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Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels

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"Capsule": *Selected ubiquitous microorganisms are important components of Cd tolerance in plants.*

Abstract

The effect of inoculation with indigenous naturally occurring microorganisms [an arbuscular mycorrhizal (AM) fungus and rhizosphere bacteria] isolated from a Cd polluted soil was assayed on *Trifolium repens* growing in soil contaminated with a range of Cd. One of the bacterial isolate showed a marked PGPR effect and was identified as a *Brevibacillus* sp. Mycorrhizal colonization also enhanced *Trifolium* growth and N, P, Zn and Ni content and the dually inoculated (AM fungus plus *Brevibacillus* sp.) plants achieved further growth and nutrition and less Cd concentration, particularly at the highest Cd level. Increasing Cd level in the soil decreased Zn and Pb shoot accumulation. Coinoculation of *Brevibacillus* sp. and AM fungus increased shoot biomass over single mycorrhizal plants by 18% (at 13.6 mg Cd kg^{-1}), 26% (at 33.0 mg Cd kg^{-1}) and 35% (at 85.1 mg Cd kg^{-1}). In contract, Cd transfer from soil to plants was substantially reduced and at the highest Cd level *Brevibacillus* sp. lowered this value by 37.5% in AM plants. Increasing Cd level highly reduced plant mycorrhization and nodulation. Strong positive effect of the bacterium on nodule formation was observed in all treatments. Results show that selected ubiquitous microorganisms, applied as enriched inocula, are important in plant Cd tolerance and development in Cd polluted soils.

Keywords: Arbuscular mycorrhizal fungus; *Rhizobium* sp; *Brevibacillus* sp; Cadmium levels

1. Introduction

Arbuscular mycorrhizal (AM) fungi improve nutrient transfer from the soil to the roots of the colonised host plant since the mycorrhizal interface developed between roots and soil is very effective in assimilating nutrients (Barea et al., 1987).

Soil contamination by heavy metals is of major importance in industrialized areas. The detrimental effects of heavy metals on soil biochemical and biological properties have been reported (Bääth, 1989). High metal concentrations in soil are toxic to bacteria and fungi but roots of most plant growing in polluted soils are colonised by AM fungi (Shetty et al., 1994a). This is an indication of the ability of AM fungi to develop tolerance to contaminants.

When metals are at toxic concentrations in soil, mycorrhizal rather than non-mycorrhizal host plants are able to colonize these polluted sites (Shetty et al., 1994a, 1994b). Thus, mycorrhizal colonization may be the key to plant survival on contaminated environments by enhancing metal resistance in plants and also by improving essential nutrients uptake. Nevertheless, metal resistance in AM fungi have not been extensively investigated in relation to their host plant (Meharg and Cairney, 2000). The co-evolution of both symbionts and other associated microbial groups in metal contaminated soils indicates the existence of adaptation strategies that enable them to survive in soil with toxic concentrations of metals (Biró et al.,

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1995; Weissenhorn et al., 1994, Weissenhorn and Leyval, 1995; Vöros et al., 1998).

AM fungi isolated from metal-contaminated soils are often more resistant to metals than those collected from uncontaminated environments (Griffioen et al., 1994; Takács et al., 2001). Also bacteria isolated from the rhizosphere in heavy metal polluted soils are more tolerant to metals, have developed resistance (Chaudry et al., 1992) and therefore are likely to play an important role in the growth, mycorrhizal development, metal tolerance and accumulation capacity exhibited by plants growing in metal contaminated soils. In fact, dual symbiosis of AM fungi and *Rhizobium* may represent an aid to the plant adaptation (Hoflich et al., 1993). Thus, the resistance of plants and associated rhizosphere microbial groups to metal-polluted niches may have co-evolved (Takács et al., 2001). Nevertheless the metal resistance or degree of benefit conferred to the plant by AM symbiosis and/or bacteria may be related to the level of pollution and the metal involved. Inoculation with suitable bacteria or/and AM fungi from contaminated soil may be beneficial for plant growth and such microbial interaction is considered as an important strategy in field remediation (Brundrett, 1996; Entry et al., 1996).

In the case of Cd, no biological or physiological functions are attributed to it and it is toxic at certain concentrations (Biró et al., 1995). But selected microorganisms were able to survive in Cd contaminated soil.

The interaction between cadmium, rhizosphere mycorrhizal fungi and bacteria in *Trifolium* plants was studied in soil contaminated with three concentrations of this metal. The microbial strains used were isolated from a long-term Cd contaminated area from a Hungarian (Nagyhorcsok) experimental field (Kádár, 1995).

In this study we have tested the effect of inoculation of *Trifolium repens* with two indigenous bacterial isolates and on AM fungus an plant growth, nutrient uptake, Cd tolerance and symbiotic development. Microorganisms were assayed in single or in dual co-inoculation in soil artificially contaminated with a range of Cd.

2. Materials and methods

2.1. Experimental design

The experiment consisted of a three-factor randomised complete block design with the following factors: (1) bacterial treatment including assays with two autochtonous bacterial species and one non-inoculated control treatment; (2) inoculation or not with an indigenous mycorrhizal inoculum; and (3) three levels of Cd added to the soil (30, 90 or 270 mg kg^{-1}). Five replicates were made for each treatment totaling 90 pots.

2.2. Soil characteristics and cadmium applications

The soil used in the greenhouse experiments was a calcareous loam (57% sand 22.3% silt and 19% clay) collected from Granada (Spain). With 1.63% organic matter and a pH of 7.2 (water). The nutrient concentrations (mg kg^{-1}) were: N (total) 2.1, P (Olsen) 1.7 and K (NH_4^- extractable) 0.8.

