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Addition of microbially-treated sugar beet residue and a native bacterium increases structural stability in heavy metal-contaminated Mediterranean soils

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ABSTRACT

A mesocosm experiment was conducted to investigate the effect of the addition of *Aspergillus niger*-treated sugar beet waste, in the presence of rock phosphate, and inoculation with a native, metal-tolerant bacterium, *Bacillus thuringiensis*, on the stabilisation of soil aggregates of two mine tailings, with differing pH values, from a semiarid Mediterranean area and on the stimulation of growth of *Piptatherum miliaceum*. Bacterium combined with organic amendment enhanced structural stability (38% in acidic soil and 106% in neutral soil compared with their corresponding controls). Only the organic amendment increased pH, electrical conductivity, water-soluble C, water-soluble carbohydrates and plant growth, in both soils. While in neutral soil both organic amendment and bacterium increased dehydrogenase activity, only organic amendment had a significant effect in acidic soil. This study demonstrates that the use of *P. miliaceum* in combination with organic amendment and bacterium is a suitable tool for the stabilisation of the soil structure of degraded mine tailings, although its effectiveness is dependent on soil pH.

1. Introduction

Mining activities have generated significant amounts of toxic waste materials that have been deposited in mine tailing impoundments. These tailings are composed of mostly silt or sand sized particles and are characterised by instability and scarce cohesion; these properties easily expose the mine tailings to water and air erosion (Mendez and Maier, 2008a). Soil surface structure stabilisation of mine tailings by establishment of a plant cover could reduce erosion, protect soil against degradation and limit the spread of metal contamination (Wong, 2003). Therefore, improved soil structure means increased water retention, nutrient uptake, drainage, aeration and root growth. However, these tailings are characterised by excessive acidity or alkalinity, high toxic-metal concentrations, poor physical structure and deficient levels of plant nutrients (Pitchel and Salt, 1998; Wong et al., 1998), which seriously limit plant growth (Ibrahim and Goh, 2004). In addition to such adverse factors, challenges to plant growth in arid environments include the lack of water and often saline conditions (Mendez and Maier, 2008b). Toxic metals can also adversely affect the number, diversity and activity of soil organisms, inhibiting soil organic matter decomposition and N mineralisation processes and consequently plant development (del Val et al., 1999; Misra, 2000). The detrimental effects of heavy metals on soil microbial activities and biomass have been reported (Bååth, 1989; Roane and Pepper, 2000). Selection of appropriate plant species which can establish, grow and colonise metal-contaminated soils is important for successful reclamation of these sites. Therefore, drought-resistant and metal-tolerant native plant species should be used in order to achieve a self-sustainable vegetation on semiarid, toxic-metal mined lands.

Bacteria isolated from metal-contaminated soils are often more resistant to metals than those collected from uncontaminated environments (Chaudri et al., 1992) and therefore are likely to play an important role in the growth, metal tolerance and accumulation capacity exhibited by plants growing in metal-contaminated soils. Thus, this resistance may be key to plant survival on contaminated soils (Copaert and Vandenkoornhuyse, 2001). Mechanisms allowing the bacteria to persist in metal-polluted media include the formation of organic metal-complexing agents (Higham et al., 1984), precipitation or redox transformation of metals (Southam, 2002). Recently, Vivas et al. (2005) reported the effectiveness of a Cd-adapted autochthonous bacterium with respect to the resistance of plants to high Cd concentration in soil. It has been shown that soil bacteria are associated with the clay and organic particles and the metallic mineral surfaces of the soil microenvironment and these associations may influence the functions and survival of microbes: for example, through storage of water for microbes and buffering against water potential fluctuations (Chenu, 1993). These associations also make an important contribution to the binding of aggregates by soil biota. To our knowledge, there are no previous studies indicating whether the inoculation of bacterial strains isolated from heavy metal-contaminated soils may enable plants to grow well under polluted conditions and thereby improve soil aggregate stability and reduce soil erosion losses.

