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ORIGINAL ARTICLE

# Effectiveness of autochthonous bacterium and mycorrhizal fungus on *Trifolium* growth, symbiotic development and soil enzymatic activities in Zn contaminated soil

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## Abstract

**Aims:** This study investigates how autochthonous micro-organisms [bacterium and/or arbuscular mycorrhizal (AM) fungi] affected plant tolerance to Zn contamination.

**Methods and Results:** Zinc-adapted and -nonadapted *Glomus mosseae* strains protected the host plant against the detrimental effect of Zn (600 $\mu$g g$^{-1}$). Zn-adapted bacteria increased root growth and N, P nutrition in plants colonized by adapted *G. mosseae* and decreased the specific absorption rate (SAR) of Cd, Cu, Mo or Fe in plants colonized by Zn-nonadapted *G. mosseae*. Symbiotic structures (nodule number and extraradical mycelium) were best developed in plants colonized by those Zn-adapted isolates that were the most effective in increasing plant Zn tolerance. The bacterium also increased the quantity and quality (metabolic characteristics) of mycorrhizal colonization, with the highest improvement for arbuscular vitality and activity. Inocula also enhanced soil enzymatic activities (dehydrogenase, $\beta$-glucosidase and phosphatase) and indol acetic acid (IAA) accumulation, particularly in the rhizosphere of plants inoculated with Zn-adapted isolates.

**Conclusions:** *Glomus mosseae* strains have a different inherent potential for improving plant growth and nutrition in Zn-contaminated soil. The bacterium increased the potential of mycorrhizal mycelium as inoculum.

**Significance and Impact of the Study:** Mycorrhizal performance, particularly that of the autochthonous strain, was increased by the bacterium and both contributed to better plant growth and establishment in Zn-contaminated soils.

## Introduction

Plant development on metal contaminated soil is relatively low as the elevated concentrations of metals in many soils restrict plant establishment and growth in those areas (Shetty *et al.* 1995). In this respect, biological processes can alleviate plant pollution stress, which improves reclamation of contaminated soil and thus they can be used to overcome this ecological problem (Hildebrandt *et al.* 1999; Leyval *et al.* 2002).

Arbuscular mycorrhizal (AM) fungi are abundant soil micro-organisms and are considered as important functional components of the soil–plant system occurring in many environments, including disturbed soils (Brundrett *et al.* 1996). In fact, AM fungi may improve plant Zn tolerance, an effect that can be related to increased plant nutrition, although additional mechanisms can also be involved (Colpaert and Vanderkoourhuyse, 2001; Whitfield *et al.* 2004). If AM fungi enhance the plant abilities to cope with environmental stresses, it is because they are present and are functioning in degraded stressed ecosystems (Brundrett *et al.* 1996; Leyval *et al.* 1997). Mycorrhizal fungi are adapted to edaphic conditions and McGonigle and Miller (1996) suggested that differences in

 

fungal behaviour and efficiency on plant development can be because of the origin of the AM fungi. Studies have focused on the diversity and tolerance of AM fungi in heavy metal polluted soils. del Val *et al.* (1999) found a reduction in the diversity and number of spores in soil with high concentrations of heavy metals, and Tonin *et al.* (2001) described that four *Glomus* species were found in the rhizosphere of metal tolerant plants but only one of them was able to colonize clover roots growing in soil supplemented with metal. However, the understanding of plant tolerance and adaptation to heavy metals in soils remains unclear and needs further investigations (Colpaert and Vanderkoourhuyse, 2001).

Fungal hyphae of selected AM inocula may have the capacity to bind metals present in roots or in the rhizosphere and this activity would decrease metal translocation from the root to the shoot, which Wasserman *et al.* (1987) proposed as mechanisms for enhancing plant tolerance. The bacterial ability for metal sequestration has also been observed (Vivas *et al.* 2005). Many studies show that combining inoculants from different microbial groups may stimulate plant growth (Barea *et al.* 1997, 2002; Toro *et al.* 1997; Marulanda *et al.* 2002). It is assumed that the beneficial effect of AM fungi coinoculated with selected bacterial strains is the result of interactions capable of increasing shoot or/and root growth, nutrient uptake and plant tolerance to stresses (Vivas *et al.* 2003b). Recently, the beneficial effect of an indigenous Cd-tolerant *Glomus mosseae* coinoculated with a Cd-adapted strain of *Brevibacillus* sp. in terms of improving plant tolerance to Cd-contamination has been reported (Vivas *et al.* 2003a,b). Nevertheless, a better understanding of the interaction between metal adapted soil micro-organisms is required for their better utilization as inoculants for practical purposes.

Adverse environmental conditions such as metal presence can negatively affect the infectivity of AM propagules (Enkhtuya *et al.* 2000). This negative effect can be compensated by rhizosphere bacteria, which are able to improve the growth of AM fungi (Vivas *et al.* 2003a,b). The bacterial inoculum can enhance not only axenic growth of *G. mosseae* but also AM-colonization (Azcón 1987, 1989). Recently, Marulanda *et al.* (2002) reported that the beneficial effect of bacterial inoculation was less relevant on AM colonization than on metabolic and physiological fungal activities that are considered as indexes of effective AM symbiosis (Tisserant *et al.* 1993; Guillemin *et al.* 1995).

Metals in the growth medium may induce changes in the length of intra- and/or extraradical mycorrhizal hyphae, reducing their infective capacity but changes in their metabolic characteristics have yet to be studied. Consequently, an interesting next step is the verification of the benefit of bacterial inoculation upon AM colonization and mycorrhizal effect in Zn-polluted soil, mediated by a metabolic and/or physiological enhancement of AM fungus functionality and extraradical mycelium development. The relative importance of mycorrhizal mycelia as propagule sources depends on the soil conditions (Jasper 1994). The mycelia network has been considered as the main source of AM propagules in a stressed ecosystem (Requena *et al.* 1996) and the low growth and viability of these propagules in Zn-contaminated soil may be a determinant factor in the survival of AMF. Zinc pollution may affect the infective capacity of AM mycelium as Enkhtuya *et al.* (2000) demonstrated.