The soil was air-dried, sieved to less than 2 mm, mixed with quartz-sand (<1 mm) at a ratio of soil:sand of 4:1 (v/v) and sterilised by steaming for three sterilization cycles (100 °C for 1 h for 3 days).

After sterilization, the soil was supplemented with 30, 90 or 270 mg kg^{-1} Cd by adding adequate amounts of an aqueous solution of $CdSO_4$. After 2 weeks of incubation (for metal stabilization) the available amounts of Cd determined using EDTA as extractant (Lakanen and Erviö, 1971) were: 13.4; 33 and 85.1 mg kg^{-1}, respectively.

2.3. Microbial soil inoculation

The soil samples for microbial inocula production were taken from the Cd-treated long-term field experiment (10 year old) at Nagyhorcsok (Hungary) (Kádár, 1995). From this soil containing the native adapted AMF and bacterial populations both microbial populations were isolated and cultivated for inocula production. The bacterial strains here selected were the two most abundant cultivable types in original Cd polluted soil (Nagyhörcsök, Hungary) (Kádár, 1995). The two bacterial strains exhibited different colony morphology and they are referred in the text as strain A or B.

The bacterial isolation was carried out following the conventional procedure: 1 g of homogeneised rhizosphere soil was suspended in 100 ml of sterile water (dilution 10^2) and 1 ml of this suspension was serially diluted to reach dilutions 10^4 to 10^7. These were plated in agar nutrient broth medium (8 g l^{-1}) and cultivated for 48 h at 28 °C.

Once selected the two most abundant bacterial types, both were independently grown in 250 ml flasks containing 50 ml of nutrient broth (8 gl^{-1}) medium in shake culture for 48 h at 28 °C.

The indigenous AM spores were isolated from Cd polluted soil (Nagyhorcsock, Hungary) by wet-sieving and decanting as described by Vilariño and Arines (1990). The fungus was identified as *G. mosseae* based on morphological observation. All the spores obtained presented similar characteristics than those of *G. mosseae* from collection thus we concluded that only this *Glomus* specie was present in the original contaminated soil.

Mycorrhizal inoculum from this endophyte was multiplied in an open pot culture of *Allium cepa* and after 6 months of plant growth the shoots were eliminated and the undergrown part (mycorrhizal roots plus

soil possessing fungal spores and mycelium) maintained by storage for 3–6 months in polyethylene bags at 5 °C. Inocula consisted of thoroughly mixed rhizosphere samples containing spores, hyphae and mycorrhizal root fragments. Twenty 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 AM inoculum to provide a general microbial population free of AM propagules.

Soil/sand mixture (4:1 v/v) was incubated with 30, 90 or 270 mg kg^{-1} of Cd (as $CdSO_4$) and appropriate pots received the mycorrhizal fungus and/or the bacterial (10^8 cfu) inocula. One millilitre of suspension of *Rhizobium leguminosarum* bv *trifoli* (10^8 cfu) was added to each pot.

2.4. Plant and growth conditions

Trifolium repens L. was grown for 3 months in pots containing 100 g of soil/sand mixture in a greenhouse under controlled climatic conditions (18–24 °C, with a 18/6 h light/dark period). Throughout the experiment plants were fertilised with 10 ml of Hewitt's nutrient solution lacking N and P (Hewitt, 1952).

2.5. Measurements

At harvest, 3 months after sowing, the dry biomass accumulation of roots and shoots, plant nutrients and metals concentrations, and symbiotic development (mycorrhizal colonization and nodulation) were determined.

Available soil–metal contents were determined using the Lakanen and Erviö (1971) method. Shoot contents (mg $plant^{-1}$) of N (micro-Kjeldahl) and P, as well as those of Pb, Cd, Ni and Zn 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 has been described (Takács et al., 2001).

The percentage of mycorrhizal root length colonised was estimated with a microcope 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 of Giovannetti and Mosse (1980). Nodule number was estimated by direct observation using a binocular microscope.

The soil–plant transfer of Cd (concentration in shoot/available concentration in soil) is given. The ratio of total element acquisition by the plants (nutrient content in shoot) in relation with total amount of available Cd in 100 g of soil (pot capacity) was also calculated.

Both bacterial isolates were cultivated at 28 °C in nutrient broth supplemented with 0, 25, 50, 75 or 100 ppm Cd as $CdSO_4$. The number of viable cells was estimated at 2-h intervals from 0 to 16h.

Since bacterium A was the most efficient increasing plant growth and nutrition and decreasing Cd concentration in plant it was selected for molecular identification and future studies.

Total DNA from bacterial isolate A was obtained as described by (Giovannetti et al., 1990) and characterised by sequence analysis of the small ribosomal subunit (16S ribosomal DNA). PCR amplification was carried out with the eubacterial primers 27f and 1495r (Lane, 1991) located respectively at the extreme 5′ and 3′ of the ribosomal rDNA sequence, which allowed the amplification of nearly the entire gene. The amplification reactions were performed in a 20-μl volume containing 0.5 μM concentrations of each primer, 100 μM dNTPs, 1× PCR buffer (Sigma, St. Louis, MO, USA), 2.5 mM $MgCl_2$, 10 ng of genomic DNA and 0.25 U Taq DNA polymerase (Sigma). A Perkin Elmer/Cetus DNA Thermal Cycler 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 pGME plasmid (Promega) for sequencing. Database searches for 16S rDNA sequence similarity using FASTA and BLAST algorithms unambiguously identified the selected bacterial isolate as a member of the genus *Brevibacillus*. The 16S rDNA sequence showed its highest similarity (more than 98%) with *B. brevis*.

