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Interactive effect of *Brevibacillus brevis* and *Glomus mosseae*, both isolated from Cd contaminated soil, on plant growth, physiological mycorrhizal fungal characteristics and soil enzymatic activities in Cd polluted soil

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Endemic isolates of bacteria and fungi were effective in bioremediation.

Abstract

The interaction between two autochthonous microorganisms (*Brevibacillus brevis* and *Glomus mosseae*) isolated from Cd amended soil increased plant growth, arbuscular mycorrhizal (AM) colonization and physiological characteristics of the AM infection (measured as SDH or ALP activities). The enhanced plant Cd tolerance after coinoculation with native microorganisms seemed to be a consequence of increased P and K acquisition and, simultaneously, of decreased concentration of Cd, Cr, Mn, Cu, Mo, Fe and Ni in plant tissue. Autochthonous microbial strains were more efficient for nutrient uptake, to immobilize metals and decrease their translocation to the shoot than reference *G. mosseae* (with or without bacteria). Indole acetic acid produced by *B. brevis* may be related to its ability for improving root growth, nodule production and AM fungal intra and extraradical development. Dehydrogenase, phosphatase and β-glucosidase activities, indicative of microbial metabolism and soil fertility, were maximized by the coinoculation of autochthonous microorganisms in cadmium polluted conditions. As a consequence, the use of native microorganisms may result very efficient in bioremediation.

Keywords: *Glomus mosseae*; *Brevibacillus brevis*; Cd pollution; SDH and ALP mycorrhizal activities

1. Introduction

Heavy metals are known to be toxic to plants and most organisms when present in soils in excessive concentrations. In metal-polluted environments, a detrimental effect not only to arbuscular mycorrhizal (AM) fungi but also to soil microbial diversity and microbial activities (indexes of microbial metabolism and of soil fertility) has been reported (Giller et al., 1998). Enkhtuya et al. (2000) determined that the length of extraradical AM mycelium was a good indicator of the negative effects of stresses in the soil. However, adapted AM fungi and rhizospheric bacteria are able to survive in sites affected by heavy metals and may increase plant growth and metal tolerance (Vivas et al., 2003a,b). Soil microorganisms may be key to plant survival on contaminated soils (Colpaert and Vandenkoornhuyse, 2001). The tolerance to metals shown by mycorrhizal plants is based on a series of mechanisms and symbiotic

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effects such as the metal binding capacity of fungal mycelium in the rhizosphere or in roots (Zhu et al., 2001).

AM fungi can adapt to varying environmental conditions (Vosátka et al., 1997) and successful growth of plants on polluted soil is highly dependent on the activity of microbial populations (Pfleger et al., 1994). Copeman et al. (1996) suggested that differences in AM fungal behaviour and effectiveness are related to the origin of AM fungi. Different AM fungi have different abilities to immobilize metals within or near the root and to reduce their translocation to the shoot (Joner et al., 2000). However, there is little evidence for intraspecific adaptative changes in fungal populations from polluted and non-polluted environments (Hartley et al., 1997). Even isolates of a given AM species may differ in their sensitivity or tolerance to heavy metals (Weissenhorn et al., 1994). In general, AM fungi isolated from contaminated soils are able to tolerate greater heavy metal concentration than strains isolated from non-contaminated soils (Weissenhorn and Leyval, 1995; del Val et al., 1999). As consequence of this, AM fungi naturally occurring in polluted soils and having a high level of metal tolerance may have a protective role ameliorating plant resistance to metal toxicity (Enkhtuya et al., 2000).

del Val et al. (1999) showed that some AM genotypes were able to survive in polluted soil due to their adaptation to heavy metal stress. Nevertheless, heavy metal presence did not always result in an increase in metal tolerance of native AM fungi (Hartley et al., 1997). In order to select efficient microbial (AM fungi and bacteria) inocula it is thought that autochthonous AM species that are able to survive under heavy metal polluted conditions, posses a high metal tolerance and are able to cope with this stress during their interaction with the host plant.

The ecological plasticity and functional diversity of these AM fungi has been recognized (Monzón and Azcón, 1996; Camprubí and Calvet, 1996), but the ecophysiological plasticity of AM fungi under polluted conditions needs to be studied.

Many studies have reported the effectiveness of AM fungi on the resistance of the host plant to Cd contamination (Weissenhorn et al., 1993; Enkhtuya et al., 2000; Joner et al., 2000). In a recent study the impact of an indigenous Cd-adapted *G. mosseae* (IM) strain and a Cd-adapted bacterium, both isolated from a Cd contaminated soil, showed the highest functional compatibility and benefit to the plant when compared with a reference *G. mosseae* (RM) strain (Vivas et al., 2003b). Moreover, the bacterium alone resulted more efficient increasing plant growth than the reference *G. mosseae* strain. Thus, we concluded that the combination of suitable symbiotic and saprophytic microorganisms consistently increased symbiotic parameters (nodule number and mycorrhizal infection) related to N and P plant nutrition, and plays an important role in Cd tolerance by plants (Vivas et al., 2003a,b).

