ELSEVIER

Soil Biology & Biochemistry 38 (2006) 2694–2704

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Nickel-tolerant *Brevibacillus brevis* and arbuscular mycorrhizal fungus can reduce metal acquisition and nickel toxicity effects in plant growing in nickel supplemented soil

A. Vivas[a], B. Biró[b], T. Németh[b], J.M. Barea[a], R. Azcón[a,*]

[a]*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*
[b]*Laboratory of Rhizobiology, Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Herman O. út 15, H-1022 Budapest, Hungary*

Received 23 February 2005; received in revised form 5 April 2006; accepted 6 April 2006
Available online 11 May 2006

Abstract

The growth of clover (*Trifolium repens*) and its uptake of N, P and Ni were studied following inoculation of soil with *Rhizobium trifolii*, and combinations of two Ni-adapted indigenous bacterial isolates (one of them was *Brevibacillus brevis*) and an arbuscular mycorrhizal (AM) fungus (*Glomus mosseae*). Plant growth was measured in a pot experiment containing soil spiked with 30 (Ni I), 90 (Ni II) or 270 (Ni III) mg kg^{-1} Ni-sulphate (corresponding to 11.7, 27.6 and 65.8 mg kg^{-1} available Ni on a dry soil basis). Single inoculation with the most Ni-tolerant bacterial isolate (*Brevibacillus brevis*) was particularly effective in increasing shoot and root biomass at the three levels of Ni contamination in comparison with the other indigenous bacterial inoculated or control plants. Single colonisation of *G. mosseae* enhanced by 3 fold (Ni I), by 2.4 fold (Ni II) and by 2.2 fold (Ni III) *T. repens* dry weight and P-content of the shoots increased by 9.8 fold (Ni I), by 9.9 fold (Ni II) and by 5.1 fold (Ni III) concomitantly with a reduction in Ni concentration in the shoot compared with non-treated plants. Coinoculation of *G. mosseae* and the Ni-tolerant bacterial strain (*B. brevis*) achieved the highest plant dry biomass (shoot and root) and N and P content and the lowest Ni shoot concentration. Dual inoculation with the most Ni-tolerant autochthonous microorganisms (*B. brevis* and *G. mosseae*) increased shoot and root plant biomass and subtantially reduced the specific absorption rate (defined as the amount of metal absorbed per unit of root biomass) for nickel in comparison with plants grown in soil inoculated only with *G. mosseae. B. brevis* increased nodule number that was highly depressed in Ni I added soil or supressed in Ni II and Ni III supplemented soil. These results suggest that selected bacterial inoculation improved the mycorrhizal benefit in nutrients uptake and in decreasing Ni toxicity. Inoculation of adapted beneficial microorganisms (as autochthonous *B. brevis* and *G. mosseae*) may be used as a tool to enhance plant performance in soil contaminated with Ni.

Keywords: Arbuscular mycorrhizal fungi; *Rhizobium* sp.; *Brevibacillus* sp.; Nickel-tolerance; Phytoremediation

1. Introduction

Soil contamination by heavy metals is an important problem in industrialised and urbanised areas. Symbiose between the arbuscular mycorrhizal (AM) fungi and approximately 80% of higher plants play an important role in enhancing the uptake of phosphorus and micronutrients and also in influencing the translocation of heavy metals and toxic elements in colonised plants (Barea et al., 1987). The interface between microbes and plant root (in the rhizosphere) may have a great influence on both the increase of nutrients uptake and the decrease of the metal toxicity (Shanableh and Karabsheh, 1996; Smith, 1994; Vivas et al., 2003a, b). Mycorrhizal colonisation has been recognised as the key to plant growth and fitness in stressed environments and in the sustainable soil–plant systems (Biró et al., 1993; Köves-Péchy et al., 1999). AM fungi and bacteria isolated from metal-contaminated soils are often more resistant to metals than those collected from uncontaminated environments (Griffioen et al., 1994;

*Corresponding author. Tel.: +34 958 18 16 00x298; fax: +34 958 12 96 00.
E-mail address: rosario.azcon@eez.csic.es (R. Azcón).

0038-0717/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2006.04.020

Leyval et al., 1991; del Val et al., 1999). Also, bacteria isolated from soils subject to long-term sewage sludge application were found to have better metal-tolerance than isolates from the unpolluted soils (Chaudry et al., 1992). Prolonged exposure to heavy metals may lead to a reduced growth rate or to the loss of several beneficial properties, such as the nitrogen-fixing ability in the case of rhizobia (Biró et al., 2001). A delay in the mycorrhizal colonisation in plants growing in contaminated soils was also reported by Koomen et al. (1990).

The effect of metals on microbial biomass and other microbial parameters are often studied in soils with a history of previous sewage sludge application (Koomen et al., 1990; Leyval et al., 1991). Sewage sludge, however, contains a mixture of various metals (Pb, Ni, Zn, Cd), thus the possibility of complex interactions among the metals cannot be excluded and it is difficult to establish the effect of individual metals. To study the basic interactions between heavy metals, plants and microbes, the use of soils containing single metal salts may be more appropriate. This usually involves adding increasing amounts of specific metal salts to the soil to produce elevated concentrations. However, application or 'spiking' of metal salts, creates a situation where the bound and the newly added metal forms may take some time to reach equilibrium with the soil components (Smith, 1994). Thus, long-term field experiments take into account the different locations and equilibria of the metals and are useful to study the effects of specific metals on abundance and function of rhizosphere microorganisms and their interaction with higher plants.

Selected metal tolerant saprophytic and symbiotic microorganisms may play an important role for plant establishment in metal contaminated soils. In the case of the dual symbiosis between AM fungi and some beneficial plant growth promoting rhizobacteria (PGPR), such as the nitrogen-fixing rhizobia, most reports identify a synergistic interaction that contributes significantly to plant tolerance to heavy metals (Biró et al., 2000; Höflich et al., 1993). Moreover, the metal resistance of microsymbionts may induce a resistance to sensitive plants growing under elevated metal concentrations (Kaldorf et al., 1999). Isolation of tolerant beneficial microorganisms from polluted soil that are able to survive and colonise the rhizosphere may enable plants to grow well in contaminated soil. In fact, selection and application of interactive microorganisms is considered as an important strategy in field remediation (Brundrett et al., 1996; Entry et al., 1996). Nevertheless, any metal tolerance or degree of benefit conferred to the plant by AM symbiosis and other PGPR will probably be related to the level of pollution and the metal involved. The plant growth promoting effect of bacteria in the presence of nickel was attributed to the bacterial ability in reducing the detrimental Ni-induced stress in plants (Burd et al., 1998, 2000). The combination of Ni-adapted AM fungal isolates with those tolerant bacteria could increase phytoremediation potential. As well as protecting food quality by limiting the uptake of potentially toxic metals co-inocula composed of resistant strains may improve plant growth and development. This would be crucial in optimising any phytoremediation effects.

The aim of this study is to determine plant-biomass, nutrition and nickel uptake from soil when inoculated with dominant indigenous, Ni metal-tolerant bacterial strains and/or AMF strains isolated from an artificially Ni-contaminated soil.

2. Materials and methods

2.1. Experimental design

The experiment consisted of a three-factor randomised complete block design of (1) bacterial treatment including assays with two indigenous Ni-tolerant bacterial species and one uninoculated control treatment; (2) inoculation with an indigenous Ni-tolerant AM inoculum, including non-inoculated control treatment; (3) three levels of Ni added to the soil (30, 90 or 270 mg Ni $SO_4\,kg^{-1}$). Five replicates were used for each treatment totalling 90 pots.