2.6. Statistical analysis

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

3. Results

The dual inoculation of mycorrhizal fungus and bacterium A increased more shoot and root biomass than the rest of microbial treatments assayed. The enhancing effect of these dual treatments was observed irrespective of the Cd level in the medium (Figs. 1–3). Nevertheless, the most positive microbial effect over non-treated control plants was observed at 33 mg kg^{-1} Cd.

Inoculation with bacterium A on mycorrhizal plants increased shoot biomass by 18% (13.6 mg kg^{-1} Cd),

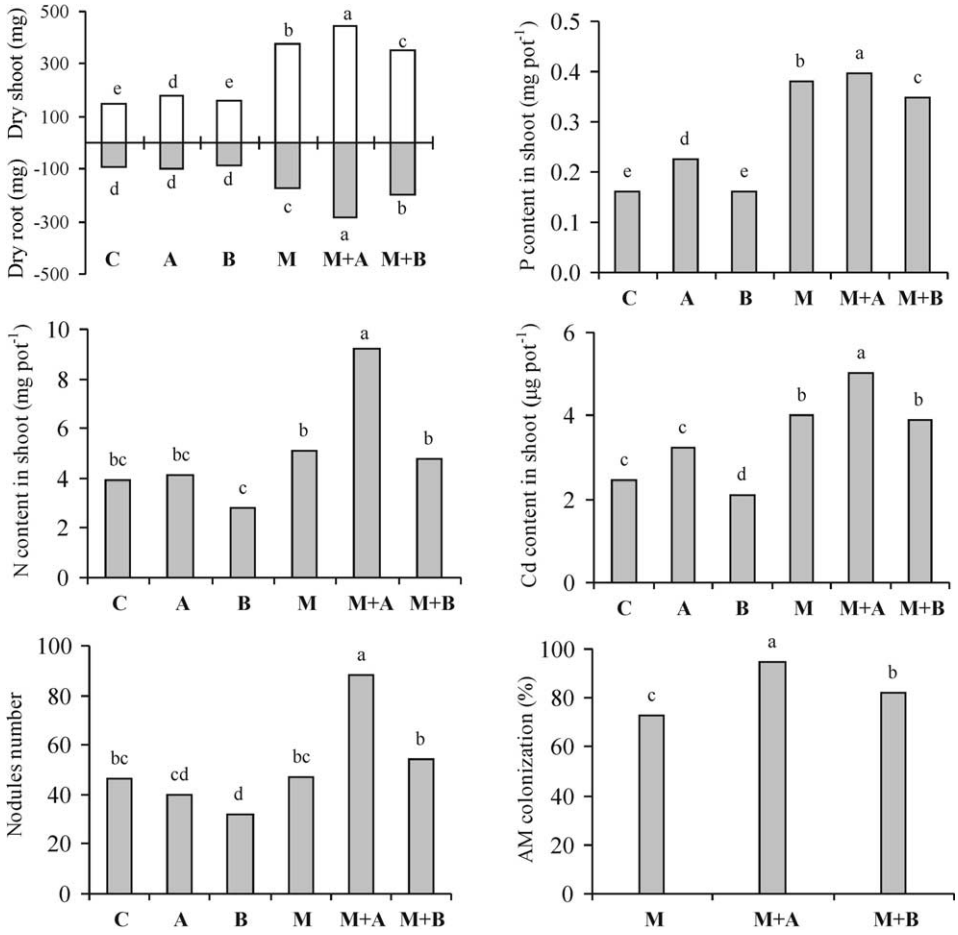

Fig. 1. Effect of the inoculation of indigenous bacteria (A or B isolates) and/or mycorrhizal fungus (M) on plant growth, cadmium (μg pot^{-1}) and nutrient content (mg pot^{-1}) and symbiotic values on *Trifolium* plants growing in soil with 13.6 mg kg^{-1} available Cd. Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($n=4$).

26% (33 mg kg^{-1} Cd) and 35% (85.1 mg kg^{-1} Cd) over single inoculated mycorrhizal plants (Figs. 1–3). In fact, *Trifolium* plants achieve further development and nitrogen and phosphorus uptake in Cd contaminated soils after co-inoculation of autochthonous AM fungus and bacterium A.

The N and P contents in shoot were differently affected by the microbial treatments according to the Cd amount in the medium. At the lowest Cd level, N content in plant was only increased by dual bacterium A-mycorrhizae inoculation. In contrast, at the highest Cd levels (33 and 85 mg kg^{-1}) AM colonization was determinant not only for P but also for N plant acquisition. (Figs. 2 and 3).

In general, the effect of *G. mosseae* colonization on N and P plant uptake was increased by bacterium A and decreased (except at 13.6 mg kg^{-1} Cd) by bacterium B (Figs. 1–3).

As the amount of available Cd in soil increased, the symbiotic structures (nodules and AM colonization) decreased in *Trifolium* roots. The number of nodules formed was stimulated by mycorrhizal colonization, particularly at the two highest level of Cd in the medium (33 and 85 mg kg^{-1}). Increasing Cd level reduced nodulation, which fell to zero in non-mycorrhizal plants in soil with 85 mg kg^{-1} Cd. The role of bacteria in the increase of nodule formation in single inoculation was evident at 33 mg kg^{-1} Cd (Fig. 2). The dual co-inoculation involving bacterium A highly increased the number of nodules of mycorrhizal plants at 13.6 and 85.1 mg kg^{-1} Cd. The beneficial effect of the inoculation of this bacterium on the extent of AM colonization was also observed at the three Cd levels (Figs. 1–3).