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The quality and productivity of P-deficient mine tailings can be improved by the combined addition of organic amendments, phosphate solubilizing microorganisms and rock phosphate to soil (Alguacil et al., 2003; Medina et al., 2006). In particular, *Aspergillus niger*-treated sugar beet waste, in the presence of rock phosphate, is an organic amendment rich in polysaccharide compounds and available P (Vassilev et al., 1995) that can be used as energy sources by heterotrophic microorganisms and as cementing and stabilising agents of soil aggregates in the reclamation of degraded mine ecosystems. Several authors have shown the capacity of sugar beet pulp to bind metals in aqueous solution, due to the carboxyl functions present in its constituents (Reddad et al., 2002). Previous studies have demonstrated the ability of *A. niger*-treated sugar beet waste, in the presence of rock phosphate, to stimulate plant growth and nutrition of *Trifolium repens* in a calcareous soil contaminated artificially with Zn in the laboratory (Medina et al., 2006). Recently, Carrasco et al. (2009) have demonstrated that the effectiveness of this type of residues for improving structural stability of neutral mine tailings is largely enhanced by soil drying.

Substrate pH affects plant growth mainly through its effect on the solubility of chemicals, including toxic metals and nutrients. It is known that, at pH 6.5, nutrient availability to plants is at a maximum and toxicity at a minimum (Harris et al., 1996). On the other hand, the biosorption of bacteria to mineral surfaces also depends on environmental pH, increasing with decreasing pH (Omoike and Chorover, 2006). We hypothesised that soil pH may influence strongly the effectiveness of metal-tolerant bacteria and organic amendments used in bioremediation and revegetation tasks involving degraded mine environments.

It is known that high metal contents in metal-contaminated wastes can cause the selection of plants growing on the sites, where the biological diversity is low. The mine tailings from the Cartagena–La Unión mining district (SE Spain) have been colonised naturally by some species, such as *Piptatherum miliaceum*, that show relative tolerance to high heavy metal concentrations. The resistance of this species to Al (Zavas et al., 1996) and Pb (Conesa et al., 2006) has been described. *P. miliaceum*, despite growing in such polluted soil, does not accumulate high metal concentrations in its shoot and, therefore, potentially could be used in the revegetation of degraded mine lands. However, knowledge of revegetation strategies involving *P. miliaceum* is still very limited.

The aim of this study was to assess and compare the effectiveness of the addition of *A. niger*-treated sugar beet, in the presence of rock phosphate, and the inoculation with a native, metal-tolerant bacterium, *Bacillus thuringiensis*, with respect to stabilising the soil aggregates of two mine tailings, of differing pH, from a semiarid Mediterranean area and, concomitantly, facilitating the establishment of *P. miliaceum* in the polluted soils. Special emphasis will be placed on examining the role of pH in the stabilising effect of the two bioremediation technologies proposed.

2. Materials and methods

2.1. Materials

The study area was located in the La Unión mine district (southeast Spain). The terrain is low lying (<400 m), but with steep slopes (20–30%) because of its proximity of the coast. The climate is semiarid Mediterranean with an annual rainfall around 250–300 mm and a mean annual temperature of 17.5 °C; the potential evapo-transpiration reaches 1000 mm y^{-1}. This zone constituted an important mining nucleus for more than 2500 years. The ore deposits of this zone have iron, lead and zinc as the main metal components. Iron is present in oxides, hydroxides, sulfides, sulfates, carbonates and silicates; lead and zinc occur in galena, sphalerite, carbonates, sulfates, and lead- or zinc-bearing (manganese, iron) oxides (Oen and Fernández, 1975). In this area two mine tailings with an age of about 50 years and with different pH were selected: an acidic mine tailing called "Brunita" (U.T.M. X686500 Y4164800 Z160, Length: 320–400 m, Width: 235 m, Height: 23 m, Volume: 850,000 m^3, IGME, 1999) and a neutral mine tailing called "Gorguel" (U.T.M. X687480 Y4162800 Z135, Length: 200–300 m, Width: 95 m, Height: 25 m, Volume: 750,000 m^3, IGME, 1999). Three soil samples were taken from each tailing. Each soil sample consisted of a mixture of 6 subsamples randomly taken from the top 20 cm depth of soil. The analytical characteristics of both mine tailings are shown in the Table 1.

