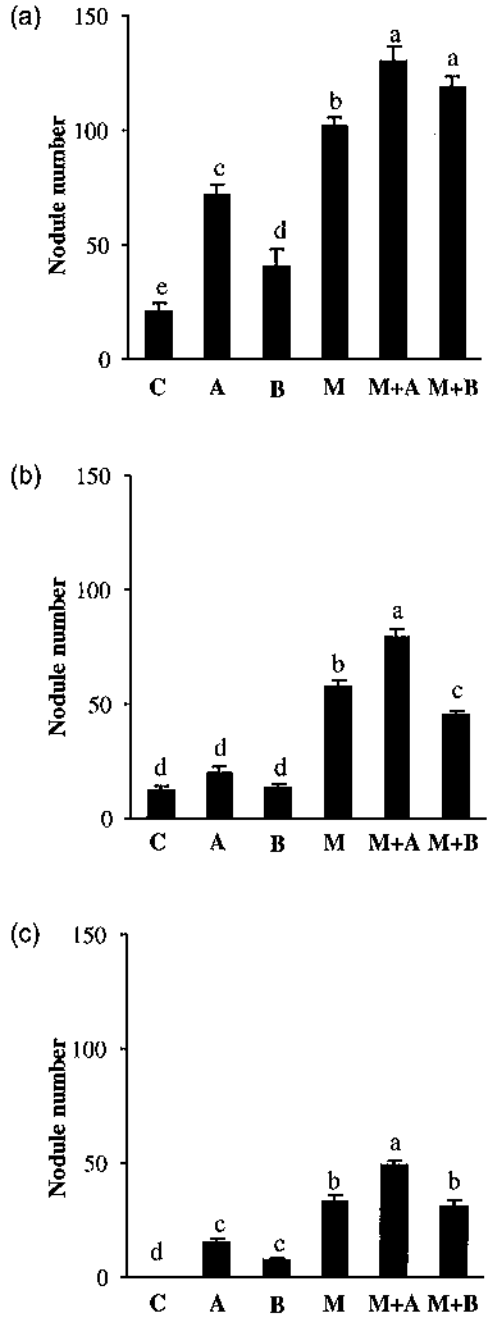


Fig. 3. Percentage of mycorrhizal root length in red clover roots cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are as described in Fig. 1. Within each lead (Pb) level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).

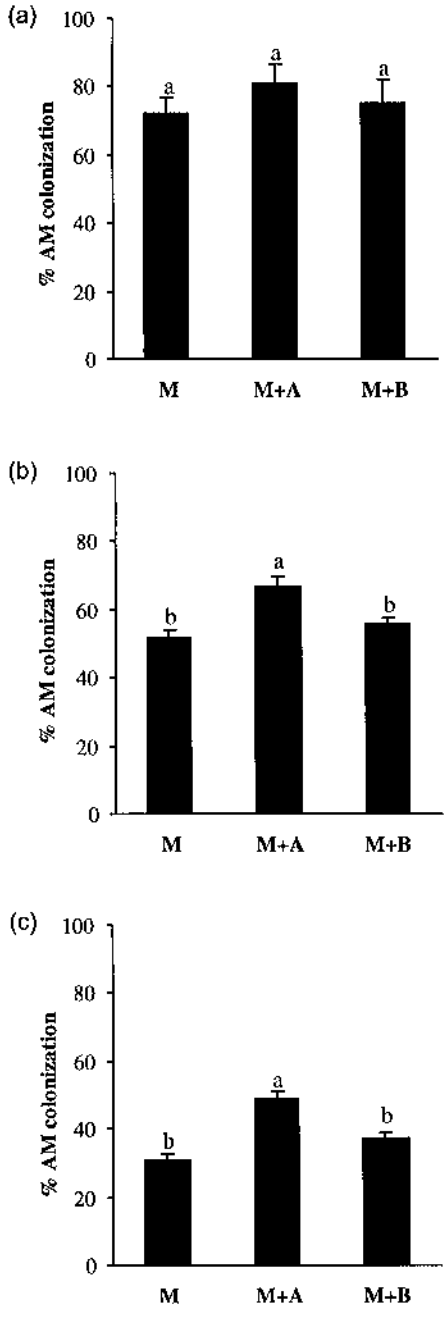
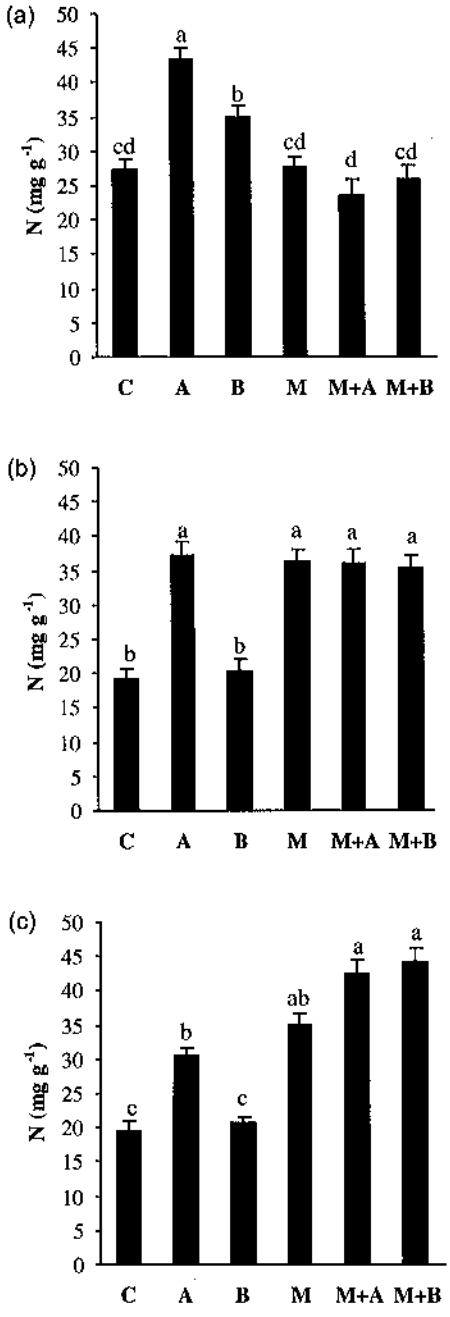