Concentrations of Cd in shoot of *Trifolium* were substantially reduced by the microbial treatments applied regardless of the Cd level in soil (Table 1).

At the lowest available Cd in soil (13.1 mg kg^{-1}) bacterial strain A, in single inoculation, was not effective decreasing Cd concentration in plant but at the two highest Cd levels it decreased such value by 218% (33 mg kg^{-1} Cd) and by 150% (85.1 mg kg^{-1} Cd). Each bacterial isolate showed different Cd tolerance since isolate A was most effective decreasing shoot Cd concentration at the highest Cd level meanwhile bacterium B only reduced this value at the two lowest (13.6 and 33.0 mg kg^{-1}) Cd levels (Table 1).

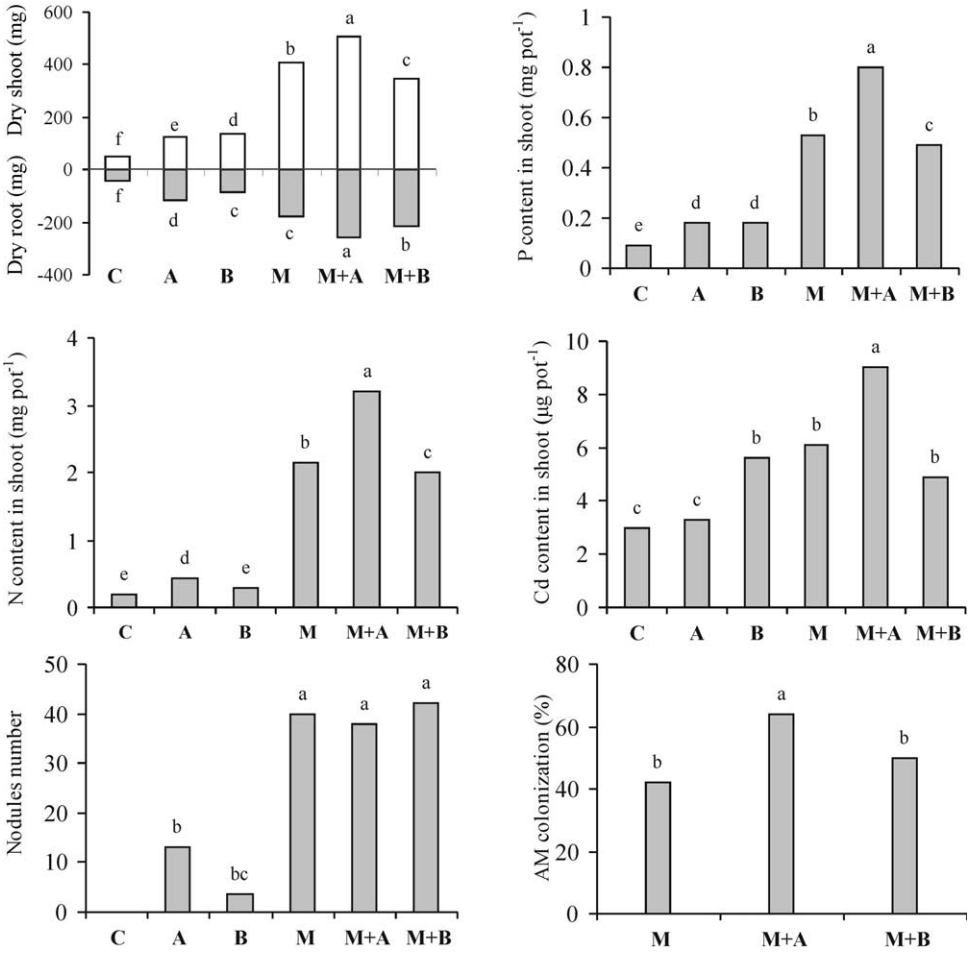

Fig. 2. Effect of the inoculation of indigenous bacteria (A or B isolates) and/or mycorrhizal fungus (M) on plant growth, cadmium (μg pot^{-1}) and nutrient content (mg pot^{-1}) and symbiotic values on *Trifolium* plants growing in soil with 33 mg kg^{-1} available Cd. Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($n = 4$).

Colonization by *G. mosseae* significantly reduced shoot Cd concentration at whatever Cd level in soil. Nevertheless, the mycorrhizal effect decreasing Cd concentration in shoot plant was enhanced by bacterium A at the highest available Cd in soil. Comparing the effect of co-inoculation with these microorganisms at the different Cd levels we can see that plant Cd concentrations were 1.46 (13.6 mg kg^{-1} Cd), 3.33 (33.0 mg kg^{-1} Cd) and 2.76 (8.51 mg kg^{-1} Cd) fold lower than in control, non-inoculated, plants (Table 1).

Remarkable results are that Cd concentration in control plants growing in soil with 33.0 mg kg^{-1} Cd matched that of the dual (M + A) inoculated plants growing in soil with 85.1 mg kg^{-1} available Cd. In the same way, the dually (A + M) inoculated plants growing in soil with 33.0 mg kg^{-1} Cd matched the Cd concentration of control plants growing in soil with 13.6 mg kg^{-1} Cd (Table 1).

The relative uptake of Cd by plants as compared to available soil Cd concentrations is shown in Table 1. Results show that in the mycorrhizal plants the transfer of Cd from the soil to *Trifolium* plants was particularly decreased at 33 mg kg^{-1} of available Cd in soil.

The transfer of Cd from the soil to the plant shoot decreased when plants were AM-colonised and at the highest available Cd in soil such decreasing effect was favoured by particular bacterial strains (Table 1).