The bacterial strain selected for this study was isolated from the experimental area. The bacterial isolation was carried out following the conventional procedure: 1 g of homogenised soil was suspended in 100 ml of sterile water and 1 ml of this suspension was diluted to reach dilutions 10^{-3} to 10^{-4}. These were plated in Petri dishes containing plate count agar medium (Oxoid) and cultivated for 48 h at 22 °C. Once selected the most abundant type was grown in nutrient broth medium (Scharlau Chemie, Spain) composed of meat and yeast extracts, peptone and sodium chloride, for 2 days at room temperature on a Heidolph Unimax1010 shaker. The bacterial strain was identified as a gram-positive bacterium member of the *B. thuringiensis* group (EEZ124). For inoculation procedures the bacterial culture was centrifuged at 2287×*g* for 5 min at 2 °C and the sediment was resuspended in sterilized tap water. The bacterial suspension contained 10^8 colony forming units (CFU) ml^{-1}.

Sugar beet residue (SB), a lignocellulosic material was dried at 60 °C and then ground to pass a 2-mm-pore screen. Portions of 15 g of SB were mixed with 40 ml of Czapek solution (agar 15.0 g L^{-1}; dipotassium hydrogen phosphate 1.0 g L^{-1}; iron(II) sulfate heptahydrate 0.01 g L^{-1}; potassium chloride 0.5 g L^{-1}; magnesium sulfate heptahydrate 0.5 g L^{-1}; sodium nitrate 3.0 g L^{-1}; sucrose 30.0 g L^{-1}; pH = 7.3) for static fermentation in 250 ml Erlenmeyer flasks. Rock phosphate (Morocco fluorapatite, 12.8% P, 1 mm mesh), was added at a rate of 0.75 g per flask. Media were sterilized by autoclaving at 120 °C for 30 min. A spore suspension of *A. niger* NB2 (1.2×10^7) was spread carefully over the surface of the media. The mixture was allowed to ferment at 30 °C for 20 days without shaking. The characteristics of the SB after fermentation were: pH, 5.3; total P, 224 μg ml^{-1}; total N, 1.2%; cellulose, 11.3%; hemicellulose, 3.1%; lignin, 4.1% and reducing sugar, 0.25 g L^{-1}.

Mature seeds of *P. miliaceum* were collected from the mine tailings. The seeds were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) for 5 min and subsequently rinsed thoroughly with sterilized water prior to a wetting treatment with sterilized water for 2 h. Seeds were placed in darkness on double layered Whatman No.1

Table 1
Chemical, biochemical, microbiological and physical characteristics of the soil used in the experiment.

	Acidic tailing	Neutral tailing
pH (H_2O)	3.6	7.7
EC (1:5, dS m^{-1})	2.4	2.5
$CaCO_3$ (%)	<5	<5
Total Organic Carbon (%)	0.3	0.4
Total N (%)	0.03	0.02
Clay (%)	10	5
Silt (%)	36	24
Sand (%)	54	71
Water-soluble C (μg g^{-1})	39	41
Water-soluble carbohydrates (μg g^{-1})	8	10
Dehydrogenase (μg INTF g^{-1})	0	6.9
Aggregate stability (%)	49.2	24.7
Fe_2O_3 (%)	37	16
Al_2O_3 (%)	4	8
Total Zn (mg kg^{-1})	1120	12,100
Total Pb (mg kg^{-1})	1431	8950
Total Cu (mg kg^{-1})	82	221
Total Cd (mg kg^{-1})	30	61
Total Ni (mg kg^{-1})	12	26

filter paper moistened with distilled water in sterilized Petri dishes until germination.

2.2. Experimental design and layout

The experiment was a mesocosm assay, conducted as a completely randomised factorial design with three factors. The first factor had two levels: addition or not of fermented sugar beet residue to the soil, the second had two levels: non-inoculation or inoculation with *B. thuringiensis*, and the third had two levels: acidic soil or neutral soil. Six replicates per treatment were carried out, making a total of 48 pots.

Four hundred grams of air-dried soil were placed in 600 ml pots. The organic amendment was mixed manually with the experimental soil at a rate of 5% (w/w). *P. milliaceum* seedlings were transplanted to the pots (three seedlings per pot). When appropriate, the soil was inoculated with 3×10^9 CFU of *B. thuringiensis* per pot. The experiment was carried out in the nursery where the average maximum temperature was of 22 °C. Plants were watered regularly with deionized water, without any fertiliser treatment. Four months after planting, plants were harvested and soil samples of the pots were taken. Soil samples were divided into two subsamples. One subsample was sieved at 2 mm and stored at 2 °C for biochemical analysis and the other subsample was air-dried at room temperature and sieved at 2 mm for physical–chemical and chemical analysis or at 0.2–4 mm for aggregate stability.

