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Significance of treated agrowaste residue and autochthonous inoculates (Arbuscular mycorrhizal fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediate soils contaminated with heavy metals

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ABSTRACT

In this study, we analyzed the impact of treatments such as *Aspergillus niger*-treated sugar beet waste (SB), PO_4^{3-} fertilization and autochthonous inoculants [arbuscular mycorrhizal (AM) fungi and *Bacillus cereus*], on the bacterial community structure in a soils contaminated with heavy metals as well as, the effectiveness on plant growth (*Trifolium repens*). The inoculation with AM fungi in SB amended soil, increased plant growth similarly to PO_4^{3-} addition, and both treatments matched in P acquisition but bacterial biodiversity estimated by denaturing gradient gel electrophoresis of amplified 16S rDNA sequences, was more stimulated by the presence of the AM fungus than by PO_4^{3-} fertilization. The SB amendment plus AM inoculation increased the microbial diversity by 233% and also changed (by 215%) the structure of the bacterial community. The microbial inoculants and amendment used favoured plant growth and the phytoextraction process and concomitantly modified bacterial community in the rhizosphere; thus they can be used for remediation. Therefore, the understanding of such microbial ecological aspects is important for phytoremediation and the recovery of contaminated soils.

1. Introduction

Heavy metals (HM) have caused serious environmental problems and may enter the ecosystem through mining, atmosphere deposition and agrochemicals as pesticides, fertilizer and anthropogenic activities (Liu et al., 1997). The metals can be extracted from the contaminated soil, translocated from soil to the plants and concentrated in shoot.

Some studies have investigated the impact of HM contamination in soils using culture methods and community-level physiological profiling (Kelly et al., 1999; Ellis et al., 2001). Traditional culture methods, however, may provide a false impression of the microbial community as many species may not be detected. Biomass and changes in respiration have also been used as indicators of soil pollution (Bååth, 1989; Chander and Brookes, 1991); however, this will not highlight changes in diversity and therefore functionality of the microbial biomass and may not be a useful indicator of the effects of contamination. Bacterial community shifts may be more helpful in assessing the impact of pollution and molecular analysis based on 16S rDNA genes of soil bacteria has opened up opportunities to investigate total bacterial populations in soils. An increasing number of studies makes use of polymerase chain reaction (PCR) based methods like denaturing gradient gel electrophoresis (DGGE) for characterising complex soil bacterial communities. The advantage of DGGE is that it recognises very small differences in the nucleotide sequence, allowing a description of the community structure expressed as band patterns on a gel that can be used for analysis of changes or shifts in populations of the predominant community members (Ovreas and Torsvik, 1998; Yang and Crowley, 2000).

For phytoremediation purpose we need a better understanding of the interactions between plant and soil microorganisms, particularly beneficial microbes as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) (Whitfield et al., 2004). AM fungi improve the growth and biomass accumulation of plants and yield mainly through the mobilization of nutrients from the soil (Toro et al., 1998) as well as this symbiosis may affect plant protection in contaminated soils (Vivas et al., 2003a). Many rhizosphere colonizing bacteria typically produce metabolites, such as siderophores, biosurfactants or organic acids that stimulate plant growth (Glick, 1995) and also may reduce metal availability in the medium. Until now mycorrhizal fungi and bacteria have been found in HM contaminated soils which is an

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indication on AM fungal and bacterial tolerance. The inoculation of autochthonous AM fungi and PGPR bacteria have shown to be an important role in bioremediation of sterilized soils artificially contaminated by HM (Vivas et al., 2003a). But the impact of the applications of both groups (AM-fungi and rhizobacteria) on the structure bacterial communities in natural soil with high grade of HM contamination has not been studied.

On the other hand, the application of the sugar-beet (SB) agrowaste positively interacts with beneficial microbial groups inoculated and these components can modify the soil's physicochemical, biological and fertility properties that improve plant establishment and growth in disturbed soils (Medina et al., 2006). Previous results suggest that the coinoculation of selected and adapted beneficial microorganisms (AM fungi plus bacteria) and the applications of agrowaste optimize plants-soil system under adverse environments. It may be an alternate way for remediation practices. However, the impact of these strategies on plant growth, bioremediation and microbial communities in a natural multicontaminated soil has not been yet considered.

The aim of this study was to test the impact of microbial inoculations and/or the application of treated SB agrowaste on plant growth, nutrition and metal extraction from a natural HM contaminated soil. In addition we test how these treatments change the microbial community composition, structure in this HM multicontaminated soil compared to PO_4^{3-} fertilization.

2. Materials and methods

2.1. Fermentation process

NB2 strain of *Aspergillus niger* was used in this study. It had previously been selected as producing citric acid on complex organic substrates as olive cake and others (Vassilev et al., 1986).