The bacterium had an important role in reducing Cd acquisition by plants and also demonstrated a plant-growth-promoting (PGP) activity that was additive to the effects of the AM symbiosis (Vivas et al., in press). This study goes one step further, and aims to investigate how the native bacterium affects the development of AM fungal extraradical mycelium and the physiological characteristics of AM colonization in order to understand the benefit of Cd-adapted autochthonous microorganisms on the plant tolerance to high Cd concentration in soil.

The present study was designed to determine, under Cd polluted conditions ($8\ \mu g\ g^{-1}$) how a bacterium isolated from a Cd polluted soil (*Brevibacillus brevis*) affects the viability and functionality of an AM symbiosis developed between either an indigenous (IM) or a reference (RM) *G. mosseae* strain. The influence of combining such two treatments on the biological properties of rhizosphere soil, measured as certain soil enzymatic activities (indicatives of soil fertility) under these Cd polluted conditions was also assessed.

2. Materials and methods

The experiment consisted of a two-factor randomized complete block design with (1) mycorrhizal treatment including assays with an autochthonous *G. mosseae* and a reference *G. mosseae* (BEG 119) fungal species and one non-inoculated control treatment; (2) inoculated or non-inoculated with an indigenous bacterial strain *B. brevis*. Five replicates were made for each treatment, totalling 30 pots.

2.1. Soil characteristics and cadmium applications

The soil used in the greenhouse experiments was a calcareous loam (57% sand, 22.3% silt and 19% clay). It contained 1.6% 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 sterilized by steaming for three sterilization cycles (100 °C for 1 h for 3 days).

After sterilization, the soil was supplemented with Cd by adding adequate amount ($18\ \mu g\ Cd\ g^{-1}$ soil) of an aqueous solution of $CdSO_4$. After two weeks of incubation (for metal stabilization) the available amount of Cd was $8\ \mu g\ g^{-1}$ determined using EDTA as extractant (Lakanen and Erviö, 1971).

2.2. Microbial inoculation of soil

Soil samples for microbial inocula isolation were taken from a 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 microorganisms were isolated and cultivated for inocula production. The bacterial strain selected here was the most abundant cultivable type in the original Cd polluted soil (Nagyhörcsök, Hungary) (Kádár, 1995), when plated in agar nutrient broth medium (8 g L^{-1}) containing meat extract (3 g L^{-1}) and peptone gelatine (5 g L^{-1}).

The bacterial isolation was carried out following the conventional procedure: briefly 1 g of homogenized 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 10^4–10^7. These were plated in agar nutrient broth medium (8 g L^{-1}) and cultivated for 48 h at 28 °C.

Once selected the most abundant bacterial type was independently grown in 250 mL flasks containing 50 mL of nutrient broth (8 g L^{-1}) medium in shake culture.

The bacterial strain, was identified as a *B. brevis* (Vivas et al., 2003a) When appropriate, pots were inoculated with 1 mL of bacterial culture (10^8 cfu mL^{-1}) grown in nutrient broth medium for 24–48 h at 28 °C of temperature.

Indigenous AM spores were isolated from Cd polluted soil (Nagyhorcsock, Hungary) by wet-sieving and decanting as described by Vilariño and Arines (1990). All the spores obtained were morphologically similar to *G. mosseae* from our current EEZ collection, thus we concluded that only this *Glomus* species was present in the original contaminated soil.

Mycorrhizal inoculum from this isolated endophyte and from a *G. mosseae* strain (BEG 119) from our collection used as reference AM inoculum, was obtained in an open pot culture of *Allium cepa*. After six months of plant growth the shoots were eliminated and the undergrown part (mycorrhizal roots plus soil possessing fungal spores and mycelium) was maintained by storage for three to six months in polyethylene bags at 4 °C. Inocula consisted of thoroughly mixed rhizosphere samples containing spores, hyphae and mycorrhizal 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 two AM inocula to provide a general microbial population free of AM propagules.

A suspension of *Rhizobium leguminosarum* bv *trifolii* was added to each pot (1 mL, 10^8 cfu per pot). This was prepared following standard procedures (Azcón, 1993).

2.3. Plant growth conditions

Trifolium repens L. was grown for three months in 350 cc pots (containing 100 g of soil/sand mixture) under greenhouse conditions (18–24 °C, with a 18/6 h light/dark period) and 70/80% relative humidity. Photoperiod was provided by fluorescent (24-F96T12VHO/CM) and incandescent (45–40 W) Sylvania and Phillips lamps, respectively. Photosynthetic photon flux (PPF) was ca. 500 μmol m^{-2} s^{-1}. Plants were fertilized throughout the experiment with 10 mL of Hewitt's nutrient solution lacking N and P (Hewitt, 1952).