Shoot Zn content was particularly enhanced in mycorrhizal plants and this effect was more evident at the highest Cd level in soil (Table 2). Zn content in non-mycorrhizal plants highly decreased with increasing Cd in soil, but differences in Zn uptake between AM and non-AM plants were more pronounced at the highest Cd level in the soil (85.1 mg kg^{-1} Cd). On the contrary, a greater decrease of Pb content was determined in AM plants than in non-AM plants at the highest Cd level. A positive effect of AM-colonization was noted on Ni plant acquisition as well. Ni content in shoot tissue tended to be the highest at 33 mg kg^{-1} Cd and at this Cd level the role of AM-colonization was important in increasing Ni in shoots (Table 2).

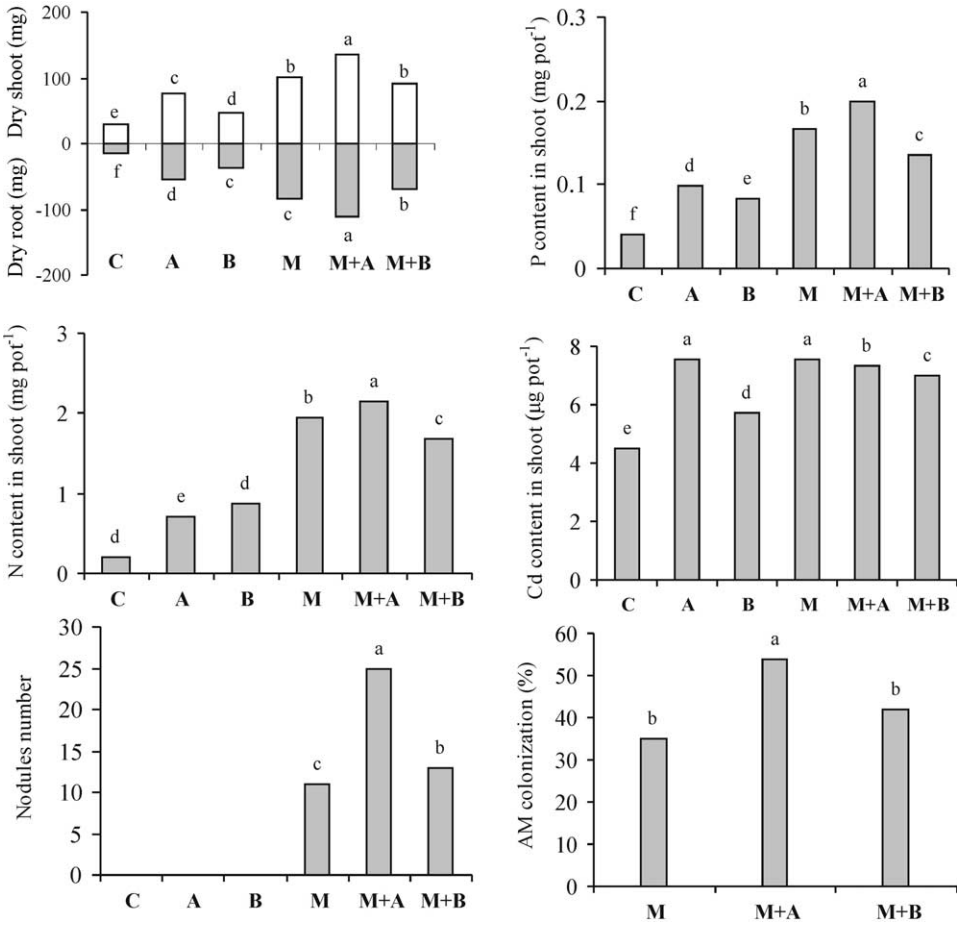

Fig. 3. Effect of the inoculation of indigenous bacteria (A or B isolates) and/or mycorrhizal fungus (M) on plant growth, cadmium (μg pot^{-1}) and nutrient content (mg pot^{-1}) and symbiotic values on *Trifolium* plants growing in soil with 85.1 mg kg^{-1} available Cd. Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($n=4$).

Table 1
Effect of the inoculation of indigenous bacteria (A or B isolates) and/or mycorrhizal fungus (M) on soil–plant Cd transfer and cadmium concentration (mg kg^{-1}) in shoot of *Trifolium* plants growing in soil under increasing cadmium levels

Microbial treatments	Available Cd (mg kg^{-1})					
	13.6		33.0		85.1	
	Cd transf.	Cd conc.	Cd transf.	Cd conc.	Cd transf.	Cd conc.
Control	1.20 a	16.3 a	1.78 a	58.8 a	2.60 a	149.6 a
A	1.30 a	17.8 a	0.82 c	27.0 c	1.20 b	99.7 b
B	0.80 b	12.9 b	1.21 b	40.0 b	1.50 ab	124.8 ab
M	0.65 c	10.7 c	0.45 d	15.0 d	0.88 c	75.6 c
M + A	0.83 bc	11.3 bc	0.30 d	17.6 d	0.64 d	54.4 d
M + B	0.82 bc	11.1 bc	0.24 d	14.8 d	0.90 c	76.3 c

Into each column values followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($n=4$).