SB a lignocellulosic material, was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10 % with 50 mL Czapek's solution (described in Fluka Chemica, catalogue no. 70185) containing (g L^{-1} of destilled water): $FeSO_4$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $NaNO_3$, 3.0; sucrose, 30; K_2HPO_4, 1.0 and a final pH of 7.3 ± 0.2 for static fermentation in 250 mL Erlenmeyer flasks. Rock-phosphate at a concentration of 1.5 g L^{-1} was added. This medium was inoculated with 3 mL of *A. niger* spore suspension (1.2×10^6 spores). Static fermentation was performed at 28 °C for 20 d.

2.2. Soil–plant experiment

The experiment consisted of a completely randomized factorial block with: (1) single mycorrhizal inoculation with autochthonous AM inoculums (AM), (2) single bacterial inoculation with autochthonous bacteria isolate from multicontaminated soil (B), (3) dual autochthonous AM inoculums plus bacteria inoculation, (AM + B), (4) PO_4^{3-} fertilization and (5) untreated control. These treatments were applied to unamended control soil or soil amended with *A. niger*-treated SB agrowaste residue. Treatments were replicated five times giving a total of 50 pots.

The multi-contaminated test soil used in the greenhouse experiment was described in Table 1. The test soil (Gorguel) was selected from Murcia province (Spain). The soil was air-dried, sieved to less than 2 mm and mixed with quart sand (<1 mm) to reach 1:1 soil/sand ratio (v/v).

The amendment was mixed at the rate of 5% with half of the soil/sand mixture and left for equilibration for 3 weeks at room temperature. Four seeds of *Trifolium repens* were sown in each pot (500 mL capacity). The seedling was thinned to two per pot 2 weeks after emergence.

Table 1
Chemical and physical characteristics of the soil text Gorguel from Murcia province (Spain).

pH (H_2O)	7.7	Total N (g kg^{-1})	0.22
EC (1:5, dS m^{-1})	2.5	Available P (μg g^{-1})	1
Aggregate stability (%)	48.7	Water soluble C (μg g^{-1})	41
Metal content (mg kg^{-1}) in soil			
Fe			*139045
Mn			8300
Al			*19385
Zn			*47695
Pb			*8555
Cu			168
Cd			*52
Ni			34
As			475
Cr			31

* Values that exceed the permissible limits (Council of the European Community, 1986).

2.3. Selection of metal tolerant microbes

The bacterial strain was isolated from the multi-contaminated soil Gorguel following serial dilutions of soil, 1 g of homogenised soil from the rhizosphere zone was suspended in 100 mL of sterile water (dilution 10^{-2}) and this suspension was further diluted to reach dilution 10^{-4}–10^{-7}. The suspension was sown on agar plates (Gryndler et al., 2000). The bacterial strains selected were the most abundant cultivable bacterial strains in such soil. For inoculation, appropriate pots were sprinkled with 1 mL (10^8 cell mL^{-1}) of this bacterial strain mixture grown in nutrient broth medium for 24–48 h at 28 °C temperature (Vivas et al., 2003c). The bacterial inoculum was applied to appropriate pots on clover seeds.

The autochthonous mycorrhizal inoculum, also coming from the multi-contaminated soil, was a stable consortium of morphologically determined *Glomus mosseae* species, from which the most abundant *G. mosseae* strain was selected. It was bulked in an open-pot culture of red clover and consisted of soil, spores, mycelia and infected root fragments. 10 g of inoculum (1 % v/v) were added to appropriate pots at sowing time just below the clover seeds. Part of the non-inoculated plants received KH_2PO_4 (as 100 g kg^{-1} P). This rate was previously used to match the effect of AM fungus on plant growth given an appropriate control for the mycorrhizal plants.

A suspension (1 mL^{-1}) of the diazotrophic bacterium *Rhizobium leguminosarum* bv. *trifolii* (10^8 cell mL^{-1}) was sprinkled over the seeds of all pots at the time of sowing.

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.

2.4. Growth conditions

The plants were grown in a greenhouse under a day/night cycle of 16–8 h, 21–15 °C, and 50% relative humidity. Photosynthetic photon flux density was 500 μmol m^{-2} s^{-1} as measured with a light meter (LICOR, model LI-188B). Water loss was replaced daily by top watering (tap water).