2.3. Plant analyses

Fresh and dry mass of shoots and roots (105 °C, 5 h) were recorded.

2.4. Soil physical–chemical and biochemical analyses

Total organic C and total N were determined with an automatic Nitrogen and Carbon Analyzer after pre-treatment with HCl to eliminate carbonates and combustion at 1020 °C. Calcium carbonate was determined using Bernard calcimeter. The texture was determined using the Robinson pipette method combined with sieving. Total metal contents were determined by nitric–perchloric digestion: 1 g of crushed sample was placed in a Kjeldahl flask, and 10 mL of concentrated HNO_3 plus 10 mL of concentrated $HClO_4$ were added. The mixture was heated at 210 °C for 90 min. When cool, the content of the tubes was filtered through an Albert® 145 ashless filter paper, and the volume completed at 50 mL by washing the Kjeldahl flasks with HCl 0.5 N several times and filtering. Soluble metal contents were determined using a 1:5 soil/water mixture (Ernst, 1996) that was shaken for 2 h. All metals were quantified using with an ICP-MS (Thermo electron corporation Mod. IRIS intrepid II XDL).

Soil pH and electrical conductivity were measured in a 1:5 (w/v) aqueous solution. In soil aqueous extracts, water-soluble carbon was determined in a Shimadzu TOC-5050A analyser of C for liquid samples. Water-soluble carbohydrates were determined by the method of Brink et al. (1960).

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 the dark. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtering through Whatman N° 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

2.5. Physical analysis

The percentage of stable aggregates was determined by the method described by Lax et al. (1994). A 4 g aliquot of sieved (0.2–4 mm) soil was placed on a small 0.250 mm sieve and wetted by spray. After 15 min the soil was subjected to an artificial rainfall of 150 ml with energy of 270 Jm^{-2}. The remaining soil on the sieve was placed in a capsule, dried at 105 °C and weighed (P1). Then, the soil was soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105 °C and weighed (P2). The percentage of stable aggregates with regard to the total aggregates was calculated by $(P1-P2)\times100/(4-P2)$.

2.6. Statistical analysis

Data were log transformed to achieve for normality. Sugar beet addition, bacterial inoculation, type of soil and their interactions effects on measured variables were tested by a three-way analysis of variance. Statistical procedures were carried out with the software package SPSS 17.0 for Windows.

3. Results

3.1. Physical–chemical properties

As observed at the beginning of the experiment, the two tailings differed greatly in the levels of total metal, which were also higher in the neutral tailing than in the acidic tailing. Neither the addition of organic amendment nor the inoculation with the bacterium had any significant effect on the concentrations of total metals, whatever the type of soil (Table 2). In both soils, the addition of organic amendment and bacterial inoculation significantly decreased the concentrations

Table 2
Total and soluble metal concentrations of rhizosphere soil of *P. miliaceum* in response to bacterial inoculation and fermented sugar beet addition four months after planting.

	Total Zn	Total Pb	Total Cu	Total Cd	Total Ni	Soluble Zn	Soluble Pb	Soluble Cu	Soluble Cd	Soluble Ni
	(mg kg^{-1})									
Acidic tailing										
Control	1095 ± 15a	1334 ± 22a	73 ± 3a	28 ± 1a	11 ± 0a	51 ± 4d	ND	0.30 ± 0.01c	0.45 ± 0.02b	1.11 ± 0.10b
Amendment (A)	1077 ± 24a	1324 ± 46a	74 ± 5a	27 ± 1a	11 ± 1a	6 ± 1a	ND	0.01 ± 0.00a	0.33 ± 0.00a	0.56 ± 0.02a
B. thuringiensis (B)	1144 ± 13a	1422 ± 34a	79 ± 2a	28 ± 1a	11 ± 0a	44 ± 0c	ND	0.27 ± 0.01bc	0.42 ± 0.09ab	0.46 ± 0.01a
A × B	1096 ± 13a	1319 ± 16a	84 ± 5a	27 ± 0a	10 ± 0a	0 ± 0a	ND	0.06 ± 0.02ab	0.34 ± 0.01ab	0.10 ± 0.02a
Neutral tailing										
Control	11,955 ± 121a	9069 ± 311a	219 ± 4a	60 ± 0ab	25 ± 2a	0.69 ± 0.17b	ND	ND	0.32 ± 0.05a	ND
Amendment (A)	12,016 ± 329a	8938 ± 147a	213 ± 10a	59 ± 2a	23 ± 1a	0.25 ± 0.02a	ND	ND	0.29 ± 0.00a	ND
B. thuringiensis (B)	12,283 ± 87a	9240 ± 111a	250 ± 9b	61 ± 0ab	25 ± 1a	0.41 ± 0.02a	ND	ND	0.29 ± 0.00a	ND
A × B	12,424 ± 133a	9486 ± 56a	229 ± 5a	62 ± 1b	24 ± 0a	0.29 ± 0.01a	ND	ND	0.29 ± 0.00a	ND