The ratio of total elements content in shoots affected by increasing total amount of available Cd in soil varied depending on microbial treatments, element and Cd level (Table 3). Differences in Ni and Zn content in shoot in relation to available Cd in soil were relatively small in mycorrhizal treatments but differences in nutrient transfer between mycorrhizal and non-mycorrhizal plants were important, particularly at 33 mg kg^{-1} Cd in soil. Nutrients in shoot decreased with increasing Cd level but such a decrease was much lower for the mycorrhizal than for non-mycorrhizal plants. The most relevant effect of bacteria A was observed for N and P in mycorrhizal plants. In the opposite way, Pb decreased more in mycorrhizal plants when Cd level increased to 85 mg kg^{-1}. Ni, N and P contents in plants were increased by AM colonization irrespective of the Cd level but differences in the soil–plant transfer of these elements between AM and non-AM plants were increased at the highest Cd level.

The growth of each bacterium in nutrient broth at increasing Cd concentrations (from 25 to 100 mg kg^{-1}) was different. Bacterium A showed a greater tolerance to Cd than bacterium B. At the highest Cd amount in the growth medium (from 75 to 100 mg kg^{-1} Cd) bacterium A reached 10^7 cfu after 6 h while bacterium B, showed less growth (it did not reach 10^6 cfu at any

Table 2
Effect of an indigenous AM fungus (M) and/or bacteria (A or B isolates) on Zn, Pb and Ni content in shoot of *Trifolium* plants grown in soil artificially contaminated with a range of Cd levels

Treatments	Content (mg)		
	Zn	Pb	Ni
13.6 mg kg^{-1} Cd			
Control	6.1 c	0.15 c	0.006 d
A	8.3 b	0.65 b	0.011 c
B	6.1 c	0.26 c	0.002 e
M	11.1 a	0.60 b	0.013 c
MA	11.0 a	0.81 b	0.017 b
MB	10.0 a	1.51 a	0.019 a
33.0 mg kg^{-1} Cd			
Control	1.42 c	0.41 d	0.08 d
A	5.26 b	1.05 ab	0.32 cd
B	6.30 b	0.27 d	0.44 c
M	11.40 a	0.71 c	1.11 b
MA	11.61 a	1.10 a	1.41 a
MB	8.85 a	0.89 bc	0.94 b
85.1 mg kg^{-1} Cd			
Control	0.69 c	0.21 bc	0.07 c
A	0.66 c	1.26 a	0.22 b
B	0.84 bc	0.11 c	0.06 c
M	3.50 a	0.17 bc	0.29 a
MA	2.82 a	0.29 b	0.20 b
MB	1.50 b	0.20 bc	0.33 a

Means followed by the same letter into each Cd level are not significantly different according to Duncan's Multiple Range Test ($n=4$).

Table 3
Ratio of element content in shoots and total available Cd per pot ($\times 10^{-2}$) in non-mycorrhizal and mycorrhizal *Trifolium* plants as affected by the bacterial inoculation (A or B isolate) and level of Cd in soil

Treatments	N	P	Cd	Zn	Pb	Ni
13.6 mg kg^{-1} Cd						
Control	290 bc	12 e	1.5 d	0.45 c	0.011 c	0.0004 d
A	300 bc	17 d	2.4 c	0.61 c	0.047 b	0.0008 c
B	210 c	12 e	1.5 e	0.45 c	0.019 c	0.0001 e
M	370 b	28 b	2.9 b	0.81 a	0.044 b	0.0009 c
MA	680 a	29 a	3.7 a	0.81 a	0.059 b	0.0012 b
MB	350 b	26 c	2.6 b	0.73 a	0.11 a	0.0013 a
33.0 mg kg^{-1} Cd						
Control	60 e	3 e	0.9 a	0.04 d	0.012 d	0.002 d
A	130 d	5 d	1.0 a	0.16 c	0.032 ab	0.009 cd
B	90 e	5 d	0.7 a	0.19 c	0.008 d	0.013 c
M	650 b	16 b	1.8 a	0.34 a	0.021 c	0.034 b
MA	970 a	24 a	2.7 a	0.34 a	0.033 a	0.043 a
MB	610 c	15 c	1.5 a	0.25 a	0.027 bc	0.028 b
85.1 mg kg^{-1} Cd						
Control	2 f	5 f	0.53 e	0.008 c	0.0024 bc	0.0008 c
A	8 c	1.1 d	0.89 a	0.008 c	0.014 a	0.0025 b
B	100 d	1 e	0.67 d	0.010 bc	0.0012 c	0.0007 c
M	230 b	1.9 b	0.89 a	0.041 a	0.0019 bc	0.0034 a
MA	250 a	2.3	0.86 b	0.033 a	0.0034 b	0.0023 b
MB	200 c	1.5 c	0.82 c	0.020 b	0.0023 bc	0.0038 a

Means followed by the same letter into each Cd level are not significantly different according to Duncan's Multiple Range Test ($n=4$).

time). At the lowest Cd amount in the medium (from 25 to 50 mg kg^{-1}) both bacterial isolates showed similar growth rate (Fig. 4).

According to the molecular identification, the bacterial strain which resulted more efficient on plant growth (bacterium A) is a member of the genus *Brevibacillus*. The 16SrDNA sequence showed the highest similarity (more than 97%) to the species *B. brevis*. Bacterium B was not identified.

4. Discussion

The aim of this study was to investigate the basic interaction between Cd contamination in soil, plant growth and nutrition and certain beneficial rhizospheric microbes adapted to Cd polluted conditions. It is known that excessive concentrations of heavy metals in soils are toxic to plants, bacteria and fungi (Bääth, 1989). Thus, elevated Cd levels reduced AM root colonization even at levels which did not yet affect plant growth (Chao and Wang, 1990, 1991).