2.5. Parameters measured

2.5.1. Biomass production, nutrients and metal concentrations

At harvest (3 months after planting) the root system was separated from the shoot and dry weights were measured after drying in a forced-draught oven at 70 °C for 2 d. Shoot concentrations of K

was determined by flame photometry and P (Olsen and Dean, 1965), as well as of Zn, Fe, Cd, Ni, Co, Mn, Mo, Al, Cr, B and Cu were also determined after wet digestion of the air-dried plant samples with $HNO_3 + H_2O_2$ by inductively coupled plasma atomic emission spectrometry (Takács et al., 2001).

2.5.2. Symbiotic development

The percentage of mycorrhizal root length infected was estimated by observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), (Phillips and Hayman, 1970) Quantification was performed using the grid-line intersect method (Giovannetti and Mosse, 1980).

2.5.3. Production of indole-3-acetic acid by the bacteria

The bacterial isolate was cultivated at 28 °C in nutrient broth. The production of indole-3-acetic acid (IAA) by this bacterium was measured by the method of Wöhler (1997). The bacterium was grown in nutrient broth and then collected by centrifugation at 1000*g* for 5 min. The bacterial pellet was then incubated at 37 °C for 24 h with 3 mL of phosphate buffer (pH 7.5) with glucose (1%) and 2 mL of L-tryptophan (1%). After incubation, 2 mL of 5% trichloroacetic acid and 1 mL of 0.5 M $CaCl_2$ were added. The solution was filtered through a Whatman filter paper No. 2.

Three milliliter of the filtrate was transferred to a test tube and 2 mL of Salper Solution (2 mL 0.5 M Fe Cl_3 and 98 mL 35% perchloric acid) was added. This mixture was incubated for 30 min at 25 °C in the dark. Then the absorbance of the resulting solution was measured at 535 nm with a Shimadzu UV-1603 spectrophotometer. The calibration curve ranged from 0.5 to 10 mg IAA L^{-1}.

2.5.4. Molecular identification of the bacterial strain

Bacterial identification was carried out by 16S rDNA cloning and sequencing as described (Vivas et al., 2003c). Database researches for 16S rDNA sequence similarity unambiguously identified the bacterium as *Bacillus cereus*.

Total DNA from the bacterial isolate selected was obtained as described by Giovannetti et al. (1990) and characterized by sequence analysis of the small ribosomal subunit (16S ribosomal DNA). PCR amplification was carried out as described previously (Vivas et al., 2003c).

2.5.5. Soil–DNA extraction

Total DNA was extracted from subsamples of 250 mg of soil by the bead-beating method according to manufacturer's instructions of the MoBio UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA), with a few modifications, including the repetition of the second step (Inhibitor Removal Solution) to remove trace concentrations of PCR inhibitors. The DNA samples were checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

2.5.6. PCR–DGGE analysis

PCR was performed with the 16S rDNA universal bacterial denaturing gradient gel electrophoresis (DGGE) primers (TIB MOLBIOL, Berlin, Germany) P1, P2, and P3 to amplify the V3 hypervariable region of 16S rDNA genes. Primer P3 contains the same sequence as P1 but with an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5′ end (Muyzer et al., 1993; Simpson et al., 1997).

Two successive amplifications were carried out following (Muyzer et al., 1993) with the following modifications: a hot start of 5 min at 94 °C; 19 cycles consisting of 94 °C for 15 s, 65–55 °C for 15 s, decreasing the temperature by 0.5 °C each cycle (touchdown), and 72 °C for 30 s; 14 cycles consisting of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final step of 10 min at 72 °C. The total reaction mixture of the first PCR consisted of 25 μL with the following ingredients: 1 μL volume (approx 10 ng) of extracted DNA, 1 μm primer P1, 1 μm primer P2, 10 μL Eppendorf Master Mix (2.5X) and sterile Milli-Q water to a final volume. The second amplification was performed by using 1 μL of the products of the first reaction as template. In this, primers P2 and P3 were used under the same conditions described above. PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

DGGE analyses were conducted using 20 μL of this latter PCR product loaded into a 40–65% urea–formamide–polyacrylamide gel. An INGENYphorU System (Ingeny International BV, The Netherlands) was run at 75 V for 17 h at 58 °C to separate the fragments. Gels were silver stained with the Bio-Rad Silver Stain according to the standard DNA-staining protocol and photographed under UV light ($\lambda = 254$ nm) using an UVItec Gel Documentation system (UVitec Limited, Cambridge, UK). The band patterns were compared in different lanes using the UVImap Analysis software (UVitec Limited, Cambridge, UK). The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric curve. Band positions were converted to R_f values between 0 and 1, and profile similarity was calculated by determining (Nei and Li, 1979) similarity coefficients (Nei and Li, 1979) for the total number of lane patterns from the DGGE gel. The similarity coefficients calculated were then used to construct a dendrogram using the unweighted pair-group method with arithmetical averages (UPGMA).