ND: Not detected. The detection limits for each heavy metal are as follows: Zn<0.01, Pb<0.05, Cu<0.01, Cd<0.001 and Ni<0.05 mg kg^{-1}.
Mean ± standard error ($n=6$).
For each parameter and for each soil type, values in columns followed by the same letter are not significantly different (Tukey, $p<0.05$).

Table 3
Physical–chemical properties, labile organic C fractions, dehydrogenase activity and metabolic index of rhizosphere soil of *P. miliaceum* in response to bacterial inoculation and fermented sugar beet addition four months after planting.

	pH (H_2O)	EC (1:5, $dS\,m^{-1}$)	WSC $mg\,g^{-1}$	WSCH $mg\,g^{-1}$	DHase ($ng\,INTF\,g^{-1}$)	DHase/WSC ratio ($\mu g\,INTF\,mg^{-1}\,C)\times 10$
Acidic tailing						
Control	3.66 ± 0.03a	3.36 ± 0.05a	55 ± 11a	20 ± 5a	3.7 ± 0.6a	0.6 ± 0.1a
Amendment (A)	4.61 ± 0.02b	3.65 ± 0.03b	105 ± 18b	45 ± 9b	46.2 ± 1.0b	4.5 ± 0.1b
B. thuringiensis (B)	3.65 ± 0.02a	3.39 ± 0.02a	46 ± 4a	17 ± 3a	3.8 ± 0.4a	0.8 ± 0.1a
A × B	4.58 ± 0.02b	3.60 ± 0.03b	101 ± 11b	45 ± 7b	44.8 ± 0.7b	4.5 ± 0.1b
Neutral tailing						
Control	7.67 ± 0.01a	3.17 ± 0.03a	45 ± 5a	14 ± 2b	15.2 ± 0.3a	3.5 ± 0.1a
Amendment (A)	8.00 ± 0.03c	3.49 ± 0.04b	69 ± 4b	17 ± 4b	175.2 ± 1.6c	25.5 ± 0.4c
B. thuringiensis (B)	7.71 ± 0.03b	3.17 ± 0.03a	42 ± 2a	10 ± 1a	26.5 ± 0.8b	6.3 ± 0.2b
A × B	8.02 ± 0.02c	3.43 ± 0.04b	79 ± 3c	16 ± 3b	160.9 ± 1.2c	20.5 ± 0.3c

EC: electrical conductivity; WSC: water-soluble carbon; WSCH: water-soluble carbohydrates; DHase: dehydrogenase activity.
Mean ± standard error ($n = 6$).
For each parameter and for each soil type, values in columns followed by the same letter are not significantly different (Tukey, $p < 0.05$).

of soluble Zn. The highest decrease in the concentration of soluble Zn was observed in the acidic soil treated with the organic amendment, recording decreases of 88% with respect to the control soil. In the acidic soil, the addition of fermented organic amendment decreased the levels of soluble Cu, Cd and Ni, whereas the bacterial inoculation only decreased the levels of soluble Ni. However, in the neutral soil the concentrations of those soluble metals were negligible and there were no significant differences between treatments.