2.2. Soil characteristics and nickel applications

The soil used in the greenhouse experiment was a calcareous loamy soil (57% sand 22.3% silt and 19% clay) with 1.63% organic matter and a pH(H_2O) of 7.2. The nutrient concentrations ($mg\,kg^{-1}$) were: N (total) 2.1; P (Olsen) 1.7 and K (NH_4extractable) 0.8. The cadmium, nickel and zinc concentration in the soil were: 0.3, 2.6 and 1.3 $mg\,kg^{-1}$, respectively. This soil was selected on the basis of its high similarity (pH, texture, organic matter and nutrients content) to the Ni-contaminated soil from Hungary. The soil was air-dried, sieved to less than 2 mm, mixed with quartz-sand (<1 mm) 4:1 soil:sand (v/v) and sterilised by steaming (100 °C for 1 h on 3 consecutive days). After sterilisation, the soil was supplemented with 30, 90 or 270 $mg\,kg^{-1}$ Ni by adding adequate amounts of an aqueous solution of $NiSO_4$ into three soil subsamples. The soil was left in a greenhouse for a 2-week period of incubation (for metal stabilisation) and then the available amounts of remaining Ni was determined using the method reported by Quevauviller et al. (1997). Those were 11.7, 27.6 and 65.8 mg Ni kg^{-1}, respectively. These three Ni contamination levels were selected to range from a low contamination level (30 mg Ni $SO_4\,kg^{-1}$), a medium contamination level (90 mg Ni $SO_4\,kg^{-1}$) and a high contamination level (270 mg Ni $SO_4\,kg^{-1}$).

2.3. Selection of metal tolerant microbes

The soil samples for microbial inocula production were taken from 10 years prior Ni-treated long-term field experiment in Nagyhörcsök, Hungary (Kádár 1995). It was a calcareous chernosem soil of RISSAC. Strains of the

most dominant bacteria-types and AM fungi were isolated from this Ni-contaminated soil containing the native microbial populations (after application of 270 mg kg^{-1} of $NiSO_4$ solution).

The experimental background of the long-term field experiment in Hungary offered the possibility of isolating different metal tolerant microbes from the Ni-polluted soils. AMF and bacterial populations were isolated from this soil for inocula production. The most dominant ecotypes of the culturable bacteria were selected from this Ni-polluted soils using a conventional serial dilution technique: 1 g of homogenised 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 gL^{-1}) and cultivated for 48 h at 28 °C.

The bacterial strains exibited different colony morphology and were refered to as strains A or B. These were the two most abundant bacterial types (A and B) growing on nutrient broth plates. 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.

Bacterial isolates were cleaned and maintained suitable for the further in vitro and microcosm applications.

Total DNA from the most efficient (in increasing plant growth and in decreasing shoot Ni concentration) bacterial isolate B was obtained and characterised by sequence analysis of the small ribosomal subunit (16S ribosomal DNA) as described in detail in an earlier study (Vivas et al., 2003a). Database searches for 16S rDNA sequence similarity using FASTA and BLAST algorithms (Lane, 1991) unambigenusly identified the selected bacterial isolate as a member of the *Brevibacillus*. The 16S rDNA sequence showed its highest similarity (more than 98%) with *Brevibacillus brevis*.

The bacterial isolates were checked in an additional "in vitro" experiment for testing the metal-tolerant abilities compared to a reference strain. Axenic nutrient broth (8 g L^{-1}) medium added with increasing Ni levels (20, 40, 60 and 80 μg Ni mL^{-1}) was used to study the comparative Ni sensitivity (as c.f.u. mL^{-1} counts) of the bacterial isolates A, B and the reference strain. Cells were grown at 28 °C in an orbital shaker (170 rev min^{-1}). Growth was monitored after 16 h by optical density (OD) at 600 mm in Spectronic 20D spectrophotometer (Spectronic instruments Inc, NY, USA). In pararelle, plates recounts from aliquots of 100 μl were determined (Fig. 1).

The indigenous AM spores were also isolated from this Ni polluted soil with a contamination level of 270 mg $NiSO_4$ kg^{-1} (Nagyhorcsock, Hungary) by wet-sieving and decanting as described by Vilariño and Arines (1990). The fungus was apparently a *Glomus mosseae* strain based on morphological observation. All the spores obtained presented similar characteristics as those of *G. mosseae* having spores of medium size (200 μm), globoses to subgloboses, sometime irregular, yellow colour, forming sporocarps (2–6 spores in each), wall composed of three different layers and funnel-shaped substending hyphae. Therefore, it was concluded that only this *Glomus* species was present in the original Ni-contaminated soil.

Mycorrhizal inoculum was multiplied in an open pot culture of plant (*Allium cepa*). After 6 months of plant growth the shoots were eliminated and the underground 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.

2.4. Microbial inoculation

Mycorrhizal inoculum adapted to the Ni-contamination consisting of soil having spores, mycelia and infected root fragments was used in the microcosm experiment. Ten grams of inoculum was 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} concentrations rates of Ni (as $NiSO_4$) and distributed in pots. Half of the pots were inoculated with the mycorrhizal inoculum containing the Ni-adapted AM inoculum.

Two bacterial strains referred to as bacterium A or bacterium B, exhibiting different colony morphology, were also used for inoculation. Both strains were the most abundant and dominant bacteria in the Ni-treated soil. Bacterial inocula (A or B) were added into half of the AM and non-mycorrhizal pots, as 1 mL per pot (6.5×10^8 cells mL^{-1}) immediately after sowing. All these microbially treated and non-treated pots were further inoculated with 1 mL suspension of the nitrogen-fixing *Rhizobium leguminosarum* bv *trifoli* (10^8 cfu) following standard procedure (Azcón, 1993).

2.5. Plant and growth conditions

Trifolium repens L. plants were grown for 3 months in 200 cm^3 pots in a greenhouse under controlled climatic conditions (18–24 °C, with a 18/6 h light/dark period). Throughout the experiment the plants were fertilised with 10 mL of Hewitt's nutrient solution lacking N and P (Hewitt, 1952) once a week. There were three cuttings performed on the shoot biomass during the 3 months of growth.

2.6. Measurements

After 3 months of growth the dry biomass accumulation of roots and shoots, plant nutrients (N and P), Ni concentrations, and symbiotic (mycorrhizal colonisation and nodulation) values were determined.