Fig. 4. Nitrogen (N) concentration in red clover plants cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are as described in Fig. 1. Within each lead (Pb) level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).



terial strains and mycorrhization decreased the amount of Pb absorbed per root weight unit. However, strain A decreased this value to a higher extent than strain B, and the co-inoculation of the AM fungi plus strain A showed the lowest Pb uptake per root weight unit (more than 50% of decrease compared with the control treatment).

Regarding other metals, such as Cd, Ni, or Zn, Table 2 shows that at the lowest Pb level in the soil, the Cd concentration in shoots only increased significantly in plants inoculated with bacterial strain B. At the medium Pb level, the concentration of Cd was higher in plants treated by both bacterial strains, especially plants dually inoculated with AM fungi and strain A, than in the uninoculated control plants. In contrast, the mycorrhiza alone or in combination with strain B did not affect this value. At the highest Pb level, again both bacterial treatments increased the Cd concentration in the plants, while the mycorrhiza alone or in combination with strain A did not affect this value.

Fig. 5. Phosphorus (P) concentration in red clover plants cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are as described in Fig. 1. Within each lead (Pb) level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).

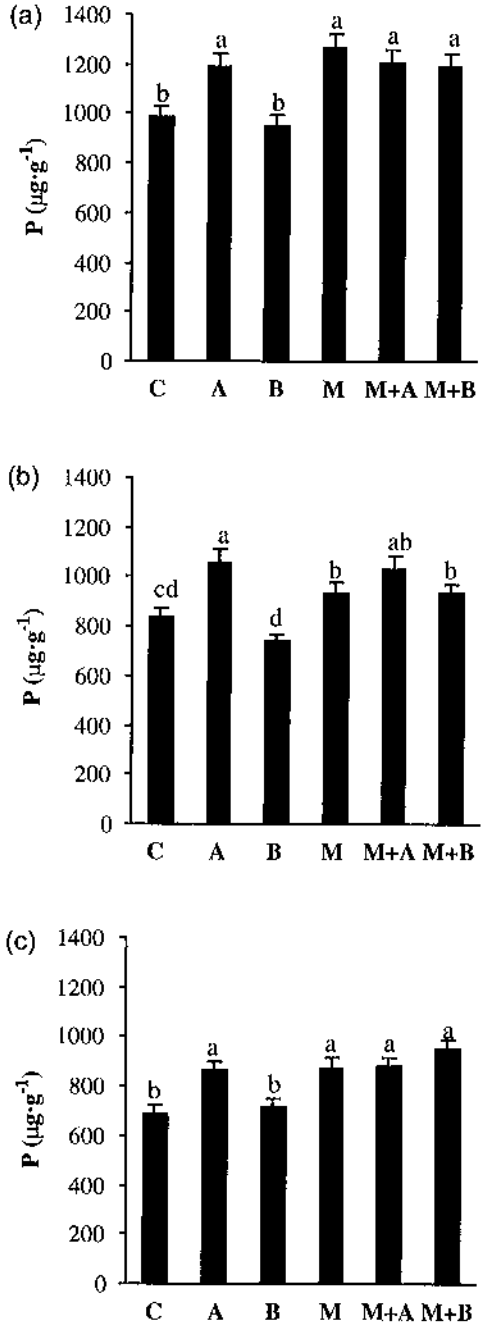
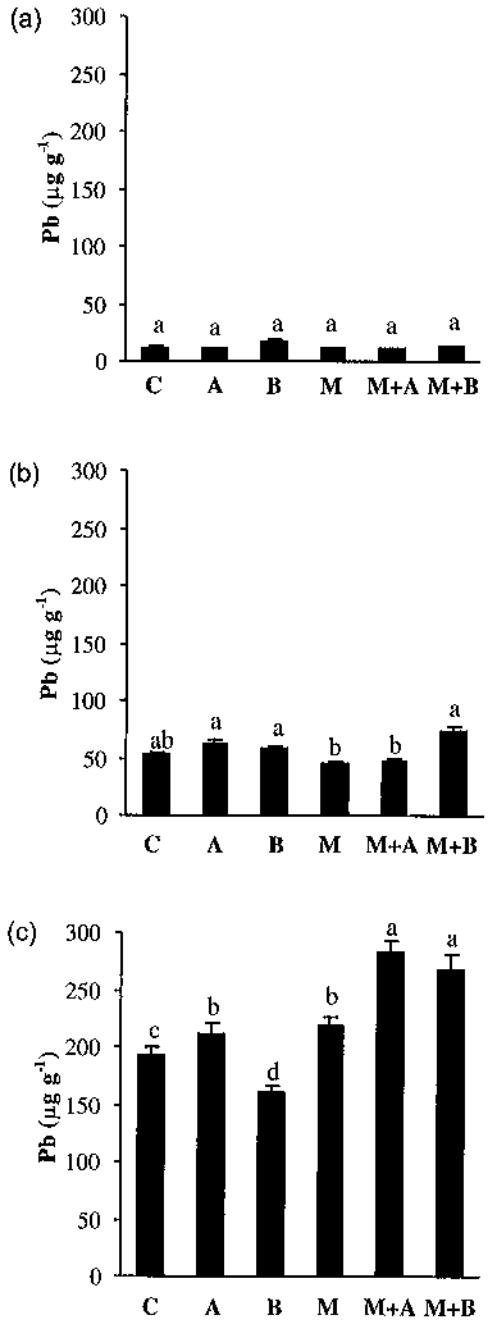


Fig. 6. Lead (Pb) concentration in red clover plants cultivated in soil amended with (*a*) 30, (*b*) 90, or (*c*) 270 mg Pb kg^{-1}. Treatments are as described in Fig. 1. Within each Pb level, bars with a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).



The Ni concentration was clearly enhanced by mycorrhizal colonization (alone or in combination with either bacterial strain) at the three Pb levels in the soil. In contrast, bacterial colonization alone had no effect on Ni concentration.

At the lowest and highest Pb levels, Zn concentration reached the highest values in the uninoculated control plants. It decreased in plants singly inoculated with AM fungi and in those dually inoculated with AM and with either bacterial strain. However, at the medium Pb level, the Zn concentration was similar in control plants and in those dually inoculated with AM fungi and strain A.

Bacterial growth under increasing Pb levels in the medium

Both bacterial isolates were grown in nutrient broth at increasing Pb concentrations, ranging from 0 mg Pb L^{-1} in the control treatment to 300 mg Pb L^{-1} (Fig. 7). The growth of

Table 1. Ratio of lead (Pb) concentration to root weight unit (mg Pb (g dw root)$^{-1}$) in red clover plants cultivated in soil amended with 30, 90, or 270 mg Pb kg^{-1}.