The influence of AM fungal origin and of a bacterial strain on the potential of two *G. mosseae* strains as AM inocula, in Zn-contaminated soil, was tested here. Plants were grown in a container with a root and a hyphal compartment (HC) separated by a mesh (50 $\mu$m pore size). The container used allows testing of the infective ability of the hyphal network of a donor plant grown in the root compartment (RC), as single inoculum source for a receiver plant, grown in the HC.

This study was carried out in two successive parts. In the first part we determined, in an RC contaminated with 600 $\mu$g Zn $g^{-1}$, the effectiveness of *G. mosseae* [autochthonous (Zn tolerant) or reference (BEG 119) strains] in interaction with a Zn-tolerant bacterium (*Brevibacillus brevis*). The effectiveness of inocula in increasing plant Zn tolerance was evaluated in terms of plant growth and nutrition, and specific absorption rate (SAR) of heavy metals. Metabolic characteristics of fungal colonization [succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) activities] were used as indicators of the vitality and activity of AM symbiosis.

Biological properties of rhizospheric soil are indicative of soil quality (Naseby and Lynch 1997) therefore, changes in phosphatase, glucosidase and dehydrogenase activities and indolacetic acid (IAA) accumulation in the rhizosphere soil under Zn-polluted conditions in response to microbial inoculation were determined in this study.

In the second part, viability, infectivity and effectiveness of detached extraradical mycelium from autochthonous or reference *G. mosseae* in Zn-polluted soil and the relevance of the bacterium affecting such AM inocula were determined in a receiver plant growing in the HC, after removing the donor plant.

## Materials and methods

### Experimental design

The experimental design consisted of a completely randomized factorial block with two factors: (i) mycorrhizal

inoculation with autochthonous or reference *G. mosseae* strains plus a nonmycorrhizal treatment and (ii) bacterial inoculation and a control treatment without any inoculation. Five replicates per treatment were made totalling 30 experimental units (two plants per unit).

### Soil, plant and pot experiment

A calcareous loamy soil from Granada province (Spain) sieved (2 mm), diluted with quartz-sand (<1 mm) (4 : 1 soil : sand v/v) and sterilized by steaming (100°C for 1 h for 3 days) was used as test soil. The soil had a pH of 7·2 (water); 1·63% organic matter, nutrient concentrations (mg $kg^{-1}$): N, 2·1; P, 1·7 ($NaHCO_3$-extractable P); K, 80. The soil texture was made up of 57·8% sand, 19% clay and 23·2% silt.

After sterilization the soil/sand mixture was supplemented with 828 $\mu$g of Zn $g^{-1}$ by adding an adequate amount (250 ml) of a 360 mmol $l^{-1}$ solution of $ZnSO_4{\cdot}7H_2O$. After 2 weeks of soil incubation (for metal stabilization) available Zn was determined according to Lakane and Ervio (1971) methodology, resulting to be 600 $\mu$g Zn $g^{-1}$.

Plants were cultivated in container of 21 cm length, 10 cm depth and 5 cm width. The experimental system was based on those described by Tobar *et al.* (1994a,b) and Ruiz-Lozano and Azcón (2000). It had two adjacent compartments separated by a nylon mesh. The RC was made with cylindrical (60 mm diameter) bags of 50 $\mu$m nylon mesh, which retains roots but allows AM hyphae to pass through, so that hyphae are able to grow from the RC to the HC. The RCs and HCs were filled with the experimental soil/sand mixture previously incubated for 15 days with 828 $\mu$g Zn $g^{-1}$ (as $ZnSO_4{\cdot}7H_2O$).

The RC was filled with 240 g of the soil/sand mixture while 550 g of this mixture constituted the adjacent HC. Seeds (four per pot and after emergence thinned to two seedlings) of *Trifolium repens* L. cv huia sown in the RC were either inoculated with the indigenous mycorrhizal inoculum of *G. mosseae* strain or a reference of *G. mosseae* (BEG 119) from collection (Nicol. and Gerd.) Gerd. and Trappe or left uninoculated (nonmycorrhizal, control). Inoculation was achieved by placing 10 g of the mycorrhizal inoculum into the RC. Both AM inocula were obtained from a stock culture and maintained by storage for 3–6 months in polyethylene bags at 5°C.

The experiment has two parts: In part I, plants were grown (for 3 months) in the RC and in part II, plants were grown (for 3 months) in the HC, after the plants from the RC were detached.

Part II of the experiment had the same experimental design as part I, i.e., to evaluate the infectivity and effectiveness of extraradical mycelia (as single source of inocula) generated by the plants grown in from the RC. Two seeds of *T. repens* were sown in the HC when the plants grown in the RC were harvested.

### Growth conditions

Plants (*T. repens*) were grown in a controlled environmental chamber under conditions of 50% relative humidity, day and night temperatures of 27 and 18°C, respectively, and a photoperiod of 14 h. Photosynthetic photon flux density (PPFD) was 503 $\mu$mol $m^{-2}$ $s^{-1}$ as measured with a light-meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied by daily weighting to maintain the required water level (100% of water holding capacity) of the test soil/sand mixture throughout the experiment. Throughout the experiment the plants were fertilized each week with 10 ml of Hewitt's (Hewitt 1952) nutrient solution lacking N and P.

### Soil micro-organisms

The Zn-adapted bacterial and AM fungal species were isolated from the Zn-treated long-term field experiment (10 years old) in Nagyhörcsök (Hungary) (Kádár 1995). Isolates were cultivated for inocula production following the conventional procedure described by Vivas *et al.* (2003b).

The indigenous mycorrhizal strain isolated from the Zn-polluted soil (Nagyhorcsock, Hungary) was the most abundant AM fungal spore in this soiland it was a *G. mosseae* strain (morphologically determined). A *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe strain (isolate BEG 119) from collection was used as reference AM inoculum. The inoculum was bulked in an open-pot culture of red clover and consisted of soil, spores, mycelia and infected root fragments having a colonization efficiency of 70% (indigenous *G. mosseae*) and of 80% (reference *G. mosseae*). Ten grams of inoculum were added to the appropriate RC at sowing time just below the clover seeds.