In this study, AM infection and nodule number decreased as available Cd in the soil increased but the beneficial effect of both AM and N_2-fixing symbioses was not supressed in highly Cd polluted soil. On the contrary, under the more stressed conditions, AM symbiosis resulted more effective enhancing nodule formation.

It is likely that the microbial strains isolated from contaminated soils are more tolerant to metals and have developed resistance (Chaudry et al., 1992). Nodulation (in the case of *Rhizobia*) is one of the most important biological processes highly sensitive to metal contamination as was here determined at 33 and 85 mg kg^{-1} of Cd. Similarly, mycorrhizal colonization, was also reduced by increasing Cd levels.

These and other evidences suggest, as it has been established, that AM fungi and bacteria can be constitutively or adaptatively resistant to high Cd concentrations (Kanazawa and Mori, 1966; Weissenhorn et al., 1994).

Metal adapted AM fungi and associated bacteria appear to have several specific strategies such as more or less sensitivity to metals that allow them to colonize soils polluted with heavy metals (Meharg and Cairney, 2000).

In this study, Cd resistant bacteria and AM fungus isolated from soil contaminated with Cd were able to grow at high Cd concentrations in soil and in culture medium (bacteria). However, different growth rate and activity was observed between both bacterial strains isolates, according to the Cd level. This effect can be

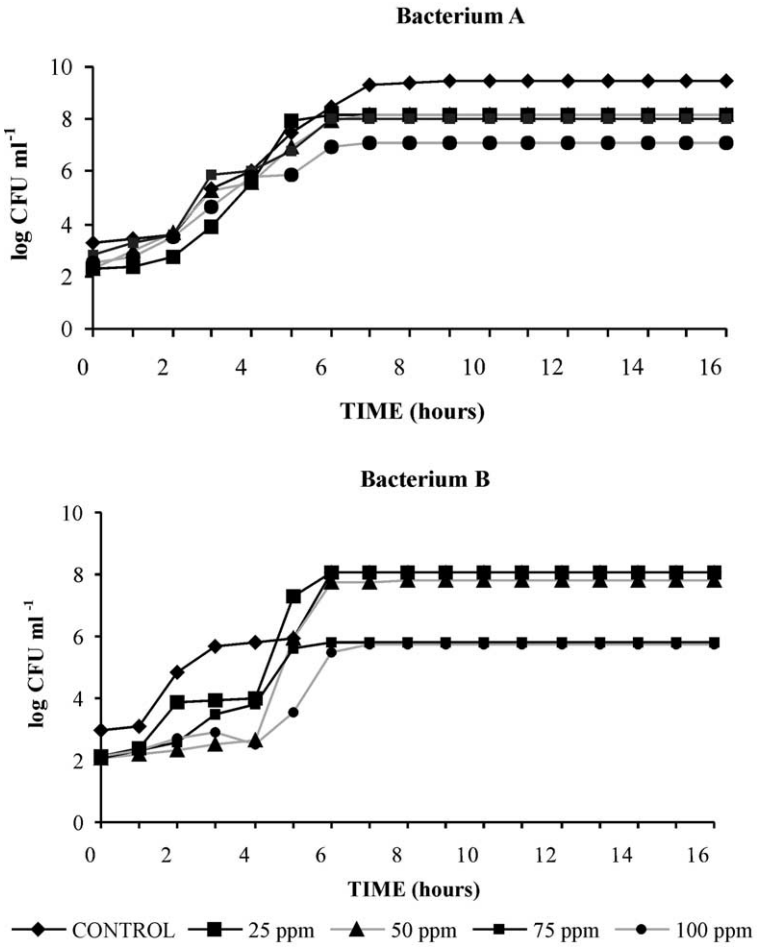

Fig. 4. Bacterial viable cells (bacterium A and B) (log cfu ml $^{-1}$) at diferent time intervals in nutrient broth supplemented with increasing Cd levels.

related with specific Cd tolerance of each strain and/or the different compatibility of these strains with rhizosphere microorganisms in general and with AM fungus and/or *Rhizobium* in particular [as symbiotic structures formation (AM-colonization and number of nodules) showed].

In culture medium, bacterium B showed to be less tolerant to Cd than bacterium A particularly at the two highest Cd levels (75 and 100 mg kg^{-1}) as evidenced by a lower number of viable cells. In vivo, only the Cd tolerant indigenous bacterial strain A successfully affected growth and nutrition of plants growing in Cd polluted soils.

Results indicate the potential importance of adapted microbial groups in Cd availability and toxicity to plants. When roots of the plants growing in Cd polluted soils were mycorrhizal, this symbiosis resulted not only in metal tolerance but also in a better plant growth on nutrition that can be increased by selected bacteria as these results evidence.

The accumulation of Cd in *Trifolium* plants depended not only on the level of Cd soil contamination but also of how microbial interactions are working in the rhizosphere. In fact, the transfer of Cd from the soil to the plant was lower in AM than in non-mycorrhizal plants at whatever Cd level. At the highest available Cd assayed (85 mg kg^{-1} Cd) bacterium A lowered this ratio by 37.5% in AM plants. Differences in the effect of bacterial strains on Cd soil–plant transfer at the highest available Cd in soil again suggest particular sensitivities or tolerance of each bacterial strain to the Cd amount in the soil.

Mycorrhizal plants increased essential nutrients and decreased Cd concentration and thus produced higher root and shoot biomass than non-mycorrhizal plants. These beneficial mycorrhizal effects were enhanced by bacterium A (*Brevibacillus* sp.). As a consequence, microbial treatments compensated the detrimental effect of Cd contamination. Bacterium A (the most Cd tolerant bacterial strain) had the best influence on AM symbiosis and on nodulation, increasing N–P uptake and enhancing Cd stress tolerance in plant. The improved Cd tolerance by particular microbial interactions is due to specific physiological mechanisms but the exact factors involved are not clearly established and there may be a combination of effects.