	Pb concn. (mg kg^{-1})		
Treatment	30	90	270
C	0.12bc	1.03a	6.60a
A	0.08d	0.74c	4.10c
B	0.17a	0.81b	5.10b
M	0.07de	0.35e	4.00c
M + A	0.05e	0.33e	3.30d
M + B	0.09cd	0.58d	4.40c

Note: Treatments were as follows: C (uninoculated control), A (bacterium A), B (bacterium B), M (mycorrhizae), M + A (mycorrhizae + bacterium A), and M + B (mycorrhizae + bacterium B). Within each Pb level, columns having a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).

both bacterial strains decreased concomitantly with an increase of Pb in the medium. However, strain A exhibited a higher tolerance to Pb than strain B. At low Pb in the medium (10 and 100 mg L^{-1}), bacterial growth reached almost 10^5 CFU after 5 h of growth, while strain B needed more than 9 h to reach a similar number of CFU. In addition, strain A produced 10^7 CFU after 10 h of incubation, while strain B only reached 10^5 CFU (100 mg L^{-1}). At higher Pb levels in the growth medium (200 and 300 mg L^{-1}), strain A reached more than 10^5 CFU after 10 h of incubation, while strain B never reached 10^4 CFU.

Pb biosorption

The Pb-adsorbing capability of strain A was 26% of the biomass dry weight, while that of a collection *Brevibacillus* strain was only 2.8% of the biomass dry weight.

Production of IAA

The production of IAA by bacterial strain A was tested against *Bacillus pumillus* (isolate B.3) and *Bacillus licheniformis* (isolate B.21) (Probanza et al. 1996; Gutierrez-Mañero et al. 1996). Strain A showed a higher production of IAA (3.8 mg L^{-1}) than both reference PGPR bacteria (average of 1.4 mg L^{-1}).

Molecular identification of the most efficient bacterial isolate

The obtained 16S rDNA sequence for strain A has been deposited in the EMBL data bank under accession number AJ457160. Data base searches for 16S rDNA sequence similarity using FASTA and BLAST algorithms unambiguously identified the bacterial isolate A as a member of the genus *Brevibacillus*. The 16S rDNA sequence from this strain showed the highest similarity (more than 97%) with that of *Brevibacillus agri* (accession no. AB039334).

Discussion

The study of synergic or antagonic effects of PGPR and AM fungi when co-inoculated is a crucial step within the framework of heavy-metal remediation strategies, but no studies have been reported about such an interactive effect. In this study, two bacterial strains isolated from a Pb-polluted soil and representing the two most abundant cultivable bacterial groups in such soil, were tested as PGPR in single or dual inoculation with a native AM inoculum from the same soil, under increasing Pb levels. Results showed that bacterial strain A increased plant growth and nutrient accumulation, as well as legume nodule numbers and mycorrhizal infection, demonstrating its plant-growth-promoting activity. In addition, this bacterial strain exhibited a higher Pb tolerance than strain B when cultivated under increasing Pb levels in the growth medium. We therefore selected this bacterial strain for molecular identification. 16S rDNA sequence analysis unambiguously identified strain A as a member of the genus *Brevibacillus*.

The mechanisms involved in the positive effect of the isolated *Brevibacillus* sp. as a PGPR are not fully known, but some authors have reported that PGPR can influence plant development not only directly (i.e., through the production of hormones, siderophores, or antibiotics or by P solubilization or asymbiotic N fixation) but also indirectly through modifications to the activity of other plant–microbe interactions, such as the mycorrhizal or the *Rhizobium* symbioses (Meyer and Linderman 1986; Azcón 1989, 1993; Garbaye 1994; Linderman 1994; Barea et al. 1996), or by inducing changes in the microbial population balance, for instance by exerting biological control against plant pathogens (Weller and Thomashow 1994). The capacity of this *Brevibacillus* sp. to produce IAA in vitro has been evidenced, and this may have contributed to the beneficial effects observed, since the production of IAA or ethylene has been proposed as a mechanism for plant growth promotion under heavy-metals stress (Pishchik et al. 2002).