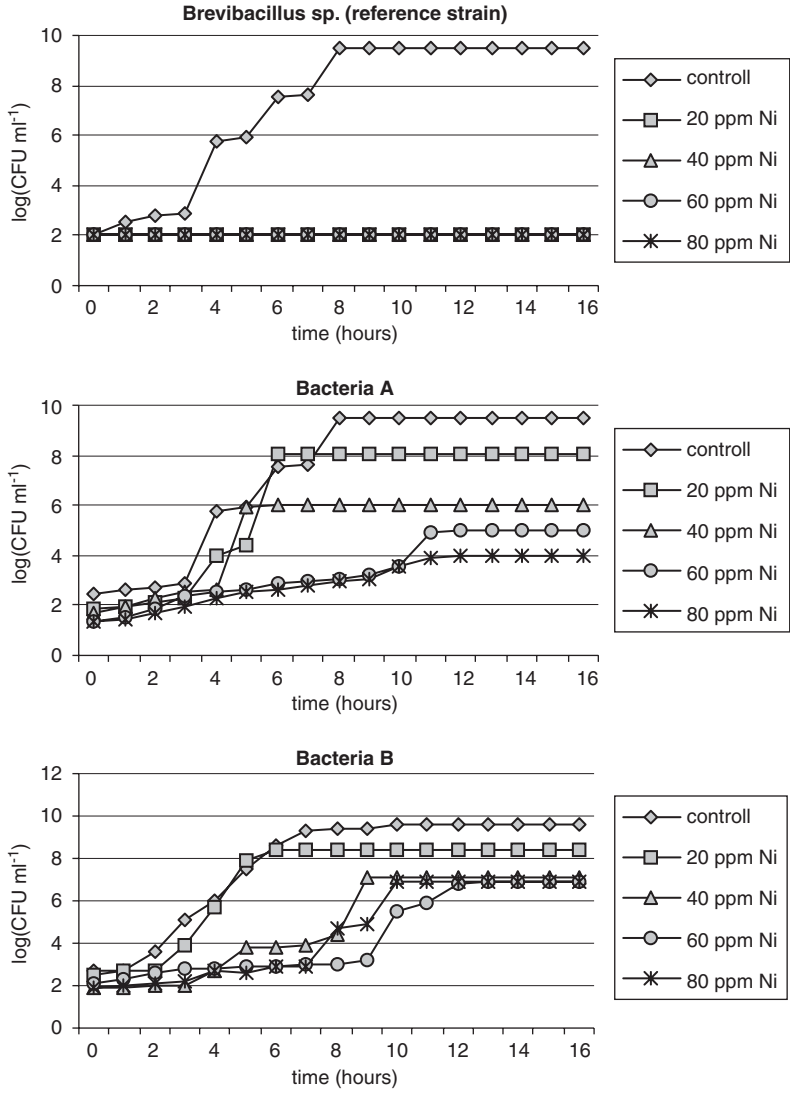

Fig. 1. Number of viable cells (log cfu mL^{-1}) at different time intervals of strain A, strain B (*Brevibacillus brevis*) and the *Brevibacillus* sp. (reference strain) grown in nutrient broth, supplemented with 0 (control), 20, 40, 60 or 80 mg Ni L^{-1}. Data correspond to the average value ($n = 4$).

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

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

Available soil–metal contents were determined using the Quevauviller et al. (1997) method. Shoot contents (mg $plant^{-1}$) of N (micro-Kjeldahl) and P; as well as Ni were 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 (Vörös et al., 2000).

The percentage of mycorrhizal root length infected was estimated by visual observation of fungal colonisation 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 numbers were estimated by direct observation using a binocular microscope. Five replicates were performed per treatment.

2.7. Statistical analysis

For each Ni 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

3.1. Plant growth as a function of microbial inoculations

The dual treatment of the native mycorrhizal fungus with the native bacteria *B. brevis* was the most effective treatment at all Ni levels (Fig. 2). As well, single application of *B. brevis G. mosseae* inocula also increased shoot and root biomass under low, medium or high Ni level in the soil (Fig. 2). Dual inoculation of *G. mosseae* plus bacterium A was a less effective treatment (Ni I) or similar (Ni II or Ni III) in increasing shoot and root biomass compared to single mycorrhizal inoculum. In contrast, the inoculation of *B. brevis* increased shoot biomass of mycorrhizal plants at all Ni levels in the medium (Fig. 2). These growth responses ranged from 12% (Ni I) untill 33% (Ni III). Therefore, as affected by the dual *G. mosseae* plus *B. brevis* inoculation, shoot and root plant biomass appeared to be about three-times greater than in non-inoculated plants independently of the Ni levels (Fig. 2).

3.2. The effect of microbial inoculations on the symbiotic developments

As the amount of available Ni in soil increased, the symbiotic structures (nodule number and AM infection) generally decreased. The nodule number, however, seems to be more affected by the Ni concentrations compared to the mycorrhizal colonisation. Nevertheless, significant differences in the length of root AM-colonised between the lowest and the highest Ni levels in the soils were found (Fig. 3).

The beneficial effect of the inoculation of *B. brevis* on the extent of AM colonisation was observed at the three levels of Ni contamination (Fig. 3). This bacterium, increased the AM colonisation by 58% (Ni I), by 60% (Ni II) and by 34% (Ni III). *B. brevis* increased the total length of AM-colonised roots by about two-times or even more over single inoculated mycorrhizal plants.

The number of nodules formed was increased by the mycorrhizal colonisation, at all Ni levels. Increasing Ni content in soil (or plant) reduced the nodule number which dropped to zero in non-mycorrhizal plants when the soil was supplied with 270 mg Ni kg^{-1}. The beneficial effect of *B. brevis* on nodule-formation was only observed at the lowest Ni level assayed (11.7 mg Ni kg^{-1}) (Fig. 3). Plants inoculated with *G. mosseae* plus *B. brevis* produced the highest number of nodule irrespectively of Ni level (Fig. 3).

In this study nodule number were reduced as much as Ni contamination increased in soil. Nodule were suppressed in non-AM inoculated control plants (at the available Ni level of 65.8 mg kg^{-1}) but this detrimental effect of Ni was overcome by the mycorrhizal inoculum applied. The inoculation of *B. brevis* raised the number of nodules up to 20 at the lowest Ni level (11.7 mg Ni kg^{-1}) and up to 11