2.4. Measurements

Three months after sowing plants were harvested, and the dry biomass of roots and shoots, nutrient and heavy metal 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 by Takács et al. (2001).

Roots were carefully washed and then divided into three batches: one was stained by the classical non-vital trypan blue (TB) staining (Phillips and Hayman, 1970) and the others were used for histochemical vital staining succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) activities in order to measure total (TB), living (SDH) or active (ALP) AM fungal development.

SDH activity was revealed according to the procedure described by Smith et al. (1990). The roots were immersed in a freshly made solution containing 0.2 M Tris–HCl pH 7.0, 2.5 M sodium-succinate hexa-hydrate, 4 mg ml^{-1} nitro blue tetrazolium, 5 mM $MgCl_2$. Root fragments were stained overnight at room temperature and then cleared for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

ALP was determined according to the procedure described by Tisserant et al. (1993). Roots were immersed in a freshly made solution containing 50 mM Tris–citric acid pH 9.2, 1 mg ml^{-1} alfa-naphthyl acid phosphate (monosodium salt), 0.05% $MgCl_2$ anhydrous, 0.05% $MnCl_2$ tetrahydrate and 1 mg ml^{-1} fast blue RR salt. Root fragments were then stained overnight at room temperature and cleared for 15–20 min in 1% active chlorine solution in sodium hypochlorite.

Mycorrhizal development was evaluated according to the method by Trouvelot et al. (1986) and expressed as

frequency of AM colonization ("*F*", root fragments showing fungal colonization), and intensity of AM colonization ["*M*", gives an estimation of the amount of root cortex that became mycorrhiza and is referred to the whole root system, while "*m*", refers only to the mycorrhizal root fraction. "*A*" is the arbuscule abundance and gives an estimation of the arbuscule richness in the whole root system, while "*a*" refers only to the mycorrhizal root fraction]. Extraradical mycelium was determined by a modification of the methods described by Joner et al. (2000) and Newman (1966).

In rhizospheric soil, acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as a substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

β-Glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M; Masciandaro et al., 1994) as substrate. This assay is based on the release and detection of PNP. Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 ml of substrate were added to 0.5 g of sample and incubated at 37 °C for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969).

Dehydrogenase activity was determined according to García et al. (1997). For this, 1 g of soil was exposed to 0.2 ml of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 °C in darkness. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtering though a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

The indole acetic acid (IAA) in rhizospheric soil was determined by a colorimetric method developed by Mitchel and Brunstetter (1939) and by Gordon and Weber (1950). Briefly, 2.0 g of air-dried soil (on an oven-dry basis) was placed in a 50 ml Erlenmeyer flask and 6 ml of phosphate buffer (pH 7.5) with glucose (1 g glucose/100 ml phosphate buffer) and 4 ml of L-tryptophan (1 g tryptophan/100 ml H_2O) were added. Such soil solutions were mixed, stoppered, and incubated at 37 °C for 24 h in the dark. For the extraction, 2 ml of 5% trichloroacetic acid solution was added to inactivate the enzymes involved in the bioassay of auxin, then 1 ml of 0.5 M $CaCl_2$ solution was added. The soil solution was filtered (Whatman No. 2). Three milliliters of filtrate was placed 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) was added. The mixture was incubated for 30 min at 25 °C in the dark. Then the absorbance of the red solution was measured with a spectrophotometer (Turner Model 350) adjusted to a wavelength of 535 nm (Wöhler, 1997).

2.5. Statistical analysis

The results were statistically evaluated by factorial analysis of variance with bacterial treatment, mycorrhizal treatment and bacterial treatment–mycorrhizal treatment interaction as sources of variation. Percentage values were arcsine-transformed before statistical analysis.

3. Results

Trifolium plant growth in the Cd polluted soil used was affected by the microbial treatments assayed. Single inoculation of the bacterium or of each of the *G. mosseae* strains [indigenous (IM) or reference (RM)] caused significant increases in shoot and root weight as well as in nodule number production (Fig. 1).

Bacterial inoculation was effective in increasing shoot and root growth by 102% (shoot) and by 148% (root). Number of nodules was also highly influenced by *B. brevis* (B). Beside this, single inoculation of RM increased plant growth by 433% (shoot) and 615% (root) compared to non-inoculated plants. However, plant biomass was much increased when root system was colonized by IM. The addition of B to the mycorrhizal plants did not significantly affect these parameters. Nevertheless, *Trifolium* plants responded to this dual treatment by reaching the maximum shoot and root growth, as well as, the highest nodule number (Fig. 1).