The protective effect of the bacteria in the presence of Pb toxicity is another important observation of this study. Plants inoculated with strain A (singly or in combination with AM fungi) grew and developed better at whatever Pb level assayed. It has been proposed that soil bacteria are associated with the clay and organic fractions of the soil microenvironment and would be expected to participate in the metal dynamics typically ascribed to these soil fractions (Mullen et al. 1989). Bacteria have a high surface area to volume ratio (Beveridge 1988) and, as a strictly physical cellular interface, should have a high capacity for sorbing metals from solutions (Mullen et al. 1989). Several investigations have shown that relatively large quantities of metallic cations are complexed by algae (Matsunaga et al. 1999), fungi (Zhou 1999), and bacteria (Mullen et al. 1989; Chen et al. 1999; Samuelson et al. 2000). Our results showed the important ability of strain A for Pb sorption (26% of the biomass weight) that may have contributed to Pb removal from soil, alleviating Pb toxicity for plants. In fact, the amount of Pb absorbed per root weight unit decreased considerably in plants inoculated with strain A or with AM fungi plus strain A. Bacterial capture systems have been described as a promising tool for immobilization and removal of heavy metal from polluted environments (Pazirandeh et al. 1998; Kotrba et al. 1999).

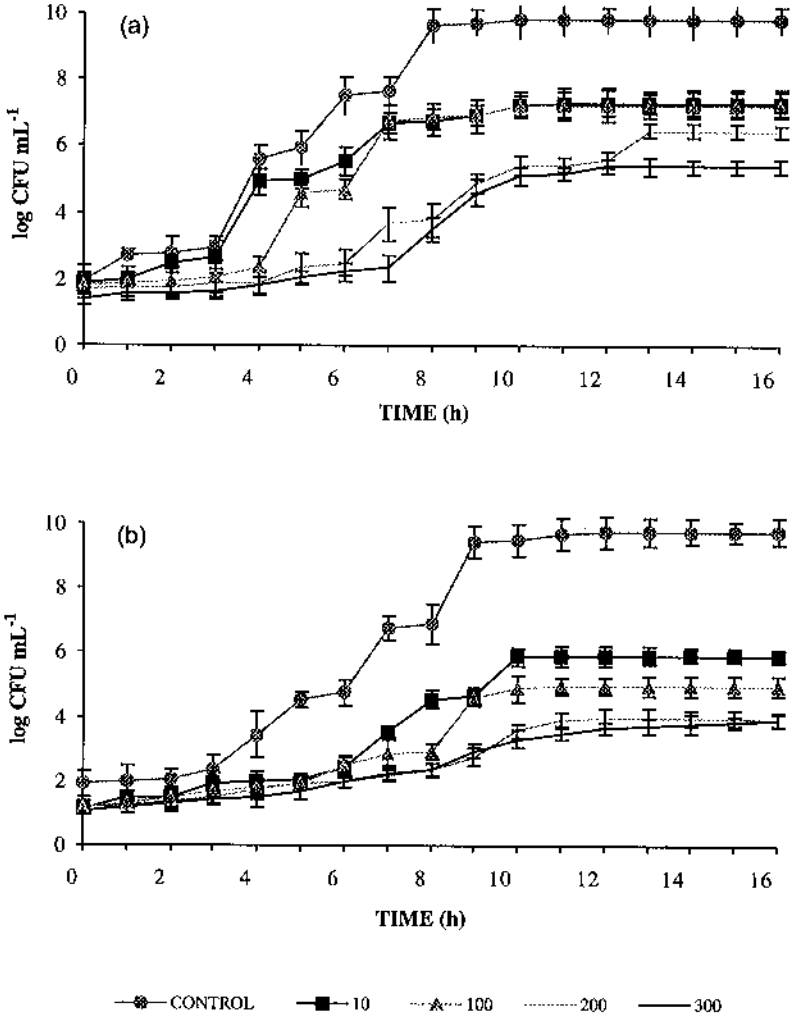
Another mechanism by which strain A could have contributed to protecting plants against Pb toxicity is by stimulation

Table 2. Cadmium (Cd), Nickel (Ni), and Zinc (Zn) concentrations (μg g^{-1}) in shoots of red clover plants cultivated in soil amended with 30, 90, or 270 mg Pb kg^{-1}.