The nonmycorrhizal containers received the same amount of autoclaved inoculum (10 g) and 2 ml of an AM inoculum filtrate, to add microbial population free of AM propagules. The infectivity and effectiveness of detached extraradical mycelium was determined in a receiver *T. repens* host plant.

The bacterial strain, later identified as a *Brevibacillus* sp. (according to the molecular method used), isolated from the Zn-contaminated soil at Nagyhörcsök (Hungary) was the most abundant bacterial type in this soil. When appropriate, RCs were inoculated with 1 ml of bacterial culture ($10^8$ CFU $ml^{-1}$) grown in 250-ml flasks containing 50 ml of nutrient broth medium (8 g $l^{-1}$) in shake culture for 24–48 h at 28°C.

The bacterium exhibited a great tolerance to Zn when it was grown in nutrient broth at increasing Zn concentrations ranging from 0 to 100 $\mu$g Zn ml$^{-1}$. At low Zn concentrations in the medium (25 and 50 $\mu$g Zn ml$^{-1}$) this strain reached almost $10^8$ CFU and at the highest Zn levels in the growth medium (75 and 80 $\mu$g Zn ml$^{-1}$) the same growth yield ($10^8$ CFU) was maintained after 10 h of incubation (Vivas *et al.* 2005).

A suspension of *Rhizobium leguminosarum* bv *trifolii* was added to all of the RCs (1 ml, $10^8$ CFU per pot). It was prepared following the standard procedure (Azcón 1993). This *Rhizobium* strain was previously selected as an efficient nodulating strain in contaminated soil (Vivas *et al.* 2003b).

### Determination of growth and symbiotic parameters

At harvest, after 90 days of plant growth in the RC, the dry biomass of shoots and roots, the nutrient and metal concentrations, and the symbiotic development (mycorrhizal infection and nodulation) were determined.

Concentrations (mg g$^{-1}$) of N (micro-Kjeldahl) and P in shoots were colorimetrically measured on an autoanalyzer according to Mingorance (2002) and K was determined by flame photometry. Shoot Pb, Cd, Ni and Zn ($\mu$g g$^{-1}$) were determined (from three different measurements made on a pooled sample containing the five replicate pots per treatment) after wet digestion of the air-dried plant samples with $HNO_3 + H_2O_2$ by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Takács *et al.* (2001).

Specific absorption rate is defined as the amount of nutrients or metal absorbed per unit of root biomass (Gray and Schlesinger 1983). It was calculated as follows:

$$\text{SAR} = \frac{\text{Plant nutrient or metal } (\mu\text{g})}{\text{Root mass (g)}}$$

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

Succinate dehydrogenase (SDH) activity was determined according to the procedure described by Smith and Gianinazzi-Pearson (1990). Briefly, the roots were immersed in a freshly made solution containing 0·2 mol l$^{-1}$ Tris–HCl pH 7·0, 2·5 mol l$^{-1}$ sodium-succinate hexahydrate, 4 mg ml$^{-1}$ nitro blue tetrazolium, 5 mmol l$^{-1}$ $MgCl_2$. Root fragments were stained overnight at room temperature and then cleared for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

Alkaline phosphatase was determined according to the procedure described by Tisserant *et al.* (1993), which confirmed the specificity of staining methods for ALP. Roots were immersed in a freshly made solution containing 50 mmol l$^{-1}$ Tris–citric acid pH 9·2, 1 mg ml$^{-1}$ $\alpha$-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 stained overnight at 37°C and cleared for 15–20 min in 1% active chlorine solution in sodium hypochlorite.

Mycorrhizal development, was evaluated by the method of Trouvelot *et al.* (1986) and expressed as frequency of AM colonization (*F*%, percentage of root fragments showing fungal colonization), intensity of AM colonization [*M*% which gives an estimation of the amount of root cortex that became mycorrhiza and is referred to the whole root system whereas *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, whereas *a*% refers to the mycorrhizal root fraction only. The method used for determination of extraradical mycelium was adapted from those described by Newman (1966) and Jones *et al.* (1998).

### Enzymatic soil activities

In rhizosphere soil samples, $\beta$-glucosidase dehydrogenase and acid phosphatase were determined. Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0·115 mol l$^{-1}$) as substrate. Two millilitres of 0·5 mol l$^{-1}$ 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 mol l$^{-1}$ $CaCl_2$ and 2 ml of 0·5 mol l$^{-1}$ NaOH were added, and the mixture was centrifuged at 15 000 ***g*** 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.

$\beta$-glucosidase was determined using *p*-nitrophenyl-$\beta$-D-glucopyranoside (PNG, 0·05 mol l$^{-1}$ ; Masciandaro *et al.* 1994) as substrate. Two millilitres of 0·1 mol l$^{-1}$ 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 at 60% of its field capacity 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 through a Whatman no 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

The indole acetic acid (IAA) in rhizosphere soil was determined by a colorimetric method developed by Mitchell and Brunstetter (1939) and by Gordon and Weber (1950). For that, 2·0 g of air-dried soil (on an oven-dry basis) were 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. These 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 were added to inactivate the enzymes involved in the bioassay of auxin, then 1 ml of 0·5 mol $l^{-1}$ $CaCl_2$ solution were added. The soil solution was filtered (Whatman no 2). Three millilitres of the filtrate were transferred to a test tube and 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 red solution was measured with a spectrophotometer (Turner Model 350) adjusted to a wavelength of 535 nm (Wöhler, 1997).

For plant growing in the HC (part B) only shoot and root dry weight and symbiotic parameters were determined following the same procedures and experimental conditions described for the part A.