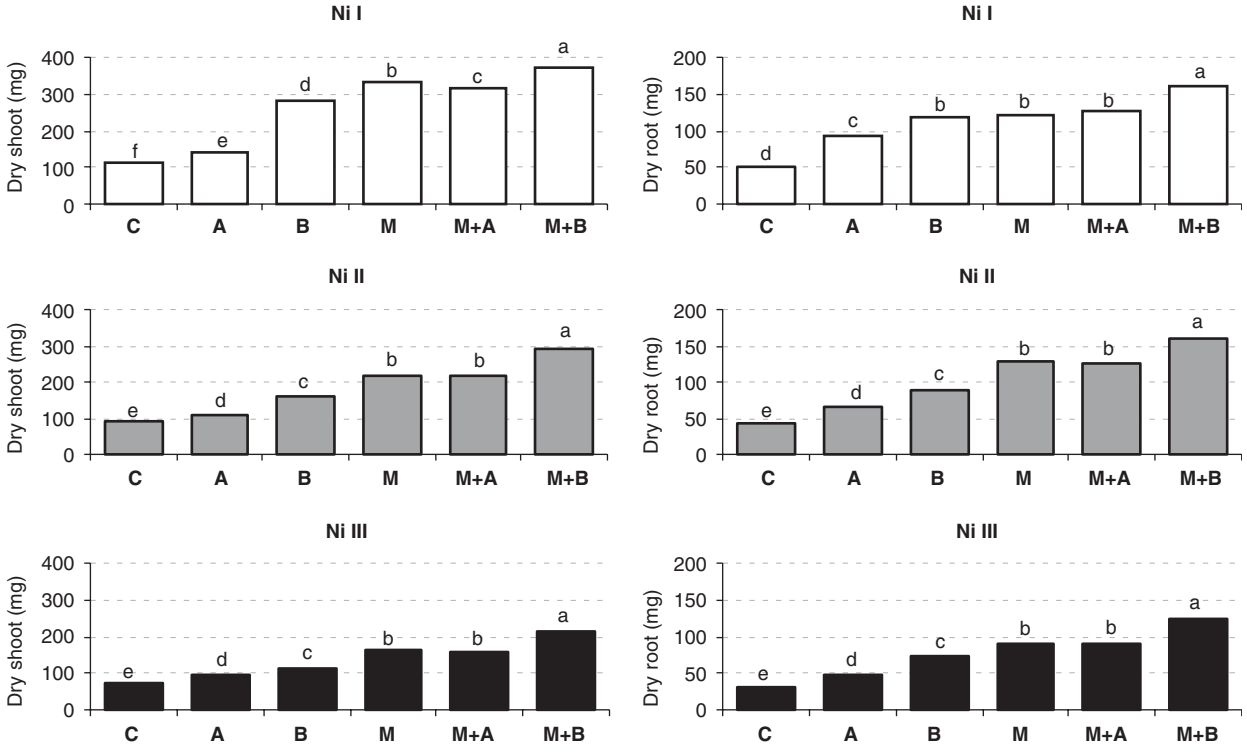

Fig. 2. Shoot and root dry weights (mg $plant^{-1}$) of red clover plants grown in soil amended with 30 (Ni I), 90 (Ni II) or 270 mg $NiSO_4$ Kg^{-1} (Ni III). Treatments are: C (Control), A (bacterium A), *Brevibacillus brevis* (bacterium B), M (indigenous mycorrhizal inoculum), M + A, M + B (Mycorrhizae + bacterium A or B). Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

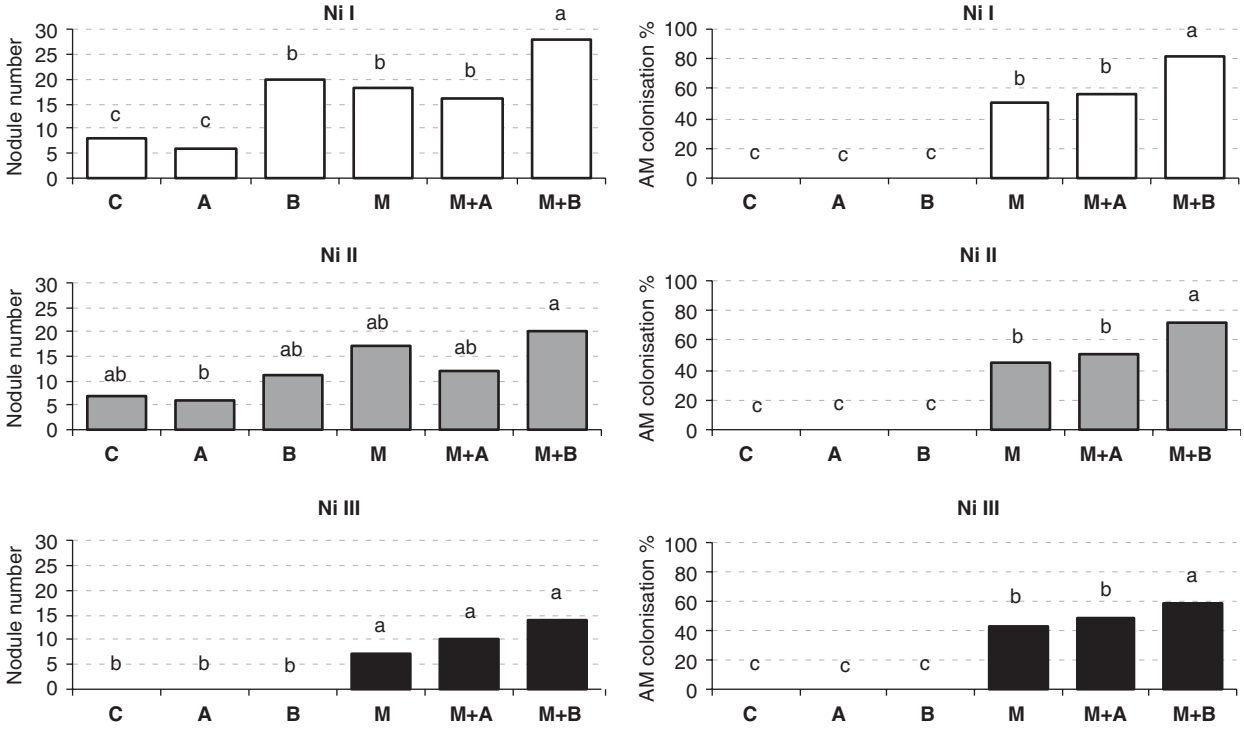

Fig. 3. Nodule numbers and percentage of mycorrhizal root length in red clover grown in soil amended with 30 (Ni I), 90 (Ni II) or 270 mg Ni SO_4 Kg^{-1} (Ni III). Treatments are designed as in Fig. 2. Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

at medium Ni level (27.6 mg Ni kg^{-1}) but at the highest Ni level (65.8 mg Ni kg^{-1}) the nodule were completely suppressed in the absence of AM-colonisation (Fig. 3). Under this high Ni level, nodule number in *G. mosseae* colonised plants were comparable to that found in non-inoculated control plants at the lowest Ni level assayed.

3.3. Plant macroelement (N, P) uptake

Shoot N and P concentration were differently affected by the microbial treatments applied at each level of Ni in soil. Concomitant with the effect of microbial inoculations in increasing P concentration, the N concentration in clover shoot was lowered in inoculated plants except by the single application of bacterium A at the lowest Ni level (30 mg kg^{-1}) applied (Fig. 4). *B. brevis* was as effective (Ni II) or more effective (Ni I) than *G. mosseae* colonisation in increasing P concentration in shoot.

Results show the great effectiveness of AM colonisation on P uptake by clover in Ni-contaminated soil (Fig. 4).

3.4. Metal uptake

Concentrations of Ni in *Trifolium* shoot were substantially reduced by the applied microbial treatments in all Ni levels in soil (Fig. 5). At the lowest available amount of Ni in soil (11.7 mg kg^{-1}) the single inoculation of bacterium A, was not effective in decreasing Ni concentration in the plant shoot but at the two higher Ni levels (27.6 or 65.8 mg Ni kg^{-1}) this bacterial strain, as the rest of applied treatments, significantly decreased Ni concentration in *Trifolium* shoot. The sensitivity of each native bacterium to Ni was different. Autochthonous isolate of *B. brevis* was the most effective in decreasing shoot Ni concentration at whatever Ni level assayed (Fig. 5).

As well, mycorrhizal colonisation by native *G. mosseae* significantly reduced Ni shoot concentration at all Ni level. Its effectiveness was similar to that observed for *B. brevis*. But this effect was improved by the coinoculation of these microorganisms that reduced plant Ni concentrations by 4.9 (Ni I), by 6.4 (Ni II), and by 6.0 (Ni III) fold compared with non-treated control plants (Fig. 5).

Comparatively, Ni concentrations in non-inoculated plants growing in soil applied with 30 mg Ni SO_4 kg^{-1} resulted similar as these found in the dual (*G. mosseae* plus *B. brevis*) inoculated plants growing in soil applied with 270 mg Ni SO_4 kg^{-1}. This result indicates that the accumulation of Ni in *Trifolium* shoot depended not only on the Ni level in soil but also on microbial interactions colonising root and the rhizosphere.

An important result from this investigation is that in coinoculated plants with native microorganisms the Ni acquisition was strongly reduced (Table 1). In fact, dual inoculum (*G. mosseae* and *B. brevis*) isolated from Ni-contaminated soil, were able to inmobilise Ni allowing plant development under Ni-polluted conditions.