	30 mg Pb kg^{-1}			90 mg Pb kg^{-1}			270 mg Pb kg^{-1}		
Treatment	Cd	Ni	Zn	Cd	Ni	Zn	Cd	Ni	Zn
C	0.060b	2.3b	73.1a	0.037c	1.6c	66.6ab	0.036c	2.7b	77.7a
A	0.040b	2.5b	58.0b	0.066b	2.3bc	60.0b	0.070a	2.5b	78.9a
B	0.090a	2.4b	64.3ab	0.066b	2.4bc	65.4ab	0.048b	2.9b	70.3a
M	0.035b	3.5a	56.0b	0.044c	3.7a	56.0b	0.045bc	3.9a	56.0b
M + A	0.040b	3.4a	41.5c	0.092a	3.1ab	78.1a	0.042bc	4.1a	30.4c
M + B	0.057b	3.7a	38.0c	0.032c	4.2a	33.1c	0.050b	4.2a	31.3c

Note: Treatments were as follows: C (uninoculated control), A (bacterium A), B (bacterium B), M (mycorrhizae), M + A (mycorrhizae + bacterium A), and M + B (mycorrhizae + bacterium B). Within each Pb level, columns having a common letter are not significantly different ($P \leq 0.05$), as determined by Duncan's Multiple Range Test ($n = 4$).

Fig. 7. Number of viable cells (log CFU mL^{-1}) at different time intervals of (*a*) strain A and (*b*) strain B grown in nutrient broth supplemented with 0 (control), 10, 100, 200, or 300 mg Pb L^{-1}. Data correspond to the average value plus the standard error ($n = 4$).



of root exudates. In this study, bacterial strain A induced a bigger root system, probably due to IAA activity. Hence, it is likely that the amount of root exudates has also increased. Root exudates have a variety of roles, including that of metal chelators (Hall 2002). In an investigation into the role of Ni-chelating exudates, it was observed that Ni-chelating histidine and citrate accumulated in the root exudates, and thus could help to reduce Ni uptake, and so may play a role in the Ni-detoxification strategy (Salt et al. 2000). Since the range of compounds exuded is wide, other exudates could play a role in tolerance to other metals, such as Pb (Hall 2002).

The positive effect of *Brevibacillus* sp. was evident not only in single inoculations but also in dual inoculations with AM fungi. In fact, this bacterial strain was compatible with arbuscular mycorrhizal symbiosis, increasing the benefits of

such symbiosis for plant growth and nutrient uptake under the adverse environmental conditions assayed here. The positive effects of AM fungal and bacterial interactions and their synergistic influence on host-plant growth have been previously demonstrated (Barea 1997; Barea et al. 2002*a*, 2002*b*). The ability of soil bacteria to stimulate the growth and the activity of arbuscular mycorrhizae has been well documented (Azcón-Aguilar et al. 1986; Mayo et al. 1986; Azcón 1987; Gryndler et al. 2000; Requena et al. 1997) and most probably involves the production of bioactive stimulatory compounds (Hrselová and Gryndler 2000). The inoculation of sporocarps of *Glomus mosseae* with a strain of *Bacillus* produced a noticeable increase in hyphal growth (Requena et al. 1999). Requena et al. (1999) hypothesized that the bacteria could be acting through a chemical signal diffused within the medium that produced increased hyphal growth. Hence, it is likely that a stimulation of extraradical mycelium production by the AM fungi also accounted for the positive effects observed. The AM mycelium has a high metal sorption capacity compared with other soil microorganisms (Joner et al. 2000), and this could have contributed to protect host plants against Pb toxicity.

In this study, both the mycorrhizal inoculum and the *Brevibacillus* sp. isolates came from Pb-contaminated soil. This is important from a practical point of view, as there is a great difference between selecting beneficial organisms for potential inoculation of plants in a sterile soil and managing to get these organisms to establish and persist in a natural soil environment. There is little point in selecting the most effective PGP organism in the laboratory unless it survives and multiplies in the field in sufficient numbers to express itself. There are many examples in the literature of the failure of introduced strains of *Rhizobium* to increase nitrogen fixation and of failures in the use of biocontrol organisms and of inconsistent plant growth stimulation by PGPR (Bowen and Rovira 1999). Hence, management of the rhizosphere involves not only introducing organisms but also inoculating indigenous organisms living in the soil.

In conclusion, we showed that the selection of indigenous soil bacteria for co-inoculation with AM fungi represents a significant factor in determining the efficiency of mycorrhizal symbiosis and that it can be of great importance in stimulating plant development under adverse environmental conditions, such as heavy-metal contamination.

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