### Molecular identification of the bacterial isolate

Bacterial identification was carried out by 16S rDNA cloning and sequencing as previously described Vivas *et al.* (2003b,c). Database searches for 16S rDNA sequence similarity unambiguously identified the Cd-tolerant bacterium as a member of the genus *Brevibacillus*.

### Statistics analysis

The results were statistically evaluated by factorial analysis of variance (ANOVA) with bacterial treatment, mycorrhizal treatment and bacterial treatment–mycorrhizal treatment interaction as sources of variation followed by Duncan's multiple range test. Percentage values were arcsine-transformed before statistical analysis.

## Results

### Part I

AM colonized plants in RCs, particularly those infected by IM, showed root and shoot biomass enhancement in relation to the control plants but the magnitude of the growth response to AM was larger when associated with B. Single B inoculation produced greater shoot and root biomass than the single inoculation with RM. Increases in plant biomass by B ranged from 125% (shoot) to 175% (root) in IM colonized plants and from 144% (shoot) to 145% (root) in plants RM colonized (Fig. 1a,b). However, some of these bacterial effects on mycorrhizal plants were not significantly different.

Similarly, the number of nodules formed was increased by the microbial treatments applied, mainly by AM colonization. The effect of B was not significant in enhancing nodule number in mycorrhizal plants. In all cases, microbial inoculation significantly increased nodule formation compared with control plants, which showed very low nodulation under such Zn-polluted conditions (Fig. 1g).

Shoot N, P, K and Zn content (Fig. 1c–f) displayed similar trends as those described for plant growth. The content of these nutrients in shoots was enhanced by the microbial treatments applied but to a greater extent in plants coinoculated with IM + B. Single inoculation of B resulted more efficient in increasing all the nutrients content than the single inoculation of reference RM. The bacterium did not significantly affect P, K or Zn acquisition in plants colonized by RM.

For Fe, Mo and Ni, the highest SAR values were observed in noninoculated control plants. Single B inoculation had the lowest SAR for Fe and Mo. The highest SAR for Cd, Cu and Mn was measured in RM-treated plants. In plants colonized by RM the coinoculation with B decreased the SAR for Cd, Cu, Fe and Mo in comparison with the single RM inoculation. Plants inoculated with single RM showed higher SAR values for Cd, Cu, Fe, Mn and Mo than plants treated with single IM. B only decreased Mn in IM colonized plants (Table 1).

As Table 2 shows, IM had a higher mycorrhizal development than the reference RM. Also, the mycorrhizal structures formed by IM showed higher vitality and activity (SDH and ALP staining) than those formed in RM-colonized roots. Bacterial inoculation increased the colonization rate, vitality (SDH) and activity (ALP) of both IM and RM fungi. Such bacterial effect was more relevant in increasing the proportion of arbuscules (%*a* and %*A*) formed in roots colonized by RM. Formation of arbuscules by the nonadapted fungus was highly reduced under this high Zn concentration.

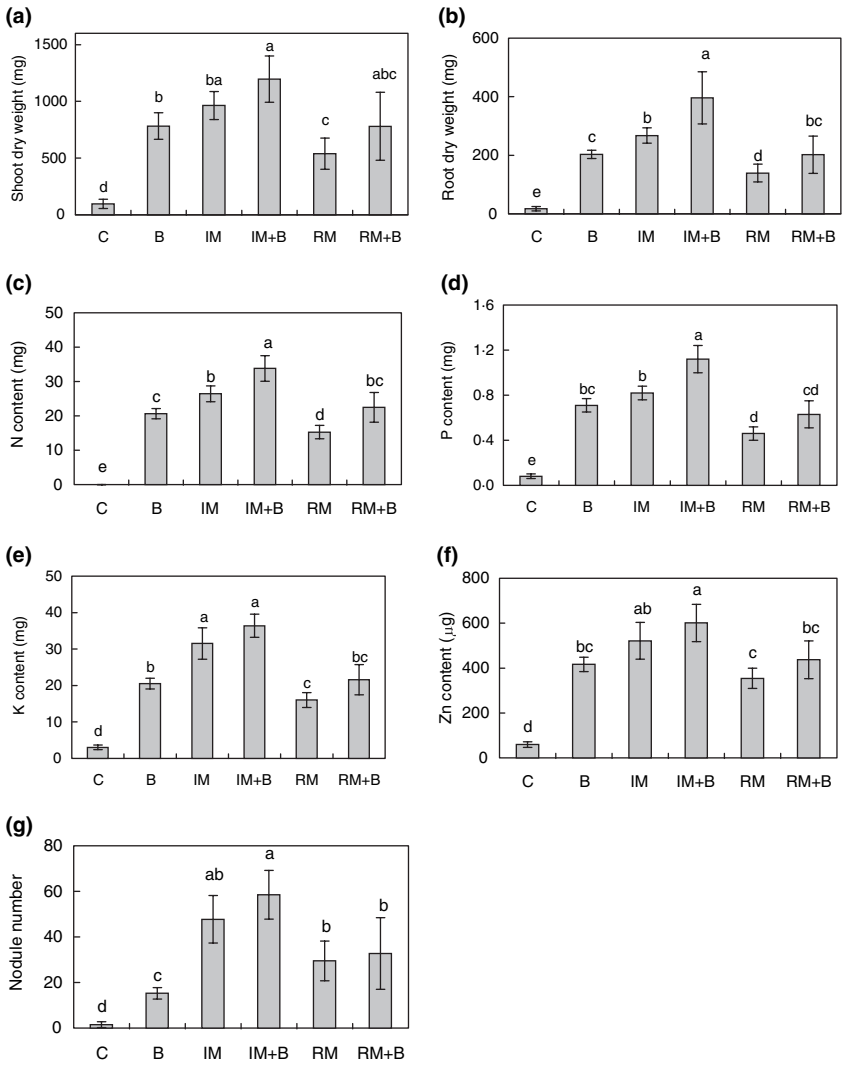


**Figure 1** Shoot and root dry weight (a, b), nutrients [N, P, K (c, d, e)] and Zn (f) content and number of nodules formed (g) in non-mycorrhizal plants [control (C) or bacteria (B) inoculated] and in mycorrhizal colonized plants [by indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM)] associated or not with bacteria (IM + B or RM + B). Plants were grown in a root compartment amended with 600 $\mu$g Zn $g^{-1}$. Vertical bars represent standard errors. Mean values (five replicates) not sharing a letter differ significantly according to Duncan's multiple range test ($P < 0{\cdot}05$).