In control plants, nodulation fall nearly to zero. However, both mycorrhizal and bacterial colonization largely increased the number of nodules formed. This effect was particularly relevant for plant infected with IM (Fig. 1)

The highest concentration of P and K in shoots was observed in plants coinoculated with autochthonous microorganisms. However, Cd concentration was not increased by this treatment (Fig. 2). Control plants showed a higher concentration of Cu and Zn than the rest of the treatments (Fig. 2). Values of Cr, Cu, Fe, Mn, Mo, Ni or Zn, in the treatments that effectively increased plant growth were also decreased (Fig. 3). A decreasing effect in the concentration of elements such as Cr, Cu, Fe, Mo and Zn was observed in plants

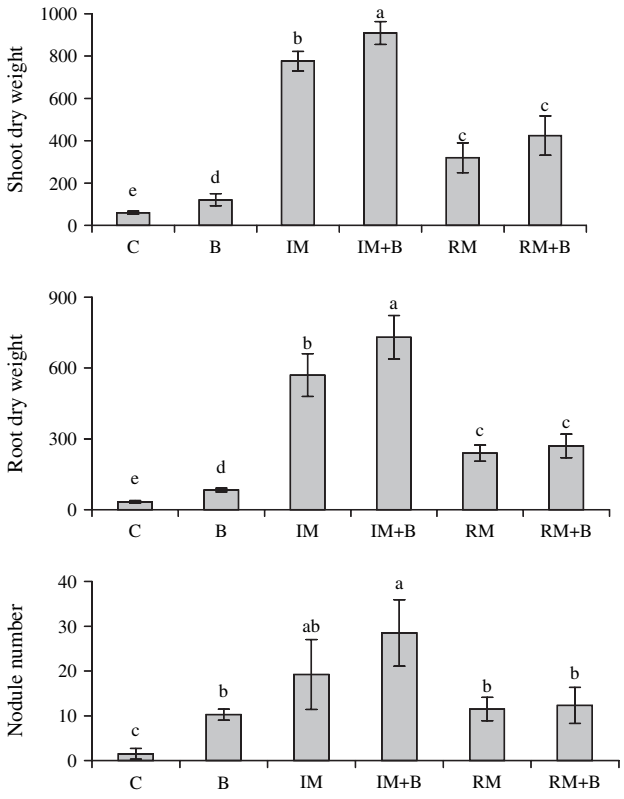

Fig. 1. Effect of indigenous (IM) or reference (RM) *G. mosseae* strains or *Brevibacillus brevis* (B) in single or in dual inoculation on shoot and root dry weight (mg) and nodule number of *Trifolium* plant cultivated in soil contaminated with 8 $\mu g\, g^{-1}$ Cd. Vertical bars represent standard errors.

colonized by single IM. The maximum concentration of Cd, Cr, Fe, Mo Mn and Ni in shoot was recorded in single bacterium inoculated plants.

The changes in the rhizosphere produced by the inoculants applied were evaluated as soil enzymatic activities and reflected the functioning of the system (Fig. 4). The highest phosphatase activity was found in the rhizosphere of plants inoculated with IM plus B. The inoculation with B increased phosphatase activity by 428% (single inoculation), by 824% (coinoculated with IM) and by 596% (coinoculated with RM). In contrast, values of β-glucosidase activity in inoculated and non-inoculated soil did not show such big differences as phosphatase activity did. β-Glucosidase activity was significantly higher in the rhizosphere of IM plus B treated plants as well (406% compared to the control). *Brevibacillus* produced a similar effect than IM on this activity and greater than RM. The coinoculation of both microorganisms was effective on this value only in the case of IM fungus.

Dehydrogenase activity was highly increased (2304%) by coinoculation of native microorganisms (IM plus B). Except for the rhizosphere of RM colonized plants, dehydrogenase activity was greater for inoculated than for non-inoculated soils. *B. brevis* had an important effect upon dehydrogenase activity in soil, increasing it by 242% (single inoculated), and by 102% (with RM) over control (Fig. 4).

Indole acetic acid (IAA) determined in rhizosphere soil was increased in all the inoculated treatments (Fig. 4), but IAA production was mainly affected by IM colonization. The bacterium was also effective in increasing this value in single and in dual inoculation with IM (Fig. 4).

The effectiveness of B on mycorrhizal colonization followed a specific pattern depending on the strain of *G. mosseae* involved and the staining method used. Regarding *F*%, IM showed higher infectivity than RM and nearly the totality of *F*% produced by IM resulted to be alive (SDH staining) and most of it was active (ALP staining). Nevertheless, the colonization frequency (*F*) developed by RM (estimated by TB and SDH staining) was 1.9 (TB) and 3.0 (SDH) fold lower than that showed by IM strain. These values increased by the inoculation with B in RM colonized plants. The effect of B on *F*% values was not generalizable for the rest of infective parameters. In contrast, "*M*" (%) and "*m*" (%) tested as SDH and ALP staining, were significantly increased by B only in association with IM.