The species richness on DGGE gels (R) was calculated as the mean number of bands present (Vivas et al., 2008, 2009). The structural diversity of the microbial community was examined by the Shannon index of general diversity H' (Shannon and Weaver, 1963) and Simpson index of dominance D (Simpson, 1949). For these analyses, each band was presumed to represent the ability of that bacterial species to be amplified (Ibekwe and Grieve, 2004; Vivas et al., 2008, 2009). The intensity of the bands was reflected as peak heights in the densitometric curve. The Shannon H' and Simpson D indexes were calculated from the following equations:

$$H' = -\sum(P_i \log P_i); \quad D = \sum P_i^2$$

$P_i = n_i/N$; n_i = height of peak and N = sum of all peak heights in the curve.

2.6. Statistical analysis

Five replicates were made per treatment and data were subjected to analysis of variance. Differences between means were analyzed by Duncan's multiple range test ($P \leqslant 0.05$). For the percentage values, the data were arcsin square transformed before statistical analysis.

3. Results and discussion

The positive effect of agrowaste as SB and microbial inoculation as tool of bioremediation on the plant growth and the impact on the rhizosphere bacterial community was determined in a natural soil from a multicontaminated area (see soil characteristics in Table 1).

The addition of PO_4^{3-} to this soil increased plant development (shoot and root growth) similarly to the dual AM + B inoculation in absence of composted SB (Fig. 1a). In un-amended soil, autochthonous single AM or B inoculation increased the root growth by 76% (AM), by 32% (B) and by 136% the dual AM + B inoculation. Comparing the control plants (without microbial inoculation and

a SB) with AM-inoculated plants differences in shoot biomass ranged from 107% (single AM) to 219% (AM + B). The plants growing in amended soils highly increased shoot and root biomass (Fig. 1a). In the SB applied soil the effectiveness on plant development of single mycorrhizal inoculation was higher than PO_4^{3-} fertilization (Fig. 1a).

Total length of AM colonized roots was highly increased by the amendment. In the natural multi-contaminated soil control plants show 20 cm of root length with AM-colonization but this symbiotic value was slightly depressed by PO_4^{3-} fertilization (Fig. 1b). Microbial inoculations increased this symbiotic development and the highest AM colonization, in soil without composted SB, was obtained in dual AM + B inoculated plants that produced 861 cm of AM colonized roots. In the SB amended soil the mycorhization was enhanced and the maximum value was observed in single AM inoculated plants (Fig. 1b) that produced 1758 cm of mycorrhizal root and similar growth responses than PO_4^{3-}-fertilization having only 228 cm of AM-colonized roots (Fig. 1b). One important effect of SB amendment was the improvement of the mycorrhizal infection as previously showed Medina et al. (2006).

The P and K content in *Trifolium* plants were widely increased by P-fertilization and particularly by SB amendment. In absence of composted SB the chemical treatment (PO_4^{3-}) was the most efficient in increasing these both nutrients in plant shoot. But in presence of composted SB, single AM inoculation was the most effective treatment in increasing the content of these nutrients [by 536% (P) and by 370% (K)] compared to plants growing in non-composted soil (Table 2).

In previous studies we used an artificially contaminated soil applying increasing amounts of single metals as Cd or Zn and the effectiveness on plant development of microbial treatments applied (selected AM fungus and/or bacteria) was a consequence of increasing plant nutrition (N, P and K) and concomitantly a decreasing metals acquisition (Vivas et al., 2003a–c; Medina et al., 2006). The plant growth enhancement by the treatments applied seems to be the result of varied and additional mechanisms as those improving nutrients acquisition and those directly related to metals quelation (Zhang et al., 2005; Wu et al., 2006).

In this study we determined that the IAA production by *B. cereus* was 6.2 μg mL^{-1}. In a previous unpublished study we determined that these bacteria were also able to reduce metals content in the medium when growing *in vitro* [in medium added of the extract (1 soil/1 H_2O v/v) of the multicontaminated test soil]. The metals reduction in the growing medium (as percentage) were

Table 2
Nutrients P, K, Fe, Zn and B (mg) and Cu, Mo (ppm) content in dry weight for shoot (g) in *Trifolium repens* as affected by composted agrowaste (SB) in non-inoculated control (C) plants, PO_4^{3-} fertilized plants and single or dual arbuscular mycorrhizal fungus (AM) and bacteria (B) inoculated plants.