The microbial inocula used here (AM fungus and/or bacteria) may confer tolerance to Cd by modifying not only specific plant physiological processes such as nutrient status and rooting development but also, in a

direct way, by affecting metal availability and uptake and changes in hormonal balance which affect root exudates, pH and physico-chemical properties of the soil (Wang and Chao, 1992). Different changes in internal transportation and/or storage, as was determined in the present study, can confer metal tolerance to the host (Turnau et al., 1993; Boddington and Dodd, 1999)

Bacteria are able for complexing relatively large quantities of metallic cations (Chen et al., 1999) and recent studies demonstrated the ability of strain A for Cd sorphion in culture medium (data not shown) which may have contributed to Cd removal from the soil, as results on Cd shoot concentration show, and to ameliorate Cd stress. In agreement with this, Kotrba et al. (1999) suggested the importance of bacteria in metal mobilization in polluted soils. As well, the production of stimulatory compounds such as IAA by bacterium A also recently checked (Wöhler, 1997) (data not shown) can be involved in the positive effects found. In fact, plant development on Cd stressed soil was dependent upon the activity of the microbial population. Under severe Cd polluted soil conditions, the survival of plants was particularly dependent of such beneficial microbial interactions and plant establishment and survival in polluted soils was impaired when AM fungi were not present (Shetty et al., 1994a, b).

An other important role of microbial inoculation on Cd polluted soils here observed was the improvement of rhizobial nodulation in legume plants. Under our experimental conditions, nodule formation decreased with an increase in Cd contamination in the soil and was suppressed in non-inoculated control plants at the available Cd levels of 33 and 85.1 mg kg^{-1}. This fact affected plant productivity since the decline in nodule formation results in a decreased N_2-fixation (Chao and Wang, 1991). The two microsymbiont colonizers of the root systems on legume plants are very sensitive to toxic metals in a free living stage (Reddy et al., 1983). Decreases in nodule size and nitrogenase activity were also determined in white clover grown in metal polluted soils (Martensson, 1992).

The increase in N uptake determined in AM-colonised and nodulated legumes has been attributed to an enhancement of N_2-fixation since nodule formation and function is stimulated by AM colonization (Barea et al., 1987) and nodules are protected by AM symbionts against the detrimental effects of abiotic stressed such as drought (Ruiz-Lozano et al., 2001; Porcel et al., 2003). The supply of available P is critical for legumes development in polluted habitats (Bethlenfalvay and Linderman, 1992). In addition to macro and micronutrients (as Zn) uptake, the mycorrhizal fungi and/or the bacteria may potentially affect, selectively, the plant uptake of the heavy metal as function of several factors (Vörös et al., 1998).

According to the permissible limits, Cd was the most toxic metals to *Rhizobium leguminosarum* bv *trifoli* (Chaudry et al., 1998). In the present study this effect was determined up to 13.6 mg kg^{-1} Cd in non-inoculated plants. Cadmiun concentrations used here as experimental approach were many times higher than the actual legislated limits.

Percentage of mycorrhizal root development was less reduced under these Cd stress conditions when associated with *Brevibacillus* sp.

Here, the AM colonization produced by a Cd tolerant isolate of *G. mosseae* protected plants against the toxic effect of excessive concentration of Cd by reducing Cd concentration in plant tissues and by increasing plant nutrition. But previous results confirmed that AM fungi did not reduce the total plant uptake of heavy metals (Heggo et al., 1990; Weissenhorn et al., 1994). The microbial associations assayed here may potentially affect the uptake of Cd in different ways depending on combination of factors such as via a hyphal uptake. AM mycelium has a particular metal sorption capacity (Joner et al., 2000) and this fungal ability accounted for the positive effects observed. Similarly, the bacterial isolates can act by direct or indirect mechanisms on the AM fungus as mycorrhizae helping organisms.

Microbially treated plants acted reducing Cd concentration and lowering the proportion of Cd soil/plant transfer, but were also able to take up more Cd (see total Cd content in plant) from the soil, mainly plants dually (M + A) inoculated at the two lowest Cd levels (13.6 and 33.0 mg kg^{-1} Cd). Thus, treated plants can also be used to facilitate remediation since microbial ability to increase Cd content in inoculated plants has the possibility to decontaminate soil in which they are growing. The use of biotechnological procedure of major interest in remediation strategies is suggested (Chaudry et al., 1992). Results from this study provide evidence that the inoculation of *G. mosseae* and *Brevibacillus* sp. (adapted microorganisms) decreased by 1.5, 3.3 or 2.8 folds Cd concentration depending on available Cd in soil and increased by two or three folds Cd uptake by *Trifolium* plants at 13.6 and 33 mg kg^{-1} of Cd, respectively. These results are consistent with reports suggesting the beneficial use of bacteria or mycorrhizal colonization in plants growing in metal contaminated sites (Shetty et al., 1994a, b).

Isolation of tolerant beneficial microorganisms (AM fungi and bacteria) from a polluted soil able to colonize and to function under such detrimental conditions may be an attractive ecological goal. Knowledge of the mechanisms by which mycorrhizal adapted fungi and bacteria alleviate heavy metal toxicity in plants growing in contaminated environments would allow the management of microbial groups with suitable characteristics to be used in bioremediation purposes. The knowledge of basic interactions between Cd level, plant sp, soil type and microbial inoculants needs additional studies that are now in progress in our group.

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