Importantly, a general stimulating role of B on vitality (SDH staining) and activity (ALP-straining) of the *A*% was assessed following RM or IM inoculation. Anyway, the most important point is that in IM plus B inoculated roots a high proportion of arbuscules was active and maintained ALP activity throughout the experiment. Meanwhile a fewer proportion of mycelium remained alive and active in plants colonized by RM at the end of the experiment under these Cd stress conditions.

Results show that, under polluted conditions, plants inoculated with native inocula (B plus IM fungus) showed a relatively high ALP activity regardless of % mycorrhization observed. The extraradical mycelium developed by IM was higher than that produced by RM strain, but non-significant effect of B increasing this fungal value was found (Fig. 5).

4. Discussion

Results reported here highlight the different intra and extraradical mycorrhizal development and also the different effects found between isolates of AM fungi on their host plant under Cd contaminated conditions. These results confirm the importance of knowing aspects related to the effectiveness and ecological meaning of autochthonous microorganisms (bacteria and/or AM endophytes) in Cd polluted soil.

According to Powell (1981) native mycosymbionts, adapted to survive under given environmental conditions, normally showed low effectiveness in terms of

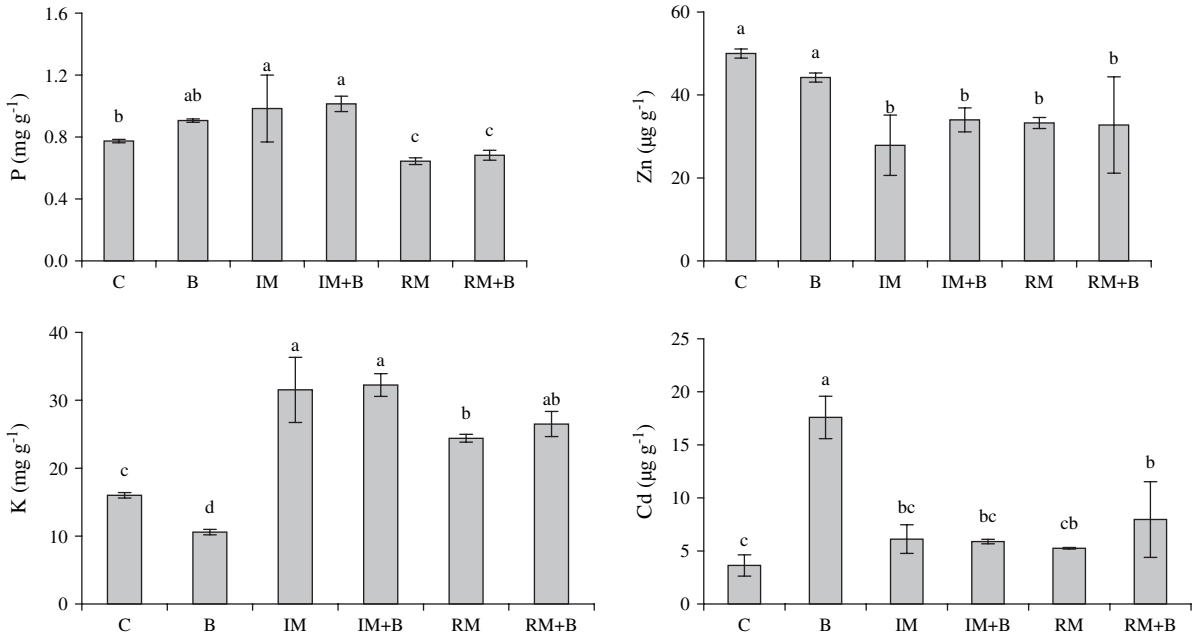

Fig. 2. Effect of indigenous (IM) or reference (RM) *G. mosseae* strains or *Brevibacillus brevis* (B) in single or in dual inoculation with IM or RM on concentration of P and K (mg g^{-1}) and Zn and Cd (µg g^{-1}) in shoots of *Trifolium* plant cultivated in soil contaminated with 8 µg g^{-1} Cd. Vertical bars represent standard errors.

plant growth and nutrition increases. In this study, the preference of the host plant for the native endophyte (as it is indicated by the highest levels of AM colonization) suggests that the autochthonous fungi are the most adapted to survive and to function in plant roots growing under Cd polluted conditions.

Results evidence that the ability of the most effective AM symbiont to alleviate Cd toxicity to the host plant was due to an increase in plant nutrition, which is also mediated by associated microbial communities (bacteria) in the rhizosphere. Such a biological factor (bacteria) promotes important changes in the functional compatibility of the symbiotic association according to the AM fungus involved.