Treatments	P (mg)		K (mg)		Fe (mg)		Zn (mg)	
	no SB	SB	no SB	SB	no SB	SB	no SB	SB
C	50g	440b	2062g	8263c	777c	144f	42c	70b
PO_4^{3-}	362cb	805a	7049dc	13,094b	949bc	356d	73b	113a
AM	126d	801a	3478f	16,332a	2099a	598c	69b	128a
B	82f	450b	2771f	11,503bc	727c	115g	46c	117a
AM + B	211c	532b	6510dc	13,248b	1400b	461d	99b	72b

	B (mg)		Cu (ppm)		Mo (ppm)	
C	3f	13b	17g	47cd	0.2f	2.4b
PO_4^{3-}	7c	21a	35d	72b	2.0b	4.4a
AM	4d	22a	40d	87a	0.5d	3.4b
B	3f	13b	23f	75b	0.1f	1.6c
AM + B	7c	14b	53c	67bc	1.4c	2.2b

Values not sharing a letter in common differ significantly ($P \leqslant 0.05$) from each other.

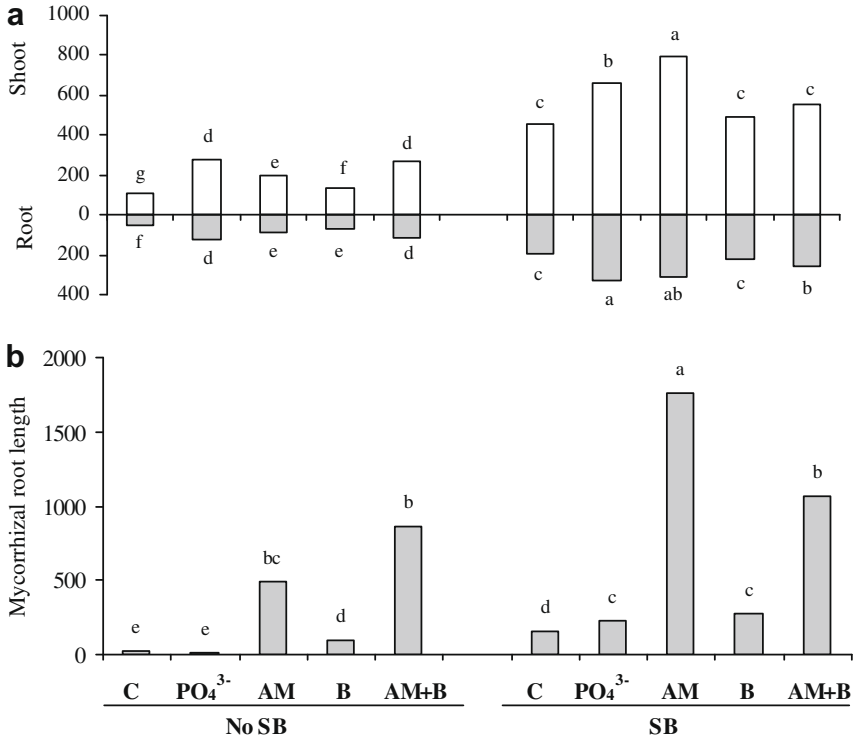

Fig. 1. *Trifolium repens* shoot and root growth (mg) [a] and total length (mm) of AM colonized root [b] in a HM multicontaminated natural soil as affected by autochthonous microorganisms [(AM fungi (AM) or/and bacteria (B)], PO_4^{3-} fertilizer with or without composted agrowaste (SB) compared to untreated control (C). Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test ($n = 5$).

6.8 (Fe), 3.5 (Mn), 4.5 (Cd) and 3.5 (Zn) after 48 h of bacterial growth. The bacterial effectiveness in reducing metals (determined after centrifugation of the growing medium and elimination of bacterial cells) may be due to the involvement of IAA production plus the bioaccumulation ability of Fe, Mn, Cd and Zn exhibited by the bacterial cells. These bacterial capabilities of *B. cereus* may be envolved in alleviating the inhibitory effects of metals on plants growing in this multicontaminated soil.

The addition of composted SB greatly decreased the Fe and increased Zn content in shoot of plants independently of microbial treatments applied (Table 2). Nevertheless, AM inoculation was particularly effective in increasing Fe content by 170% (without SB) and by 316% (with SB). The AM inoculation also increased Zn content by 63% (without SB) and by 84% (with SB) and similarly behaved PO_4^{3-} fertilization (Table 2). The lack of bacterial effect on Zn, Fe, Mn and Cd content in plant agreed well with the biosorption showed by this bacteria in the medium with soil extract (without SB). Therefore, the IAA production did not affect shoot promotion.

The plants PO_4^{3-} fertilized extracted more P, K, B, Mo, Cd, Cr, and Ni (without SB) than AM inoculated plants while AM-colonized plants with SB accumulated more K, Fe, Cu, Al, and Cr content than PO_4^{3-} fertilized plants (Tables 2 and 3).