**Table 1** Specific absorption rate (SAR) (mg $g^{-1}$) of elements (Cd, Cr, Cu, Fe, Mn, Mo and Ni) in nonmycorrhizal plants [control (C) or bacteria (B) inoculated] and in mycorrhizal colonized plants [by indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM)] associated or not with bacteria (IM + B or RM + B). Plants were grown in a root compartment contaminated with 600 $\mu$g Zn $g^{-1}$

| Microbial treatments | Cd | Cr | Cu | Fe | Mn | Mo | Ni |
|---|---|---|---|---|---|---|---|
| C | 4·1c | 2·8b | 33·9b | 3669a | 2537bc | 16·4a | 6·3a |
| B | 4·6c | 0·5b | 38·2b | 648c | 2267cd | 6·8c | 1·8b |
| IM | 14·5b | 5·6b | 36·2b | 1400bc | 2931bc | 4·7d | 3·4ab |
| IM + B | 11·0b | 13·2a | 32·8b | 2077b | 1626d | 4·2d | 4·6ab |
| RM | 25·6a | 3·3b | 47·3a | 2268b | 3840a | 10·5b | 2·3b |
| RM + B | 13·9b | 3·1b | 32·5b | 1015c | 3184ab | 6·4cd | 1·3b |

For each parameter means values (five replicates) not sharing a letter differ significantly ($P < 0{\cdot}05$).

No mycorrhizal colonization was observed in the uninoculated plants. IM produced a more abundant extraradical mycelium than RM. The stimulating effect of B particularly affected RM extraradical fungal growth (the less effective and infective endophyte and that showing the lower extraradical mycelia) (Fig. 2a).

IAA accumulation in the rhizosphere was improved by single inoculations (AM fungi or bacteria) but it was the greatest in soil dually inoculated with autochthonous Zn-adapted micro-organisms (Fig. 2e). Likewise, all the measured soil enzymatic activities increased, to the highest extent, by the combined inoculation of autochthonous micro-organisms. Nevertheless, all the microbial treatments increased phosphatase, $\beta$-glucosidase and dehydrogenase activities in the rhizosphere (Fig. 2b–d). B also had a positive effect (not always significantly different) when associated with RM.


 

**Table 2** Root colonization in root compartment amended with 600 $\mu$g Zn $g^{-1}$ by indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM) associated or not with native bacteria (B) after Trypan blue (TB), succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) staining. AM values measured (as % and total) were: *F* (colonization frequency), *M* (colonization intensity in the whole root system), *m* (intensity in the root fraction) *A* (arbuscule abundance in the whole root system) and *a* (arbuscule abundance in the mycorrhizal root fraction)

| Mycorrhizal treatment | *F* | *M* | *m* | *a* | *A* |
|---|---|---|---|---|---|
| % | | | | | |
| *TB staining* | | | | | |
| IM | 66·3b | 23·5ab | 31·0ab | 72·8a | 17·1ab |
| IM + B | 83·5a | 31·0a | 36·7a | 74·8a | 24·1a |
| RM | 49·5c | 9·8c | 16·3c | 54·8b | 5·4c |
| RM + B | 62·2b | 18·7b | 25·8b | 66·8ab | 13·9b |
| *SDH staining* | | | | | |
| IM | 57·8ab | 6·5b | 9·9a | 39·2b | 13·2a |
| IM + B | 65·4a | 7·7ab | 12·6a | 54·8a | 14·3a |
| RM | 41·4c | 5·3b | 11·8a | 45·7ab | 1·7c |
| RM + B | 53·4b | 11·2a | 14·2a | 55·7a | 9·2b |
| *ALP staining* | | | | | |
| IM | 50·0a | 3·9a | 4·5c | 26·5b | 6·5b |
| IM + B | 53·8a | 5·2a | 7·2b | 42·3a | 9·4a |
| RM | 30·1b | 1·9b | 4·1c | 12·7c | 1·2c |
| RM + B | 32·5b | 5·6a | 10·1a | 40·6a | 4·9b |
| Total | | | | | |
| *TB staining* | | | | | |
| IM | 177·7b | 63·0b | 83·1b | 195·1b | 45·8b |
| IM + B | 330·7a | 122·8a | 145·3a | 296·2a | 95·4a |
| RM | 68·8d | 13·6d | 22·6d | 76·2d | 7·5d |
| RM + B | 125·6c | 37·8c | 52·1c | 134·9c | 28·1c |
| *SDH staining* | | | | | |
| IM | 154·9b | 17·4c | 26·5b | 105·0b | 35·4b |
| IM + B | 215·4a | 30·5a | 49·9a | 217·0a | 56·6a |
| RM | 57·5d | 7·4d | 16·4c | 63·5c | 2·4d |
| RM + B | 107·9c | 22·6bc | 28·7b | 112·5b | 18·6c |
| *ALP staining* | | | | | |
| IM | 134·0b | 10·4b | 12·1c | 71·0b | 17·4b |
| IM + B | 213·0a | 20·6a | 28·5a | 167·5a | 37·2a |
| RM | 41·8d | 2·6c | 5·7d | 17·6c | 1·7b |
| RM + B | 65·6c | 11·3b | 20·4b | 82·0b | 9·9c |

For each parameter means values (five replicates) not sharing a letter differ significantly ($P < 0{\cdot}05$).

### Part II

No roots were detected outside the mesh (HC). Thus, the AM inoculum in HC was exclusively AM mycelium detached from AM colonized plants in RC.