The functional compatibility was tested, in physiological terms, as abilities involving both symbionts (Smith and Gianinazzi-Pearson, 1988) and their effect on the development of both symbiotic partners. The colonization rate and production of extraradical mycelium indicate a particular affinity of the symbiotic interaction. A close relationship seems to exist between the level of AM colonization, the quality of this infection (in terms of vitality and activity of AM colonization) and the plant growth response. These values show that the native *G. mosseae* is the best inoculant for improving plant development in this Cd polluted soil, particularly when associated with the native *Brevibacillus*.

Big differences were found in the compatibility between each strain of *G. mosseae* and *B. brevis* in terms of metabolic fungal activities (SDH and ALP). Such enzymatic values are indexes of active metabolic fungal performance which tend to decrease and even disappear under Cd polluted conditions. But the bacterium compensated the detrimental Cd effects. Thus, an important conclusion derived from these data is the relevance of using adapted effective and infective AM fungi in order to enhance plant nutrition which improves the ability of the plant to become established and to cope with the Cd pollution. Available data highlight the importance of enhancing AM potential by coinoculation with adapted native bacterial species.

Results from this study support the view that non-native *G. mosseae* was also able to colonize plant roots and to promote plant growth under Cd polluted conditions (Enkhtuya et al., 2000) Nevertheless, it is assumed that microbial population non-inhabiting the same rhizosphere is not developed together (as reference *G. mosseae* and *B. brevis*) and any association between them may not be functionally compatible.

In the present study, *B. brevis* behaved as a plant growth promoting rhizobacterium (PGPR) (Kloepper, 1992) but its positive effect on root biomass was greater than that observed on the shoot. Also as mycorrhizae helper bacterium (Garbaye, 1994a,b) it promoted mycorrhizal colonization (quantitatively and qualitatively) mainly in interaction with autochthonous *G. mosseae*.

Azcón et al. (1978) and Azcón (1987) described the stimulation of mycorrhizal root colonization by IAA-producing bacteria since growth promoting compounds were able to stimulate the plant susceptibility to AM