In non-treated control soil, the high metal concentration and limited nutrient content may be responsible for the low plant biomass production. The plant growth enhancement caused by the treatments applied seems to be the result of varied nutritional and/or HM tolerance factors (Zhang et al., 2005; Wu et al., 2006). One relevant factor related to with the success of phytoremediation/extraction processes of multi-contaminated soils is to obtain a high plant biomass with abilities for acquiring metals from soil. Regarding results, plant biomass and metals acquisition by plant growing in this multi-contaminated soil highly increased compared to control, non-treated plants, when amended with composted agrowaste and when plants were microbial inoculated with AM and/or B.

Such biological manipulation can be considered as a promising alternative for phytoremediation extraction purpose. The phytoextraction efficiency of plants was affected by treatments applied as PO_4^{3-} fertilizer, microbial inoculations and/or SB amendment. It was different according to the metal involved as results shown. This is an indication on each one of the treatments applied (chemical and/or biological) affecting differently the translocation value for each metal. In fact, SB amended soils showed a higher phytoextraction for most of metals (Fe, Zn, B, Cd, Cr and Ni) particularly associated with AM inoculum (Table 3).

In general, the SB amendment increased the plant's content of most of the analyzed elements (B, Cu, Mo, Cd, Cr and Mn) but Al decreased or did not change by the SB amendment application (Tables 2 and 3). In non-amended soil, the most effective applied treatments in improving elements content in plants were: single B inoculation for Al, PO_4^{3-} fertilizer for B, Cd, Cr, Mo and Ni, being Cu and Mn more accumulated in dual AM + B inoculated plants (Tables 2 and 3). In the case of SB amended soil, plants inoculated with single B increased in the highest extent Al uptake but the highest Mn and Mo contents were determined in PO_4^{3-} fertilized plants (Tables 2 and 3). In SB amended soil, single AM inoculated plants matched B, Cd and Ni shoot content with PO_4^{3-} fertilized plants and increased value of Cu content (Tables 2 and 3). The dual (AM + B) inoculation was the treatment that more increases Cr content by plants in SB amended soil (Table 3). These results indicated that phytoextraction was enhanced by the composted SB application (except for Al) and the microbial inocula applied highly improved this effect for whatever metal analyzed.

The HM exert a negative influence on the soil's microorganisms (Khan, 2005; Biró et al., 2007) which are considered an index to test soil pollution because the HM have a deleterious influence on cell functioning and thus in soil microbial community. Soil quality is related to the rhizosphere microbial groups but few studies have considered the effect of the mycorrhizal and SB amendment on the structure of the bacterial communities and diversity (Maliszewska-Kordybach and Smreczak, 2003; Zhang et al., 2006). Nevertheless, soil application of SB amendment resulted in a practical method because of its advantages in increasing soil fertility in this degraded soil. Metabolizable C compounds from this SB amendment must be applied to the microbes to ensure their growth and activity. However, no information is available about the effect of the soil application of treated agrowastes which include alternative carbohydrate sources on the environmental structure and function of microbial community compared to conventional PO_4^{3-} fertilized used in parallel.

In this study, the bacterial-community profiles were generated from DGGE of the amplified soil DNA (Fig. 2a). The dendogram analysis by homology (Fig. 2b) and the Shannon's diversity index (Table 4) show that the SB amendment modified the structure of the bacterial community. We found two groups forming a distinct cluster in a UPGMA dendrogram: One group included the treatments without amendment, that enclosed three subgroups. (a) Untreated control C and PO_4^{3-} fertilized treatments, (b) AM treatment, and (c) treatments inoculated with the bacteria B (B and AM + B) (Fig. 2b). Nevertheless, in the presence of SB amendment, the control and microbial inoculation with AM + B formed two very distinct groups, which indicate a significant structural difference between these two treatments (Fig. 2b).

R index indicates the species richness on DGGE gels calculated as the numbers of bands present (Ampe and Miambi, 2000; Vivas et al., 2008). With the exception of the treatment inoculated with *B. cereus* the SB amendment increased these values in the treatments (Table 4). Treatments inoculated with AM and AM + B plus SB amendment increased by 233% (AM) and by 123% (AM + B) when compared with their respective treatments (AM and AM + B) without amendment. In the non-inoculated (C or PO_4^{3-}) or B inoculated soils the effect of SB amendment was not (control) or less (PO_4^{3-}) relevant (Table 4).