The organic amendment and the interaction between the organic amendment and the soil type had a significant effect on soil pH and electrical conductivity (Table 4). In both soils, the addition of organic amendment significantly increased soil pH (one unit in acidic soil and 0.33 units in neutral soil) and electrical conductivity (Table 3). The physical–chemical parameters of the soils only inoculated with the bacterium showed values similar to the corresponding control soils.

The water-soluble C and water-soluble carbohydrates values were increased with the addition of the organic amendment in both soils (Tables 3 and 4), particularly in the acidic soil. Increases in water-soluble C and carbohydrates of 91% and of 125%, respectively, were observed in the amended acidic soil with respect to the non-amended soil. The inoculation with *B. thuringiensis* had no significant effect on values of water-soluble C, in either soil (Table **4**). However, the bacterial inoculum slightly decreased the water-soluble carbohydrates in the neutral soil. The interaction of organic amendment and *B. thuringiensis* inoculation had a positive effect on the water-soluble C of the neutral soil (Table **4**), values being higher than when each was applied individually.

Table 4
Three factor ANOVA (bacterial inoculation, fermented sugar beet addition and type of soil) for all parameters studied in the rhizosphere soil of *P. miliaceum* four months after planting.

	Amendment A	*Bacterium* B	Soil S	A × B	B × S	A × S	A × B × S
pH	<0.001	NS	<0.001	NS	0.003	<0.001	NS
EC	<0.001	NS	<0.001	NS	NS	0.011	NS
WSC	<0.001	NS	<0.001	0.014	0.020	0.027	NS
WSCH	<0.001	NS	<0.001	NS	NS	<0.001	NS
Aggregate stability	<0.001	<0.001	<0.001	0.014	0.020	0.027	NS
Dehydrogenase	<0.001	NS	<0.001	NS	NS	0.049	NS

P significance values.
EC: electrical conductivity; WSC: water-soluble carbon; WSCH: water-soluble carbohydrates. NS: not significant.

3.2. Biochemical parameters

In the neutral soil, the addition of fermented sugar beet and bacterial inoculation increased the dehydrogenase activity significantly (Table 3), the organic amendment (about 11.5-fold higher than control soil) being more effective than the bacterium (about 1.7-fold higher than control soil). The dehydrogenase activity of the acidic soil was increased significantly by the addition of fermented residue but not by bacterial inoculation. The effect on dehydrogenase activity produced by the organic amendment was independent of bacterial inoculation but was significantly dependent on soil type (Table 4).

The dehydrogenase/WSC ratio (metabolic index) was significantly higher in the neutral soil than that in the acidic soil (Table 3). In both soils, the organic amendment was the most effective treatment for increasing the dehydrogenase/WSC ratio, producing increases of more than 600% with respect to the control soils. The bacterial inoculation only had a positive effect on metabolic index in the neutral soil.

3.3. Aggregate stability

The effect of the organic amendment and bacterium on soil aggregate stability was strongly dependent on soil type (Table 4). In both soils, the fermented organic amendment and *B. thuringiensis* treatments significantly increased aggregate stability compared to their corresponding controls, the increases being more noticeable in the neutral soil, for both experimental factors (Fig. 1). In the neutral soil, the highest increase was observed in the amended soil followed by the soil inoculated with *B. thuringiensis*. However, in the acidic soil there were no significant differences between the two factors with regard to increasing soil structural stability. In both soils, the interaction of fermented sugar beet and *B. thuringiensis* produced even higher percentages of stable aggregates than each treatment applied separately (Table 4), recording increases of 38% in the acidic soil and of 106% in the neutral soil compared with their controls (Fig. 1).

3.4. Plant growth parameters

The addition of organic amendment stimulated significantly greater growth of the plants of *P. miliaceum* than did inoculation with *B. thuringiensis*, in both soils (Table 5). The effect of both factors depended significantly on soil type (Table 4). It is worth noting that only the organic amendment permitted an acceptable growth of the plants in the acidic soil. The increase in shoot biomass produced by the