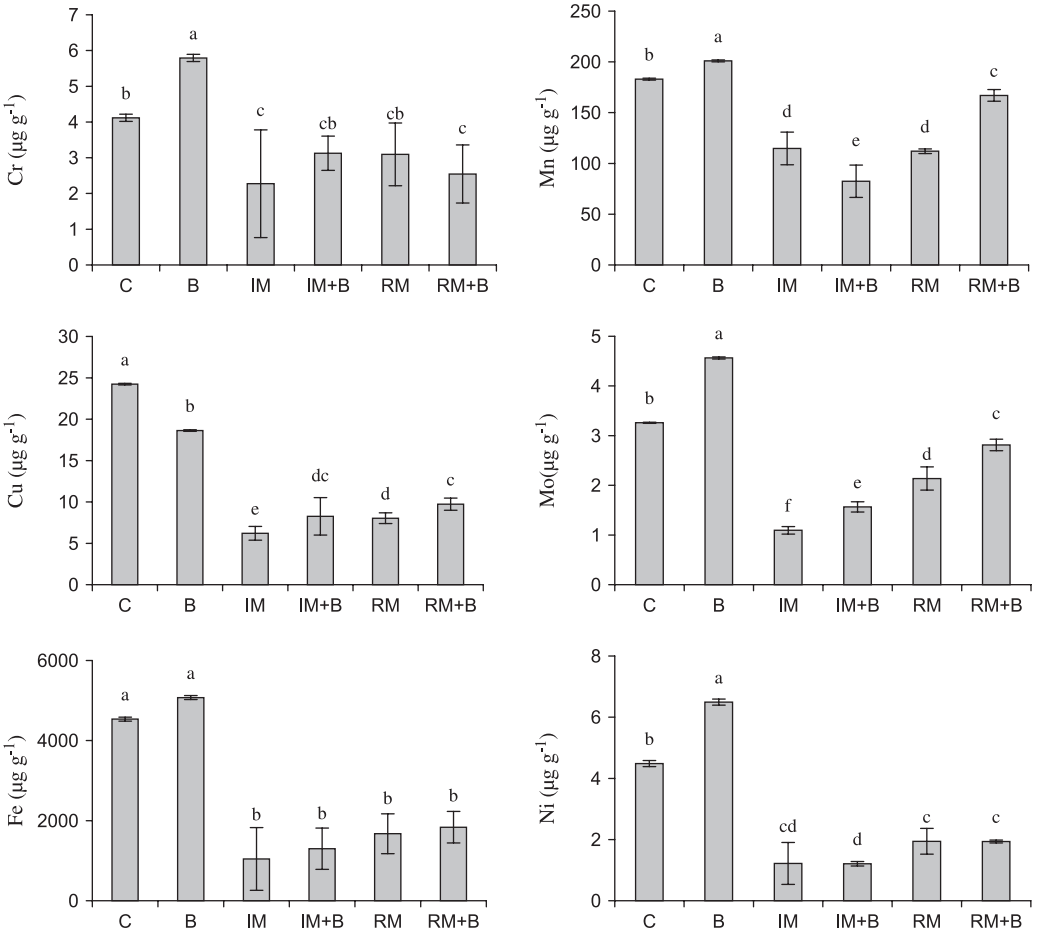

Fig. 3. Effect of indigenous (IM) or reference (RM) *G. mosseae* strains or *Brevibacillus brevis* (B) in single or in dual inoculation with IM or RM on Cr, Cu, Fe, Mn, Mo and Ni (μg g^{-1}) in shoots of *Trifolium* plant cultivated in soil contaminated with 8 μg g^{-1} Cd. Vertical bars represent standard errors.

infection and the growth of extraradical AM mycelium. The increased amount of IAA in the rhizosphere of *B. brevis* inoculated plants may be correlated to the effect of this bacterium not only on root growth and nodule formation but also on the stimulation of level and physiological status of mycorrhizal colonization.

In previous studies the interactive effects of some *Glomus* sp.–bacterium sp. were tested in environments without stress (Toro et al., 1997; Barea et al., 2002; Marulanda et al., 2002).

The highest P and K concentrations in plants coinoculated with autochthonous microorganisms might explain why the shoot and root growth of *Trifolium* plants increased in treated plants. As well, effect of these two inocula on decreasing the uptake of metals may contribute to the plant growth effect found.

The reduced concentration of metals in colonized plants by AMF could be partly attributed to a dilution effect linked to the increased plant growth. However, most of the results found here cannot be explained by such mechanism since Cd concentration in plants colonized by whatever *G. mosseae* (IM or RM) strain was quite similar while the growth of native *G. mosseae* colonized plants was twice higher than that reached in reference *G. mosseae* colonized plants. Similar effect was found with respect to plant concentration of other metals. This may explain the highest effectiveness of autochthonous microorganisms on plant growth: concentrations of Mn, Cu, Mo or N decreased and a dilution due to an increase yield could have occurred.

In this study, shoot of mycorrhizal plants did not show lower Cd concentration than the corresponding non-inoculated plants as it was evidenced in a previous study (Vivas et al., 2003a,b). Nevertheless, in those previous studies the amount of Cd applied was 30 μg Cd g^{-1} and in this study the Cd amount was reduced to 8 μg Cd g^{-1}. According to Chen et al. (2001) the effect of AM formation on Zn uptake changes with increasing Zn addition and below the critical Zn application rate the Zn uptake was enhanced while above this level Zn translocation to the shoot decreased. A similar effect may have occurred with Cd in this study.

Several mechanisms may be involved in the enhancement of plant Cd tolerance by the coinoculation of