As results show bacterial diversity H′ was positively increased by the treatments applied particularly AM inoculation with SB (Table 4). The Shannon's diversity index (H′) is defined as the proportional abundance of species in a community. We found that these values decreased in AM inoculated plants and particularly when both AM and B inocula were applied. These results

Table 3
Elements Al, Cd and Cr (mg); Mn and Ni (μg) content in dry weight for shoot (g) of *Trifolium repens* as affected by composted agrowaste (SB) in non-inoculated control (C) plants, PO_4^{3-} fertilized plants and single or dual arbuscular mycorrhizal fungus (AM) and bacteria (B) inoculated plants.

Treatments	Al (mg)		Cd (mg)		Cr (mg)	
	no SB	SB	no SB	SB	no SB	SB
C	69c	14f	0.2f	0.4c	2.1f	5.5d
PO_4^{3-}	79c	37d	0.4c	0.6a	17.4	10.8c
M	78c	65c	0.3d	0.6a	7.9c	12.3b
B	164a	131a	0.2f	0.5b	5.3d	8.4c
AM + B	103b	36d	0.4c	0.5b	9.2c	17.6a

	Mn (μg)		Ni (μg)	
C	282f	579c	1.7c	1.4d
PO_4^{3-}	419d	1041a	3.3a	3.4a
AM	359d	841b	2.1c	4.1a
B	277f	640c	2.7bc	2.4a
AM + B	446d	729b	2.8bc	3.1a

Values not sharing a letter in common differ significantly ($P \leqslant 0.05$) from each other.