SAR for Ni decreased in AM-colonised plants and such value was affected in a highest extent by the inoculation of

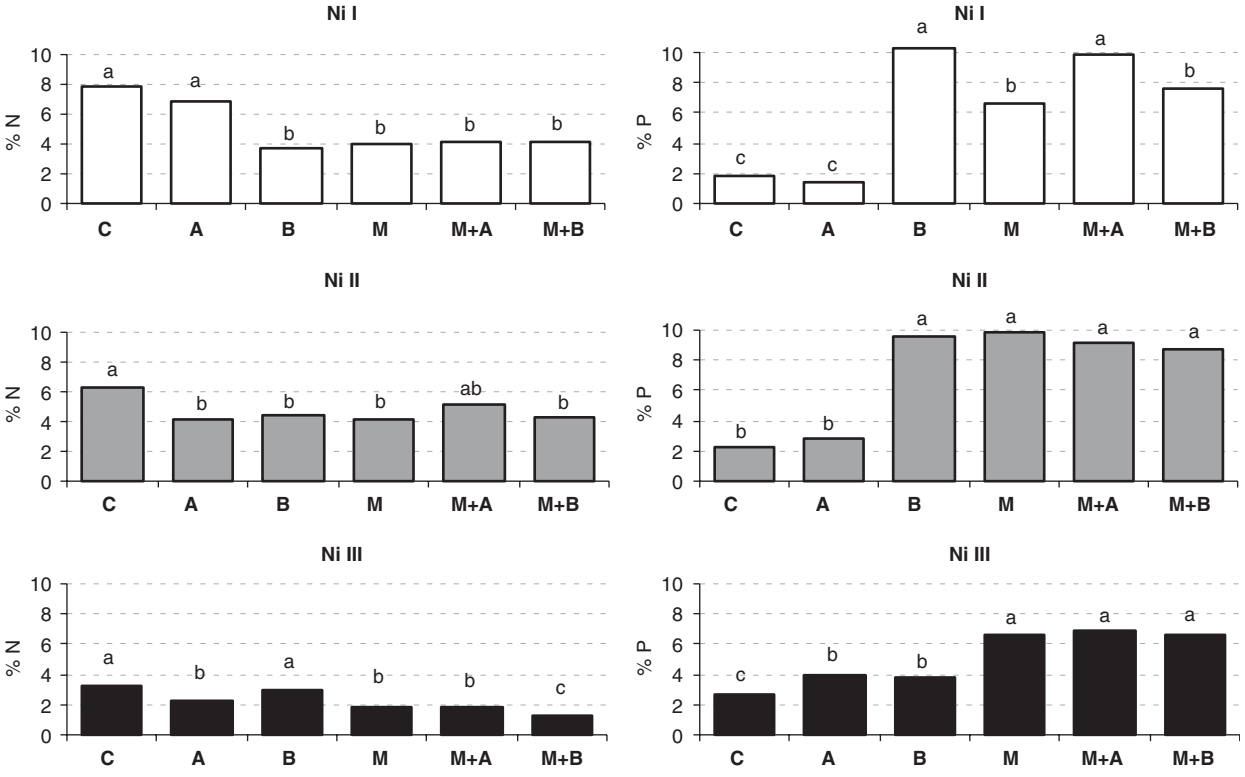

Fig. 4. N and P concentration in red clover plants grown in soil amended with 30 (Ni I), 90 (Ni II) or 270 mg Ni SO_4 Kg^{-1} (Ni III). Treatments are designed as in Fig. 1. Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

B. brevis particularly at the highest available Ni in soil (Table 1). In fact, Ni absorbed per unit of root was affected by the microbial treatments applied. Shoot Ni acquisition per unit of root was several folds lower in treated plants (Table 2). This effect was maximised in dual *G. mosseae* plus *B. brevis* colonised plants and became strongest as the available Ni in soil increased. The SAR values for Ni always increases as Ni availability in soil increases. Major differences in SAR between treatments were noted when Ni increased from 11.7 to 65.8 mg kg^{-1}. Microbial treatments applied decline SAR values for Ni being maximum in control plants. Bacterial inoculation, in particular *B. brevis*, reduced SAR values.

In addition, the ratio of total Ni/P content in shoots was affected by the amount of available Ni in soil and depended on microbial treatments applied (Table 2). Dually *B. brevis* plus *G. mosseae* inoculated plants were highly effective lowering this ratio.

3.5. Bacterial Ni-tolerance

The most effective bacterial strain was taxonomically characterised as *B. brevis*. The less Ni tolerant bacterium A, with a lower effectiveness on plant growth than *B. brevis*, under Ni-polluted soil, was not identified.

Bacterial isolates were grown in nutrient broth at increasing Ni concentrations, ranging from 0 mg Ni L^{-1} in the control treatment to 80 mg Ni L^{-1} (Fig. 1). The growth of bacterium A decreased concomitantly with an increase of Ni in the medium. However, *B. brevis* exhibited a higher tolerance to Ni than bacterium A. At low Ni level in the medium (20 mg L^{-1}) both bacteria reached 10^8 cfu mL^{-1} after 6 h of growth. Nevertheless *B. brevis* produce 10^7 cfu mL^{-1} while strain A only reached 10^6 cfu mL^{-1} in medium added of 80 mg Ni L^{-1}.

B. brevis showed a higher tolerance to Ni than bacterium A and than the reference *Brevibacillus* sp. strain (Fig. 1). In fact, at 60 and 80 mg Ni L^{-1}, the highest Ni amounts in the growing medium used, *B. brevis* reached 10^7 cfu mL^{-1} after 10 h, while bacterium A, under such Ni concentrations, showed a lower growth. At the lowest Ni amount in the medium (from 0 to 20 μg mL^{-1}) both bacterial isolates showed a similar growth rate. The reference *Brevibacillus* sp. resulted highly sensitive at whatever Ni concentration used. Although the growth intensity of the reference strain after 8 h of growth was similar to that of the metal-tolerant isolates in the metal-free culture medium this reference Ni non-adapted bacterium strain proved to be highly sensitive to the applied Ni-levels since Ni added to the culture medium inhibited its growth severely (Fig. 1).

4. Discussion

The aim of this study was to investigate the effect of basic interactions between Ni contamination and certain beneficial rhizosphere microbes adapted to the Ni-polluted

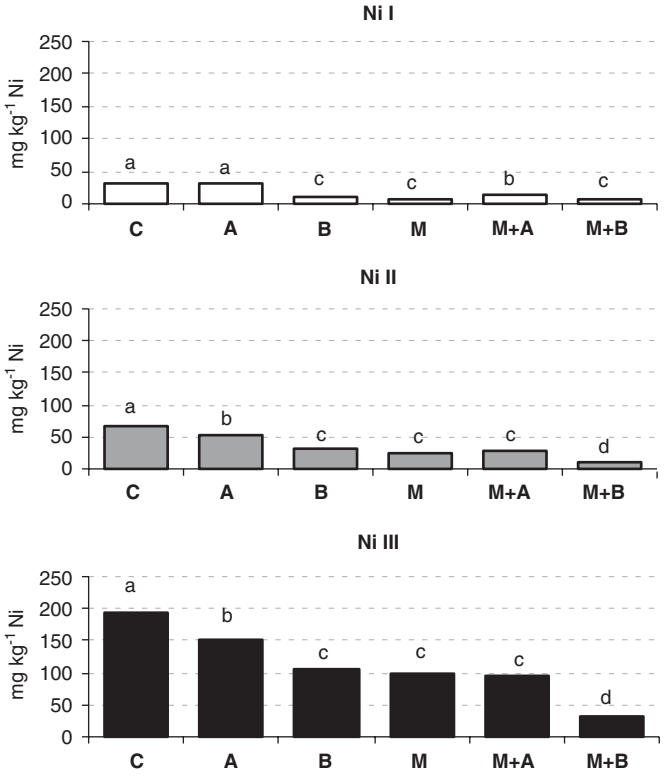

Fig. 5. Ni concentration in red clover plants grown in soil amended with 30 (Ni I), 90 (Ni II) or 270 mg Ni SO_4 kg^{-1} (Ni III). Treatments are designed as in Fig. 1. Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