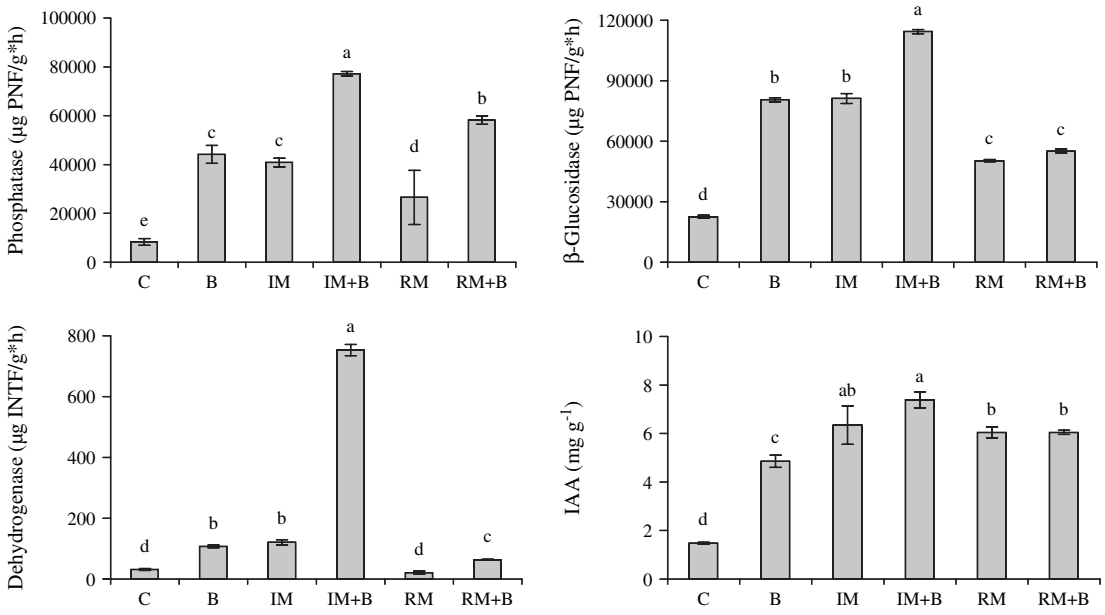

Fig. 4. Effect of indigenous (IM) or reference (RM) *G. mosseae* strains or *Brevibacillus brevis* (B) in single or in dual inoculation with IM or RM on phosphatase, β-glucosidase, dehydrogenase activities and indole acetic acid (IAA) content in rhizospheric soil of *Trifolium* plant cultivated in soil contaminated with 8 μg g^{-1} Cd. Vertical bars represent standard errors.

microorganisms, e.g. the effect in increasing nutrients (P and K) and in decreasing concentration of metals (Cd, Cr, Mn, Cu, Mo, Fe and Ni). The greater intra and extraradical mycorrhizal development and activity by autochthonous microbial strains increased nutrients uptake. As well, the protective effect of AM colonization against Cd toxicity has been explained by the possibility that much Cd was retained in the mycorrhizal roots and thus the translocation to the shoots was inhibited (Weissenhorn and Leyval, 1995). Both mechanisms seem to be responsible for the plant growth effect found here.

In this Cd contaminated soil, rhizosphere from non-inoculated plants showed reduced enzymatic activities which is an indicator of the perturbations caused to the ecosystem functioning under polluted conditions

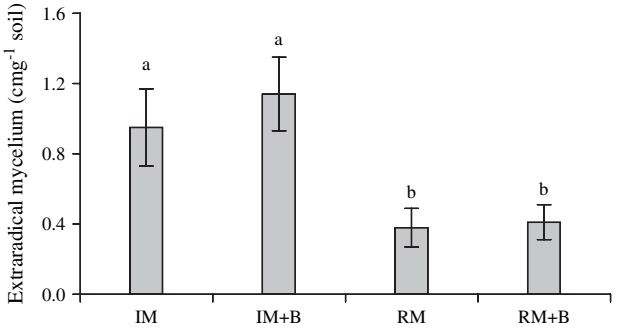

Fig. 5. Extraradical mycelium production by indigenous (IM) or reference (RM) *G. mosseae* strain inoculated or not with *Brevibacillus brevis* (B) in soil contaminated with 8 μg g^{-1} Cd. Vertical bars represent standard errors.

(Naseby and Lynch, 1997). The increase of enzymatic phosphatase, β-glucosidase and dehydrogenase activities in the rhizosphere of inoculated plants could be due to the effect of nutrient leakage from roots (quantitative and/or qualitative changes in root exudates). Microbial inoculated plants showed an increased root system that, in turn, increased carbon and nutrient leakage to the rhizosphere zone. Nevertheless, values of root growth did not correlate with those of enzymatic activities determined here. In fact, root growth was lower in single *B. brevis* inoculated plants than in reference *G. mosseae* colonized *Trifolium* but whatever enzymatic activity determined here was greater in the rhizosphere of the bacterial inoculated plants. These results support the idea that microbial inoculation by specific bacterium and/or AM fungi produced qualitative, more than quantitative, changes in the composition of root exudates (Kotari et al., 1991).

Enzymatic changes detected here also suggest a direct effect of microbial inoculation on microbial populations in the rhizosphere. Enzyme activities involved in P cycle are inversely related to P availability (Tadano et al., 1993; Azcón and Barea, 1997). When P is a limiting nutrient, its demand increases and, as a result, there is an increase in phosphatase activity in the rhizosphere, as occurred in native *G. mosseae* plus *Brevibacillus* coinoculated plants.

In polluted soils, the size of microbial population is highly reduced. Oxireductase enzymes, such as dehydrogenase, are an indication of microbial metabolism in soil (Skujins, 1976). As well, glucosidase has a key role in regulating the availability of plant nutrients

through the mineralization processes (Degens et al., 1996) and in our study it was improved in the rhizosphere of plants with the highest growth. Such stimulating effects are indicative of a better biological functioning and fertility of the inoculated soil. Thus, the effectiveness of these treatments on plant growth could also be the result of an indirect effect through changes on microbial composition in the rhizosphere (Medina et al., 2003).

This study can lead to practical applications since microbial inoculation may be very important to maximize plant growth and nutrition as a biotechnological approach to recover polluted environments. The combined application of effective microbial groups can be used for reclamation of polluted soil. In this study, the most effective inocula for improving crop yield consisted of native microorganisms, but this research topic deserves further investigations.

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