Regarding the effectiveness and infectivity of mycelium from IM or RM in Zn-contaminated soil, differences between both mycorrhizal mycelia used as inocula, were also found. Similarly to that observed in the RC, the mycelial inoculum from donor plants increased growth and nodule numbers in receiver plants growing in Zn-contaminated soil in the HC. However, the bacterial effect on the mycorrhizal inoculum potential was mainly observed in the enhancement of root development when IM was the fungus involved (Fig. 3b).

Mycorrhizal colonization (TB) and SDH or ALP-ase activities in receiver plant were lower when the AM inoculum was mycelia from RM (Table 3). AM colonizations by mycelium from IM + B treated plants in RC showed a greater proportion of %A and %M (TB), %a (SDH) and %F, %M (ALP) compared to mycelia from single IM. In the case of mycelium from RM + B treated donor plants only increased %F (TB staining) in receiver plant compared to mycelium from single RM (Table 3). In general, the total amount of root colonization and that showing active fungal metabolism (SDH and ALP activities) in receiver plants (HC with 600 $\mu$g Zn $g^{-1}$) was increased when IM + B were applied in the donor plant. The generalized stimulating role of B on vitality and activity of fungal mycelium from IM is an important aspect accounting for the interaction, which was not observed when RM was involved (Table 3).

## Discussion

The infective ability of the extraradical mycelium was affected by the origin of the mycorrhizal fungus involved in mixture and not with bacterial inoculum. Thus, the physiological characteristics of AM-colonization formed in the host plant may have importance in Zn-contaminated soil.

Ours results indicate the potential of microbial inoculations to improve growth and nutrition in Zn-stressed plants and the abilities of AM fungi, from different origins and Zn adaptation, to colonize and to affect plant growth. Both *G. mosseae* strains assayed protected (in terms of growth) the host plant against the detrimental effects of Zn excess. But *G. mosseae*, autochthonous Zn-adapted endophyte, was the most efficient fungus in increasing plant growth and symbiotic parameters. Microbial inoculation did not decrease Zn content in the plant but it increased N, K and particularly P content and changed values of SAR for Cd, Cr, Cu, Mn, Co and Fe. These microbial effects on plant nutrition and mineral (micro/macronutrients) balance seem to be related to plant Zn tolerance.

Differences in behaviour and efficiency between AM fungi under metal-polluted conditions can be related to their origin because of their adaptation to edaphic conditions. In any way, different and specific susceptibility to heavy metals among AM fungi isolates from the same metal-polluted soil was reported by del Val *et al.* (1999). The mechanisms involved in the amelioration of metal stress are probably complex (Leyval *et al.* 2002).

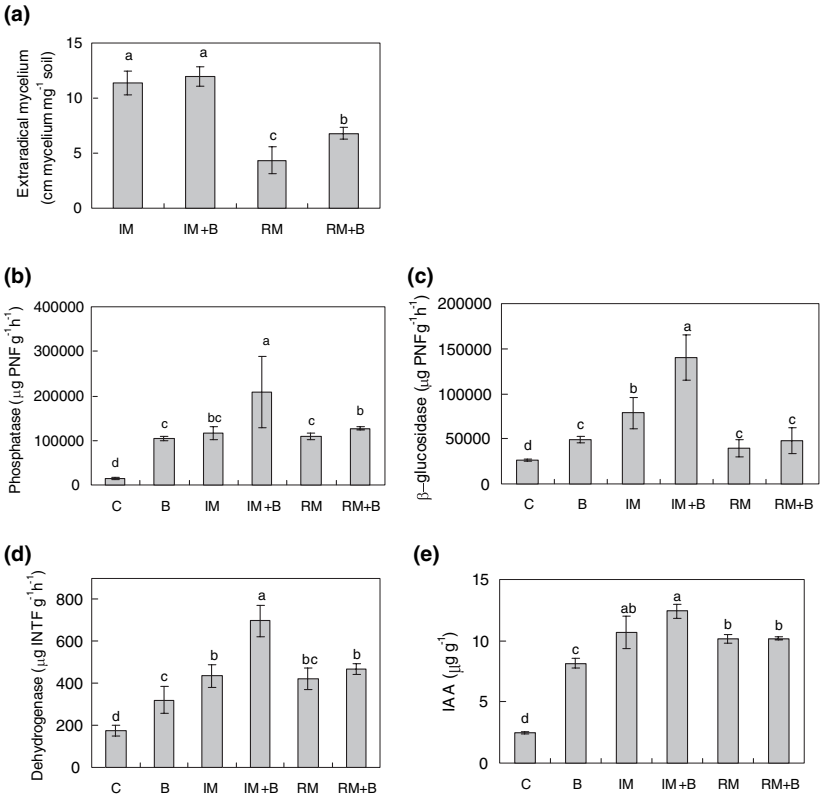


**Figure 2** Extra-radical mycelium developed by mycorrhizal plants (a), enzymatic activities (b–d) and indol acetic acid (IAA) concentration (e) in rhizosphere soil from nonmycorrhizal soil [control (C) or bacteria (B) inoculated] and in mycorrhizal colonized soil [by indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM)] associated or not with bacteria (IM + B or RM + B). Plants were grown in a root compartment amended with 600 $\mu$g Zn g$^{-1}$. Vertical bars represent standard errors. Mean values (five replicates) not sharing a letter differ significantly according to Duncan's multiple range test ($P < 0{\cdot}05$).