Table 1
Effect of native bacteria A or B (*Brevibacillus brevis*) and/or native AM inoculum M (*Glomus mosseae*) on specific absorption rate (SAR) values for Ni in plants growing under increasing available Ni in soil

Microbial treatments	Available Ni (mg kg^{-1})		
	11.7	27.6	65.8
C	0.070a	0.139a	0.450a
A	0.046b	0.092b	0.310b
B	0.022cd	0.067c	0.166c
M	0.022cd	0.040d	0.177c
M + A	0.032c	0.047d	0.166c
M + B	0.015f	0.018f	0.056d

Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

conditions on plant growth and nutrition. Although Ni is in general known as an essential microelement (Pais, 1992), the excessive concentrations of this and other metals in soils are toxic to plants, bacteria and fungi. Thus, elevated Ni levels might reduce AM-root-colonisation even at levels which did not yet affect plant growth (Chao and Wang, 1990). These evidences suggest that microorganisms are far more sensitive to heavy metal stress than plants growing on the same soils.

Table 2
Effect of native bacteria A or B (*Brevibacillus brevis*) and/or native AM inoculum M (*Glomus mosseae*) Ni/P ratio in shoot of plants growing under increasing available Ni in soil

Microbial treatments	Available Ni (mg kg^{-1})		
	11.7	27.6	65.8
C	17.60a	29.90a	58.30a
A	21.00a	19.30b	34.90b
B	0.93c	3.25c	19.80c
M	1.20b	2.43d	10.90d
M + A	1.31b	3.04c	10.00d
M + B	0.87c	1.17f	3.66f

Within each Ni level, columns having a common letter are not significantly ($P = 0.05$) different as determined by Duncan's Multiple Range Test ($n = 5$).

Metal-adapted microorganisms appear to have several strategies (such as the low sensitivity to metals) that allow them to colonise soils polluted with heavy metals (Meharg and Cairney, 2000). In this study, Ni resistant bacteria and an AM fungus were able to grow at high Ni concentrations in soil and in culture medium (bacteria). However, different growth rate and activity was observed between each one of the selected autochthonous bacterial isolates although both of them were found to be the most abundant ecotype in the Ni-spiked soil. According to the viable cell-counts from each bacterial strain determined along time at increasing Ni levels in the culture media, bacterium A proved to be more sensitive than *B. brevis* to the highest Ni levels applied (60 and 80 mg kg^{-1}). Similarly, *B. brevis* affected growth and nutrition of plants growing in Ni-polluted soils more successfully than bacterium A. Plant development on Ni-amended soil showed to be dependent upon the activity of selected microbial population in the rhizosphere as reported by Shetty et al. (1994) and Garbaye (1994). No previous information on the influence of native bacterium associated with native AM fungi on plant tolerance to Ni pollution has been reported. In this study, the formation of symbiotic associations (AM infection and nodulation) decreased as available Ni in the soil increased but the beneficial effect of the symbionts (*G. mosseae* and *Rhizobium*) was not decreased in the highest Ni-polluted soil. On the contrary, under the more stressed Ni conditions, the AM symbiosis resulted more effective in enhancing growth values and in decreasing Ni acquisition particularly when associated with *B. brevis*. This study showed that at whatever Ni level supplied to the soil, inoculated plants have a decreased amount of Ni absorbed per unit of root mass. A retention of this metal in the root zone would result in a lower amount transported to shoot as evidenced in inoculated plants.

Simon et al. (2001) reported the potential importance of Ni availability on toxicity to plants. But regarding results here presented the detrimental effect of Ni could be reduced by the interactive effect of selected beneficial microbes in the soil–plant system. When roots of the plants

growing in Ni-polluted soils were mycorrhizal-colonised this symbiosis reduced Ni uptake per mg of root and this decreased SAR for Ni seems to be the main effect involved in the improved plant biomass in inoculated plants. Microbial coinoculation compensates the detrimental effect of Ni contamination. The interactive effect was more efficient when appling preselected metal-tolerant bacteria (Nicks and Chambers, 1998; Biró et al., 1993). It is likely that the microbial strains isolated from contaminated soils are more tolerant to metals and have developed resistance (Chaudry et al., 1992; Takács et al., 2000).

Soil pollution results in a progressive degradation of vegetation cover and also soil quality by change of microbial activities (Mikanová et al., 2001). Our results showed that mycorrhizal colonisation increased essential nutrients (in plants), such as phosphorus but decreased Ni concentration and produced higher root and shoot biomass than non-mycorrhizal plants. This beneficial mycorrhizal effect was further enhanced by *B. brevis*.

The survival of applied autochthonous bacterial inocula was not analysed here. But regarding previous information on their colonising abilities in *Trifolium* rhizosphere soil, under similar growth conditions, no differences between native strains were found. These results suggested that the particular effectiveness of each native bacterial strain, as inocula, was not linked to its survival characteristics in this contaminated soil.

Results from this study provide evidence about the effectiveness of the inoculation of adapted AM fungus (*G. mosseae)* and rhizosphere bacteria as *Brevibacillus* strain in decreasing Ni concentration and uptake but this microbial activity changed depending on available Ni in soil. In addition, these treatments increased P uptake by *Trifolium* plants by more than ten times (Ni I and Ni II). These results are consistent with reports suggesting the beneficial use of bacteria or mycorrhizal colonisation in plants growing in metal contaminated sites (Shetty et al., 1994).

Weissenhorn et al. (1994) established that AM fungi and bacteria can be constitutively or adaptatively resistant to high metal concentrations. Here, differences in Ni adaptation and effect between the two bacterial isolates were found. At the highest Ni concentrations used, the Ni resistant *B. brevis* grew better than bacterium A. Garbaye (1994) and Turnau and Mesjasz-Przybylovicz (2003) suggested that amelioration of pollution stress tolerance by the inoculation of microbial groups may be due to specific physiological and nutritional effects depending on the plant and microbial groups associated.

N_2-fixation by *Rhizobium* is one of the most important biological processes but highly sensitive to metal contamination as it was determined at the highest Ni level used. Similarly, mycorrhizal colonisation was also reduced by increasing Ni levels. By that, an important role of microbial inoculation on Ni-polluted soils observed is the ability to improve symbiotic associations as AM colonisation and rhizobial nodulation in legume plants.

The factors involved in improving nodule-formation under such stress conditions may be a combination of effects as the supply of available P to nodule which is critical for legume-development in polluted habitats. The bacteria inoculation could, in addition, facilitate the plant root development by altering the hormonal balance (Burd et al., 2000). Thus, mycorrhizal-colonisation particularly in *B. brevis* inoculated plants was very effective in increasing nodulation at whatever Ni level. The knowledge of basic interactions between Ni level, plant species, soil type and microbial groups is still limited.

According to Charudhry et al. (1998), Ni was among the most toxic metals to *R. leguminosarum* bv *trifoli.* The reduction in nodule size and nitrogenase activity were also determined in white clover grown in metal-polluted soils (Märtensson, 1992). In the present study, this detrimental effect of Ni on nodulation was determined up to $27.6\,mg\,Ni\,kg^{-1}$ in non-inoculated plants.