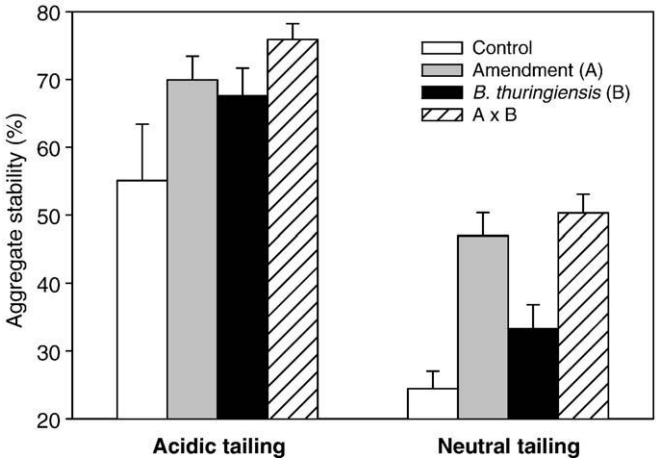

Fig. 1. Percentage of stable aggregates of rhizosphere soil of *P. miliaceum* in response to bacterial inoculation and fermented sugar beet addition four months after planting ($n=6$). Bars represent standard errors.

addition of the fermented organic amendment was higher in the acidic soil than in the neutral soil. The bacterial inoculation only produced a slight increase in the growth of plants in the neutral soil, but conferred an additional benefit when combined with fermented residue addition. The root biomass of plants grown in both soils was increased only by the addition of organic amendment, particularly in the neutral soil.

4. Discussion

4.1. Changes in structural stability of rhizosphere soil in response to organic amendment addition and bacterial inoculation

The present study confirms the ability of an autochthonous, metal-adapted bacterial strain and of microbially-treated sugar beet to promote soil aggregate stability of rhizosphere soil of *P. miliaceum* in two semiarid, degraded mine tailings. The two experimental factors assays differed markedly in their stabilising abilities and deserve further discussion.

The improvement of structural stability produced by the addition of an organic residue to soil is attributed to various organic cementing agents. These include the microorganisms themselves and the products of microbial synthesis and decomposition of organic materials (Lynch and Bragg, 1985). Polysaccharides, which are mostly by-products of the microbial activity developed in the C-containing substrate, are considered as the main temporary aggregate-stabilising agents (Roldán et al., 1994). The biological transformations that the SB underwent during fermentation increased the quantity of polysaccharides and available P. It is worth noting that in a previous experiment carried out in the both acidic and neutral tailings but without plant, we did not notice the effect of this residue on percentage of stable aggregates under watering conditions (Carrasco et al., 2009). Differential effect of the organic amendment on soil aggregate stability in presence of plants could indicate that the added microbial biomass positively interacted with the microbial population developed in the rhizosphere soil of *P. miliaceum*. Comparing both studies, we can also point that rhizosphere aggregate stability did not differ with that recorded in the soil without plant. These results support the fact that the microbial biomass associated with the rhizosphere of *P. miliaceum* was not too effective for the stabilisation of the soil structure of degraded mine tailings and so may be necessary to reactivate it by appropriate additions of organic amendments. In the current study, stimulation of rhizosphere microbial activity and growth prompted by the addition of the organic amendment could be responsible for the improvement of structural stability observed in the amended soils, as suggested by Roldán et al. (1994). Increased biological activity was revealed by the increases in dehydrogenase activity and the levels of labile C fractions of the amended soils. The metabolic potential (Masciandaro et al., 1998; Benitez et al., 1999), calculated as the ratio between the size and activity of the viable microbial community (dehydrogenase activity) and the sources of energy for microorganisms (water-soluble carbon concentration), was also increased by the addition of fermented sugar beet, in both tailings. The increased values of this index in the amended soils suggest the potential recovery of microbial metabolism.