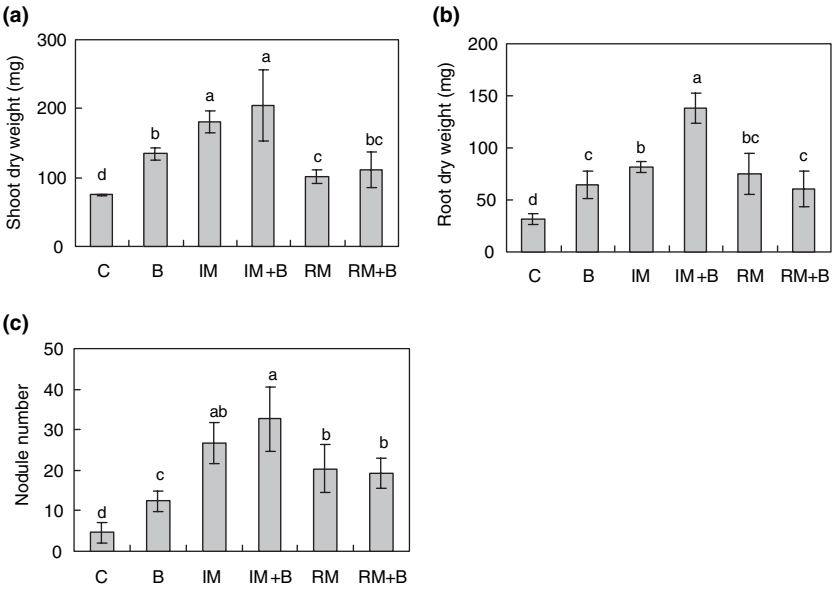


**Figure 3** Shoot and root dry weight (a, b) and number of nodules formed (c) in nonmycorrhizal plants [control (C) or bacteria (B) inoculated] and in mycorrhizal colonized plants [by indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM)] associated or not with bacteria (IM + B or RM + B). Plants were grown in a root compartment amended with 600 $\mu$g Zn g$^{-1}$. Vertical bars represent standard errors. Mean values (five replicates) not sharing a letter differ significantly according to Duncan's multiple range test ($P < 0{\cdot}05$).

Here the effect of inocula cannot be explained by Zn hyphal sequestration, root inmobilization or Zn tissue dilution as Zn uptake by mycorrhizal plants was higher than in noninoculated plants. These results are different than those reported by Heggo *et al.* (1990) or Zhu *et al.* (2001) that found a decreased Zn uptake by AM-colonized soybean plants growing in Zn (400 $\mu$g g$^{-1}$) polluted soils. Nevertheless, the amount of available Zn (600 $\mu$g g$^{-1}$) in the experimental soil used here may be determinant for the mycorrhizal effect on Zn uptake found in this study (Zhu *et al.* 2001).


 

**Table 3** Root colonization in hyphal compartment amended with 600 mg Zn $g^{-1}$, by mycelium from indigenous *G. mosseae* (IM) or by reference *G. mosseae* (RM) associated or not with native bacteria (B) in RC after Trypan blue (TB), succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) staining. AM values measured (as % and total) were: *F* (colonization frequency), *M* (colonization intensity in the whole root system), *m* (intensity in the root fraction), *A* (arbuscule abundance in the whole root system) and *a* (arbuscule abundance in the mycorrhizal root fraction)

| Mycorrhizal treatment | *F* | *M* | *m* | *a* | *A* |
|---|---|---|---|---|---|
| % | | | | | |
| *TB staining* | | | | | |
| IM | 63·8a | 13·4b | 22·2a | 54·8a | 1·83b |
| IM + B | 67·3a | 16·9a | 22·4a | 57·7a | 12·37a |
| RM | 37·6c | 9·1c | 2·3b | 14·2b | 0·80c |
| RM + B | 46·1b | 11·4bc | 2·5b | 14·7b | 1·57bc |
| *SDH staining* | | | | | |
| IM | 41·6a | 0·4a | 1·3a | 19·3b | 0·37a |
| IM + B | 46·7a | 0·5a | 1·4a | 31·6a | 0·36a |
| RM | 30·4b | 0·3b | 1·1a | 4·3c | 0·14a |
| RM + B | 28·0b | 0·3b | 1·5a | 5·1c | 0·12a |
| *ALP staining* | | | | | |
| IM | 21·7b | 0·2b | 1·0a | 2·7a | 0·01a |
| IM + B | 30·1a | 0·3a | 1·0a | 3·8a | 0·02a |
| RM | 13·0c | 0·1c | 0·6a | 0·6b | 0·05a |
| RM + B | 12·7c | 0·1c | 0·5a | 0·2b | 0·03a |
| Total | | | | | |
| *TB staining* | | | | | |
| IM | 51·1b | 10·7b | 17·7b | 43·9b | 1·46b |
| IM + B | 94·2a | 23·7a | 31·3a | 80·7a | 17·30a |
| RM | 30·1c | 7·3c | 2·0c | 1·8d | 0·60c |
| RM + B | 27·6c | 6·8c | 1·5c | 8·8c | 0·90c |
| *SDH staining* | | | | | |
| IM | 33·3b | 0·3b | 1·0b | 15·5b | 0·30b |
| IM + B | 65·4a | 0·6a | 2·0a | 44·2a | 0·50a |
| RM | 24·3c | 0·2c | 0·9b | 3·4c | 0·11c |
| RM + B | 16·8d | 0·2c | 0·9b | 3·1c | 0·07c |
| *ALP staining* | | | | | |
| IM | 17·4b | 0·2b | 0·8b | 2·2b | 0·01c |
| IM + B | 42·2a | 0·4a | 1·4a | 5·4a | 0·28a |
| RM | 10·4cd | 0·1c | 0·5c | 0·5c | 0·04b |
| RM + B | 7·6d | 0·1c | 0·3c | 0·1d | 0·02c |

For each parameter means values (five replicates) not sharing a letter differ significantly ($P < 0·05$).

The impact and activity of selected microbes (AM fungi and/or bacteria) was relevant in reducing the damage of Zn stress on plant growth and nutrition. They help the plant to tolerate Zn-polluted conditions, presumably until a critical threshold value.