Differences in the Ni plant acquisition by the coinoculation with each one of the two bacterial isolates according to Ni level assayed can be related to their specific Ni sensitivity and/or the different compatibility between each bacterial strain and *G. mosseae* or *Rhizobium*. Microbial compatibilities also may be involved in the different effectiveness found under these Ni-polluted conditions used.

Mycorrhizal root development when associated with *B. brevis* was less reduced by Ni stress conditions. This fact could be particularly relevant for improving plant growth and nutrient acquisition. Different strategies might be involved in preventing plant toxicity damage. Changes in metal uptake and/or internal transportation storage can confer metal tolerance to the host plant (Hildebrandt et al., 1999; Scholeske et al., 2004) as was also determined in the present study. The microbial inocula here used (AM inoculum and/or bacteria) seem to confer tolerance to Ni not only by modifying specific plant physiological processes (Burd et al., 2000) but also by affecting metals availability and uptake. Changes in root exudates, pH and physico-chemical properties of the soil (Grichko et al., 2000) may be involved and such changes could reduce metal root uptake or translocation from root to shoot tissue. The benefit that legume plants obtain from the AM symbiosis under Ni-polluted conditions are more relevant in coinoculation with *B. brevis*.

A decrease in the relative uptake of Ni was described for species of the *Alyssum* genus as much as the soil was contaminated (de Varennes et al., 1996).

Differences in the effect of bacterial strains and/or mycorrhiza on Ni soil/plant transfer at the three levels of available Ni in soil suggest not only the particular sensitivities of each strain to the Ni amount in the soil but also the highest effectiveness of dual inoculations reducing such value (Appane et al., 1996). A consequence, particular compatibilities was found at each Ni level between each one of these bacteria with symbiotic microorganisms involved. In addition, the intensification

of the AM colonisation (as affected by the bacterium) has been suggested as a strategy for decreasing heavy metal uptake by the host (Hildebrandt et al., 1999). In this and previous studies (Vivas et al., 2003a, b), AM-colonised roots might have reduced the total plant uptake of heavy metals. Both, the metal tolerant mycorrhizal inoculum of *G. mosseae* and *B. brevis* protect the plants against the toxic effect of excessive concentration of Ni by reducing Ni concentration in plant tissues. The microbial associations assayed here may potentially affect the uptake of Ni in different ways depending on combination of factors such as via a hyphal quelation, or sequestation in the vacuolar membran vesicles (Nishimura et al., 1998). Similarly, the bacterial isolates can act inmobilising metal by a direct mechanism as demonstrated for Cd (Vivas et al., 2005) and/or as mycorrhizae helping organisms improving extraradical biomass of the AM fungus (Garbaye, 1994; Vivas et al., 2003c).

The current research in biotechnology includes investigations that use plants to facilitate remediation (Vosatka et al., 1999). According to the results presented here, microbial treatments plants acted in reducing Ni concentration thus we recommend the inoculation of specific (tolerant) beneficial microbial populations cooperating in a coordinated way (compatible symbiotic and saprophyte strains) as a biotechnological procedure of major interest in soil-recuperation strategies (Chaudry et al., 1992).

Knowledge of the mechanisms involved in how mycorrhizal fungi and the bacteria alleviate heavy metal toxicity in plants growing in contaminated environments will allow the management of these microbial groups with suitable characteristics to be used in bioremediation purposes.

Acknowledgements

A. Vivas wants to thank the Fundación Gran Mariscal de Ayacucho (Venezuela) for the scholarship. The project was further supported by the CSIC-HAS bilateral agreement, the Hung. National Research Fund (OTKA T046610) and the COST Action 8.38 and the EU-fp 5 QLK (Mycorem) program, which are highly acknowledged. Thanks for the long-term experimental background to I. Kádár (RISSAC, Budapest).

References

Appane, V.D., Gazsó, L.G., St. Pierre, M., 1996. Multiple metal tolerance in *Pseudomonas fluorescens* and its biotechnological significance. Journal of Biotechnology 52, 75–80.

Azcón, R., 1993. Growth and nutrition of nodulated mycorrhizal and non-mycorrhizal *Hedysarum coronarium* as a results of treatments with fractions from a plant growth-promoting rhizobacteria. Soil Biology & Biochemistry 25, 1037–1042.

Barea, J.M., Azcón-Aguilar, C., Azcón, R., 1987. VA mycorrhiza improve both symbiotic N_2-fixation and N uptake from soil as assessed with a ^{15}N technique under field conditions. New Phytologist 106, 717–721.

Biró, B., Vörös, I., Köves-Péchy, K., Szegi, J., 1993. Symbiont effect of *Rhizobium* bacteria and VAM fungi on *Pisum sativum* in recultivated mine spoils. Geomicrobiology Journal 11, 275–284.

Biró, B., Köves-Péchy, K., Vörös, I., Takács, T., Eggenberg, P., Strasser, R.J., 2000. Interrelation between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa at sterile, AMF-free or normal soil conditions. Journal of Applied Soil Ecology 15, 159–168.

Biró, B., Tiricz, H., Morvai, B., 2001. Investigations on the vitality, resistance and diversity of metal-adapted and non-adapted *Rhizobium* strains. Acta Microbiologica et Immunologica Hungarica 48, 156–157.

Brundrett, M., Bougher, N., Dell, B., Gove, T., Malajczuk, N., 1996. Working with Mycorrhizas in Forestry and Agriculture. Australian Centre for International Agricultural Research, Canberra, Australia, (374pp.).

Burd, G, Dixon, D.G., Glick, B.R., 1998. A plantgrowth-promoting bacterium that decreases nickel toxicity in seedlings. Applied and Environmental Microbiology 64, 3663–3668.

Burd, I.G., Dixon, D.G., Glick, B.R., 2000. Plant growth promoting bacteria that decrease heavy metal toxicity in plants. Canadian Journal of Microbiology 46, 237–245.

Chao, C.C., Wang, Y.P., 1990. Effects of heavy metals on the infection of vesicular arbuscular mycorrhizae and the growth of maize. Journal of Agricultural Association of China 153, 34–45.

Charudhry, T.M., Hayes, W.J., Khan, A.G., Khoo, C.S., 1998. Phytoremediation—focusing on accumulator plants that remediate metal-contaminated soils. Australian Journal Ecotoxicology 4, 37–51.

Chaudry, A., McGrath, S.P., Giller, K.E., 1992. Metal tolerance of isolates of *Rhizobium leguminosarum* bv. *trifolii* from soil contaminated by past applications of sewage sludge. Soil Biology & Biochemistry 24, 83–88.

de Varennes, A., Torres, M.O., Coutinho, J.F., Rocha, M.M.G.S., Neto, M.M.P.M., 1996. Effects of heavy metals on the growth and mineral composition of a nickel hyperaccumulator. Journal of Plant Nutrition 19, 669–676.

Entry, J.A., Reeves, D.W., Mudd, E., Lee, W.J., Guertal, E., Raper, R.L., 1996. Influence of compaction from wheel traffic and tillage on arbuscular mycorrhizae infection and nutrient uptake by *Zea mays*. Plant and Soil 180, 139–146.

Garbaye, J., 1994. Helper bacteria, a new dimension to the mycorrhizal symbiosis. New Phytologist 128, 197–210.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytologist 84, 489–500.

Gray, J.T., Schlesinger, W.H., 1983. Nutrient use by evergreen and deciduous shrubs in Southern-California. 2. Experimental investigations of the relationship between growth, nitrogen uptake and nitrogen availability. Journal of Ecology 71, 43–56.