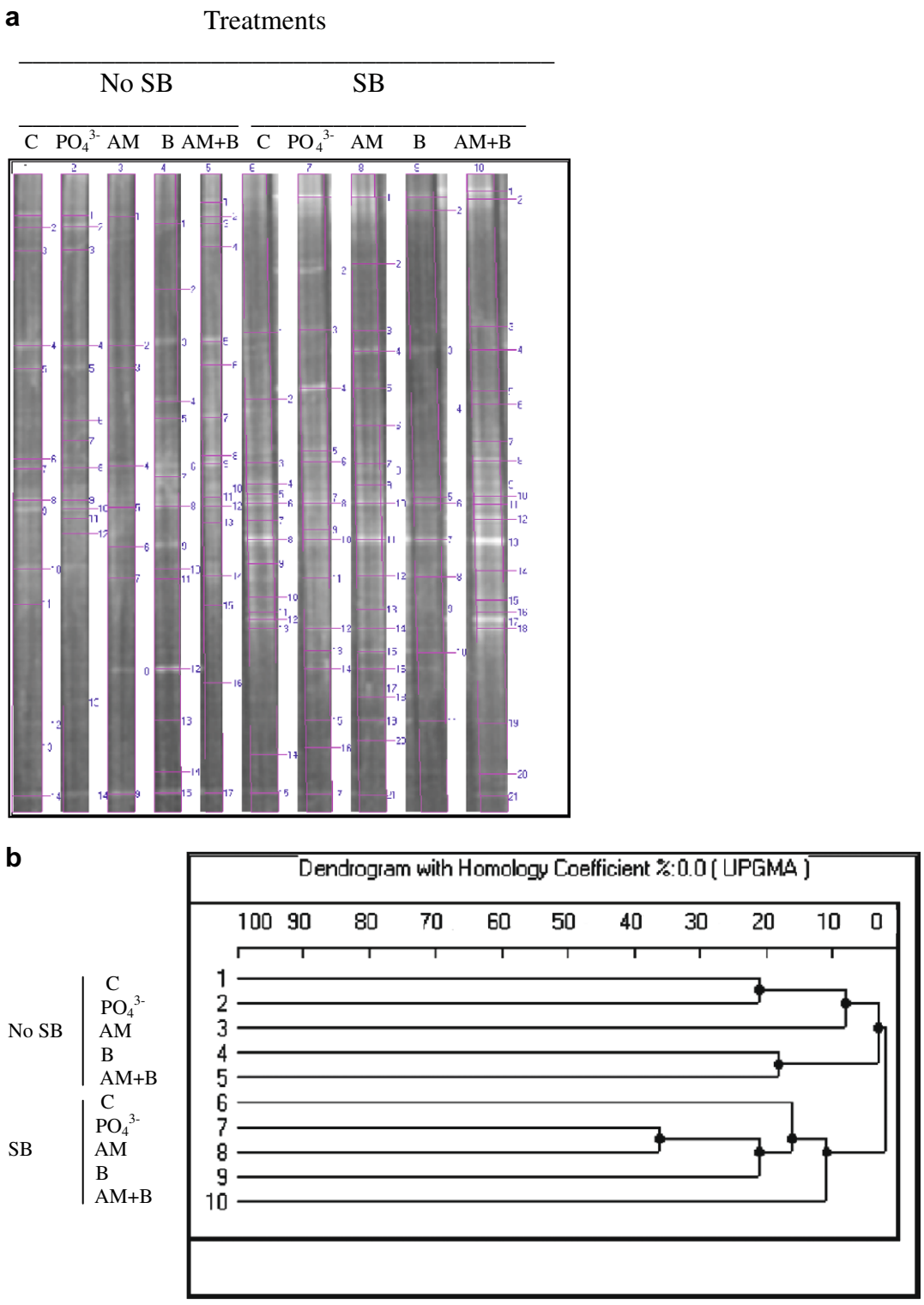

Fig. 2. DGGE profiles [a] from the rhizosphere of HM multicontaminated natural soil samples and cluster Analysis of bacterial DGGE profiles [b] as affected by composted agrowaste (SB) application in non-inoculated control (C) plants, PO_4^{3-} fertilized plants and single or dual mycorrhizal fungus (AM) and bacteria (B) inoculation.

would indicate a decrease or even disappearance of some species in the community when massive introduction of others microorganisms (AM and B) are done. Curiously, microbial inoculations (AM, B or AM + B) decreased H′ diversity index in non-SB amended soil. The negative effect of massive incorporation (inoculation) of particular microbial groups on the structure of the bacterial community is not surprising in our opinion (Table 4 and Fig. 2a). The positive effect of SB amendment (possibly as an extra C source) on such value compensated the decrease in the structure caused by these microbial treatments and reached the highest bacterial structure values in presence of biological treatments applied.

Table 4
Species richness (*R*), Shannon (*H'*), Simpson (*D*) diversity indexes values for DGGE profiles in non-inoculated control (C), PO_4^{3-} fertilized and single or dual arbuscular mycorrhizal fungus (AM) and bacteria (B) inoculated in soil.

Treatments	No SB			SB		
	R	*H'*	*D*	*R*	*H'*	*D*
C	14	2.6	0.04	15	2.5	0.08
PO_4^{3-}	14	2.5	0.05	17	3.3	0.03
AM	9	2.0	0.03	21	4.3	0.04
B	15	2.2	0.08	12	3.6	0.07
AM + B	17	1.6	0.03	21	3.5	0.03

Biodiversity index (*H*) as well as dominance concentration index (*D*) significantly varied with the presence of SB amendment (Table 4). The inoculation of AM caused the highest bacterial diversity (4.3) in the presence of the SB amendment. The microbial inoculation of AM or B and particularly AM + B did not affect the structure of the bacterial community in non-amended soil. Although, in SB amended soils such biological treatments greatly increased (*H'*) value by 215% (AM), 163% (B) and 218% (AM + B), when compared with these treatments in non-amended soil (Table 4). The Simpson index of dominance (D) was affected only in non-inoculated control SB-amended soil, where it increased 100% the "*D*" (0.04–0.08) value (Table 4). This indicates the presence of the predominant bacterial group.

This study evidences that SB amendment is a suitable tool for increasing and changing the bacterial community in rhizosphere of the multicontaminated soil used. Microbial properties of this multicontaminated soil such as biodiversity and dominance index increased by the application of the treated SB agrowaste and concomitantly favoured the plant development. An interesting result is that PO_4^{3-} fertilization and single AM inoculation (used in parallel) similarly promoted plant biomass but only AM inoculation increased microbial diversity in the presence of SB amendment.

Singh et al. (2008) suggest that AM fungi affect the bacterial community associated with the roots of the plants where high numbers of bacteria are associated with the extraradical mycelium of colonizing AM fungi, but their functions and in situ activities are largely unknown and most have never been characterized. Artursson et al. (2005) studied the impact of *G. mosseae* inoculation on the active bacterial communities (in soil by using a molecular approach) and they found that the dominant bacterial species were activated as a result of *G. mosseae* inoculation but mostly were uncultured bacterial and *Paenibacillus* species.

The highest metals accumulation in AM inoculated plants is highly interesting since AM inoculation may be used as a phytoremediation practice in most of natural multi-contaminated soils (Gohre and Paszkowski, 2006; Hildebrandt et al., 2007). The microbial treatments applied and SB amendment used also improved physiological plant status and these plant factors may enhance plant HM tolerance which has relevance in phytoremediation.

Ours results are indicative of the positive effect of the amendments and beneficial microorganisms as AM fungi and bacteria, on the microbial soil status and the relevance of such biotechnological management on bioremediation in multi-contaminated soils. In futures studies we are interested in understanding the main microbial activities and mechanisms involved in the positive effects found in this study. Mechanisms that govern the interaction and selection of bacterial species in the multicontaminated soil may be considered in future proposals for bioremediation strategies.

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