In the case of AM-colonization by the Zn-adapted *G. mosseae* strain, the bacterium increased the arbuscular vitality and activity (%*a* and %*A*) more than the colonization intensity (%*m* or %*M*). No previous information has been reported on the role of autochthonous bacteria in increasing the physiological characteristics of mycorrhizal colonization under Zn-polluted conditions. The more efficient AM fungal functioning resulted in greater plant growth and nutrition (Tisserant *et al.* 1993). However, the improvement of AM colonization and active fungal metabolism of reference *G. mosseae* when coinoculated with the bacterium apparently did not significantly contribute to increase shoot plant biomass. The selective effect of the autochthonous bacterium in the interaction with each *G. mosseae* strain (more or less metal adapted) has been previously reported (Vivas *et al.* 2003c). Nevertheless, in this study differences in functional compatibilities in the interaction between the bacterium and each *G. mosseae* strain according to their microbial origin were found (Monzón and Azcón 1996). The available data highlight that native Zn-adapted *G. mosseae* has the greatest effectiveness on plant development and the highest potential for new AM colonization particularly when associated with the bacterium.

Plants colonized by reference *G. mosseae* showed the highest SAR for metals as Cd, Cu, Mn or Mo. SAR gives an indication about the rate of metal usage (Koide 1993) and the coinoculation with the bacterium highly decreased SAR values for these metals. Such microbial activity may have physiological relevance on the host plant and may be involved in the positive effects observed on plant performance.

The arbuscular richness (%*a* and %*A*) shows the functioning of the AM symbiosis as it is considered to be the interactive structure link between the plant and the fungus. Plants coinoculated with autochthonous *G. mosseae* and the bacterium seem to be more Zn tolerant (in terms of root growth and N, P nutrition) than single AM-colonized plants. Probably, the bacterial effect in increasing arbuscule content is a mechanism involved in this tolerance.

In general, the enhancement in the level of AM colonization and its metabolic characteristics in coinoculated plants did not strictly determine the inoculum ability to improve plant growth and nutrition in Zn-supplemented medium (particularly in reference *G. mosseae* colonized plants) as proposed by Tisserant *et al.* (1993) and Guillemin *et al.* (1995). This microbial AM fungi plus bacterium interaction was previously observed also under unpolluted conditions (Marulanda *et al.* 2002). In agreement with Smith *et al.* (1994), mycorrhizal efficiency was not closely related to the extent of colonization and no generalization can be made on the correlation between fungal enzyme activities and symbiotic effectiveness as shown here.

In this study, the mycorrhizal helper bacteria (MHB) effect was strongest than the plant growth promoting rhizobacteria (PGPR) effect. Thus, the bacterial effectiveness on AM plants did not seem to be because of mechanisms operating via nutritional status in the plant in spite of its

effect on the physiological and metabolic status of *G. mosseae* (autochthonous or BEG 119). The IAA accumulated in the rhizosphere soil from inoculated plants could suggest that this hormone production by the bacterium could contribute in the mycorrhizae helper effects found (Azcón 1987; Puppi *et al.* 1994). The ability of bacteria to produce auxin may also regulate root formation in mycorrhizal plants as Alvarez *et al.* (1989) suggested. However, in the rhizosphere of the plants colonized by reference *G. mosseae* (BEG 119) the bacterium did not significantly improve IAA in soil or root growth.

Significant changes in enzymatic activities found in the rhizosphere of native *G. mosseae*-colonized plants could be attributed to the greater root development observed. Nevertheless, in reference *G. mosseae* (BEG 119) some activities were higher (dehydrogenase and IAA production) or similar (phosphatase) than in single bacteria treated plants while root growth was greatest in single bacterial inoculated plants.

The bacterium increased all enzymatic activities in soil inoculated with autochthonous *G. mosseae.* Curiously, this inoculated soil ought to be more depleted in available nutrients than the control soil as it supported the highest plant biomass and nutrient acquisition and a lack of nutrients in the medium reduces the microbial populations responsible for such enzymatic activities (Germida and Walley 1996). Thus, the highest enzymatic values in rhizosphere soil from these dually inoculated plants are probably because of the stimulating effect of root exudates (Bowen and Rovira 1999). In the highly Zn-polluted soil used here, plants have a greater necessity for an active soil/plant system and this was achieved by the inoculation of beneficial autochthonous microbes (Hildebrandt *et al.* 1999).

The extraradical mycorrhizal mycelium was presumably affected by the Zn supplied to the experimental soil and by the microbial treatments applied in the RC. In the HC, the sole source of AM inoculum was the mycelium (and eventually spores) developed from the root grown in the RC.

Extraradical mycelium seems to be an important source of AM inoculum under Zn-polluted conditions. Here, both *G. mosseae* sp. showed different abilities to form extraradical mycelium for colonizing the receiver plant.

When the level of AM colonization and its physiological activities are shown in terms of concentration (%), a diluting effect occurs by the enhancement of root growth as observed in donor and receiver plants treated with *Brevibacillus* and autochthonous *G. mossea*.

General data emphasize that bacterial inoculation increased not only AM colonization but also the physiological and metabolic status of both *G. mosseae* irrespective of the ecotype used. Bacterial stimulation had a greater relevance on biological characteristics of the rhizosphere soil than on plant growth and nutrition, as Marulanda *et al.* (2002) and Vivas *et al.* (2003a) also reported. These results confirm previous findings on the beneficial effect of the bacterium and/or mycorrhizae inoculation on plants growing on metal-polluted environments (unpublished results) and extend the information concerning changes in the infectivity and physiological characteristics of the mycelial network generated by each *G. mosseae* ecotype in presence of the selected bacterium. The level of colonization and to the highest extent SDH and ALP, active infection by native *G. mosseae* was also stimulated in receiver plants because of bacterial inoculation of donor plants.

This study demonstrated not only the protective effect of AM fungi and/or bacteria (autochthonous isolates particularly) on *Trifolium* plants growing in Zn-polluted soil but also the diversity in the function and response of the AM symbiosis regarding *G. mosseae* strains Zn adaptation. These results highlight that the most adapted microbial strains (fungi and/or bacteria) tend to cope and to prevail in Zn-contaminated soil and microbial interactions seem to be crucial for plant survival in Zn-amended soil. Further research on microbial phytoremediation effect and on the relationship between vitality and/or activity of AM colonization, the potential of AM inoculum for further colonizations, and how this relationship is affected by biological factors are in progress.

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