Grichko, V.P., Filby, B., Glick, B.R., 2000. Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb and Zn. Journal of Biotechnology 81, 45–53.

Griffioen, W.A.J., Ietswaart, J.H., Ernst, W.H.O., 1994. Mycorrhizal infection of an *Agrostis capillaris* population on a copper contaminated soil. Plant and Soil 158, 83–89.

Hewitt, E.J., 1952. Sand and Water Culture Methods Used in the Study of Plant Nutrition. Technical Communication No. 22. Commonwealth Agricultural Bureau, Bucks, UK (p. 241).

Hildebrandt, U., Kaldorf, M., Bothe, H., 1999. The zinc violet and its colonisation by arbuscular mycorrhizal fungi. Journal of Plant Physiology 154, 709–717.

Höflich, G., Glante, F., Kühn, G, Hickisch, B., 1993. Phyto-effective symbiosis in pea. Zentralblatt fur Mikrobiologie 148, 48–54.

Kádár, I., 1995. Contamination of the Soil–Plant–Animal–Man Foodchein by Chemical Elements in Hungary. Ministry of Environmental Protection and Land Management, Budapest (in Hungarian).

Kaldorf, M., Kuhn, A.J., Schröder, W.H., Hildebrandt, U., Bothe, H., 1999. Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. Journal of Plant Physiology 154, 718–728.

Koomen, I., McGrath, S.P., Giller, K., 1990. Mycorrhizal infection of clover is delayed in soils contaminated with heavy metals from

past sewage sludge applications. Soil Biology & Biochemistry 22, 871–873.

Köves-Péchy, K., Biró, B., Vörös, I., Takács, T., Osztoics, E., Strasser, R.J., 1999. Enhanced activity of microsymbiont-host systems probed by the OJIP test. In: Garab, G. (Ed.), Photosynthesis, Mechanisms and Effects. In: Proceedings of the XIth International Congress on Photosynthesis, Budapest, Hungary, August 17–22. Kluwer Academic Publishers, Dordrecht, pp. 2765–2770.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Wiley, New York, pp. 115–147.

Leyval, C., Berthelin, J., Schöntz, D., Weissenhorn, I., Morel, J.L., 1991. Influence of endomycorrhizas on maize uptake or Pb Cu, Zn and Cd applied as mineral salts or sewage sludge. In: Farmer, J.G. (Ed.), Heavy Metals in the Environment. CEP Consultants, Edinburgh, pp. 204–207.

Märtensson, A.M., 1992. Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. Soil Biology & Biochemistry 24, 435–445.

Meharg, A.A., Cairney, J.W.G., 2000. Co-evolution of mycorrhizal symbionts and their hosts to metal-contaminated environments. Advances Ecological Research 30, 69–112.

Mikanová, O., Kubát, J., Mikhailovskaya, N., Vörös, I., Biró, B., 2001. Influence of heavy metal pollution on some biological parameters in the alluvium of the Litavka river. Rostlinna Vyroba 47, 117–122.

Nicks, L., Chambers, M.F., 1998. A pioneering study of the potential of phytomining for nickel. In: Brooks, R.R. (Ed.), Plants that Hyperaccumulate Heavy Metals. CAB, Wallingford, UK, pp. 313–326.

Nishimura, K., Igarashi, K., Kakinuma, Y., 1998. Protongradient-driven nickel uptake by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. Journal of Bacteriology 180, 1962–1964.

Pais, I., 1992. Criteria of essentiality, beneficiality and toxicity. What is too little and too much? In: Pais, I. (Ed.), IGBP Symposium of the Hungarian Academy of Sciences "Cycling of nutritive elements in geo- and biosphere". Univ. Hort. Food Ind., Budapest, Hungary, pp. 59–77.

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society 55, 159–161.

Quevauviller, P., Rauret, G., Rubio, R., López-Sánchez, J.-F., Ure, A., Bacon, J., Muntau, H., 1997. Certified reference materials for the quality control of EDTA—and acetic acid—extractable contents of trace elements in sewage sludge amend soils (CRMs 483 and 484). Fresenius Journal of Analytical Chemistry 357, 611–618.

Scholeske, S., Maetz, M., Schneider, T., Hildebrandt, U., Bothe, H., Povh, B., 2004. element distribution in mycorrhizal and non-mycorrhizal roots of the halophyte *Aster tripolium* determined by proton induced X-ray emission. Protoplasma 223, 183–189.

Shanableh, A., Karabsheh, A., 1996. Stabilisation of Cd, Ni and Pb in soil using natural zeolite. Journal of Hazardous Materials 45, 207–217.

Shetty, K.G., Hetrick, B.A.D., Figge, D.A.H., Schwab, A.P., 1994. Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environmental Pollution 86, 181–188.

Simon, L., Szegvári, I., Prokisch, J., 2001. Enhancement of chromium phytoextraction capacity in fodder radisch with pycolinic acid. Environmental Geochemistry and Health 23, 313–316.

Smith, S.R., 1994. Effect of soil pH on availability to crops of metals in sewage sludge treated soils. I. Nickel, copper and zinc uptake and toxicity to ryegrass. Environmental Pollution 85, 321–327.

Takács, T., Biró, B., Vörös, I., 2000. Cd, Ni and Zn affecting the species diversity of arbuscular mycorrhizal fungi in Hungarian. Agrokemia es Talajtan 49, 465–478.

Turnau, K., Mesjasz-Przybylovicz, J., 2003. Arbuscular mycorrhiza of *Berkheya coddii* and other Ni-hyperaccumulating members of *Asteraceae* from ultramafic soils in South Africa. Mycorrhiza 13, 185–190.

del Val, C., Barea, J.M., Azcón-Aguilar, C., 1999. Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage-sludge contaminated soils. Applied Soil Ecology 11, 261–269.

Vilariño, A., Arines, J., 1990. An instrumental modification of Gerdeman and Nicolson's method for extracting VAM fungal spores from soil samples. Plant and Soil 121, 211–215.

Vivas, A., Azcón, R., Biró, B., Barea, J.M., Ruiz-Lozano, J.M., 2003a. Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Canadian Journal of Botany 49, 577–588.

Vivas, A., Vörös, I., Biró, B., Campos, E., Barea, J.M., Azcón, R., 2003b. Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus *G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels. Applied of Soil Ecology 24, 177–186.

Vivas, A., Marulanda, A., Gómez, M., Barea, J.M., Azcón, R., 2003c. Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels. Soil Biology & Biochemistry 35, 987–996.

Vivas, A., Barea, J.M., Azcón, R., 2005. *Brevibacillus brevis* isolated from cadmium- or zinc-contaminated soils improves in vitro spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations. Microbial Ecology 49, 416–424.

Vosatka, M., Jansa, J., Regvar, M., Sramek, F., Malcova, R., 1999. Inoculation with mycorrhizal fungi—a feasible biotechnology for horticulture. Phyton-Annales Rei Botanicae 39, 219–224.

Vörös, I., Biró, B., Takács, T., Köves-Péchy, K., Bujtás, K., 2000. Effects of arbuscular mycorrhizal fungi on heavy metal toxicity to *Trifolium pratense* in soils contaminates with Cd, Zn and Ni salts. Agrokemia es Talajtan 47, 277–288.

Weissenhorn, I., Glashoff, A., Leyval, C., Berthelin, J., 1994. Differential tolerance to Cd and Zn of arbuscular mycorrhizal (AM) fungal spores isolated from heavy metal-polluted and unpolluted soils. Plant and Soil 167, 189–196.