The effectiveness of the organic amendment for increasing aggregate stability was notably higher in the neutral tailing than in the acidic one. This result reflects the fact that the ability of the organic amendment to act as a soil-aggregating agent is highly dependent on environmental pH. Initially, the two tailings differed greatly in the percentage of stable aggregates, it being higher in the acidic tailing. The size and stability of soil aggregates are related to clay and silt mineral composition and content (Caravaca et al., 2002). In fact, the highest contents of clay and silt were recorded in the soil with highest aggregate stability, i.e., in the acidic soil. The presence of iron oxide minerals in soils has also a favourable effect on soil physical properties, increasing aggregate stability. As already mentioned, these tailings are rich in iron oxides, particularly in the acidic tailings (Conesa, 2005). Solution chemistry influences the ionizable functional groups on iron (hydr)oxides, which has implications for the adsorption reaction. The extent to which Fe oxides stabilise soil aggregates is higher in acidic soils, since they are able to form bonds with negatively charged soil particles (Rhoton et al., 2003). Likewise, these soils presented a major proportion of Fe oxides, as mentioned above. The pH increased with the organic amendment treatment in both soils but this increase was not sufficient to induce a dispersion of the aggregates formed by Fe oxides in the acidic tailing, which could be due to that this tailing is also richer in clay particles. The differences in aggregate stability between the two tailings were reduced after the addition of organic amendment, as a consequence of greater improvement in the neutral tailing. It is likely that iron oxides are more important than added polysaccharides and reactivation of microbial activity, in terms of water-stable aggregates for acid soils. In contrast, the beneficial effect of the organic amendment on aggregation was

Table 5
Shoot and root biomass of *P. miliaceum* seedlings in response to bacterial inoculation and fermented sugar beet addition four months after planting.

	Shoot	Root
	(mg dw plant^{-1})	
Acidic tailing		
Control	2.2 ± 1.1a	6.4 ± 4.5a
Amendment (A)	200.8 ± 28.0b	54.0 ± 32.0b
B. thuringiensis (B)	2.6 ± 0.4a	17.2 ± 10.8a
A × B	185.7 ± 80.0b	71.5 ± 37.0b
Neutral tailing		
Control	3.9 ± 3.0a	4.0 ± 2.0a
Amendment (A)	134.0 ± 90b	46.7 ± 28.0b
B. thuringiensis (B)	4.7 ± 1.7a	4.1 ± 3.0a
A × B	225.0 ± 43.0b	65.0 ± 14.7b
	Shoot	Root
Amendment (A)	<0.001	<0.001
B. thuringiensis (B)	NS	NS
Soil (S)	NS	NS
A × B	NS	NS
B × S	NS	NS
A × S	0.009	NS
A × B × S	NS	NS

NS: not significant.
Mean ± standard error ($n=6$).
For each parameter and for each soil type, values in columns followed by the same letter are not significantly different (Tukey, $p<0.05$).

more pronounced in the neutral tailing because the contribution of Fe oxides as aggregating agents is less relevant when pH is neutral or alkaline.

The organic amendment was only clearly more effective than bacterial inoculum in the neutral tailing. However, in the acidic tailing the stabilising effect of the two experimental factors was similar. The combination of organic amendment and bacterial inoculation also produced higher increases in structural stability in the neutral soil than in the acidic soil. These differences may be related to different mechanisms involved in the aggregation and stabilisation of soil aggregates depending on environmental pH.

The ability of bacteria to be associated with soil mineral surfaces is known to be related to the production of extracellular polymeric substances (EPS) by such bacteria (Omoike and Chorover, 2006). It has been observed that many species from the genus *Bacillus*, such as *B. megaterium* (Davidson et al., 1996) and *Bacillus subtilis* (Omoike and Chorover, 2006), produce EPS. EPS functional groups are mostly protonated at pH <2, but become progressively negatively charged with increasing pH. Likewise, proton adsorption and desorption at mineral surface hydroxyl groups exhibit strong pH dependence. Omoike and Chorover (2006) recorded that the adsorption of EPS from *B. subtilis* to goethite decreased with increasing pH from 3 to 9 because of progressive proton dissociation of both goethite and ionizable EPS functional groups. The secretion of EPS, primarily composed of polysaccharides and proteins, is stimulated by the presence of toxic metals and may act as a "sticker", binding adjacent cells due to its adhesive nature (Fang et al., 2002; Guibaud et al., 2005). In addition to their great ability to complex heavy metals, it has been reported that EPS play a significant role in the formation and function of microbial aggregates, including adhesion phenomena, matrix structure formation and microbial physiological processes such as protecting cells against predation, desiccation (Roberson and Firestone, 1992) or harmful effects of heavy metals (Guibaud et al., 2005). We have demonstrated that our strain of *B. thuringiensis* produces EPS under culture conditions (data not shown) and presumably it may also have produced EPS in soil. These are probably composed primarily of proteins since no changes in the concentration of water-soluble carbohydrates were detected, in either soil. Fang et al. (2002) also found that the EPS produced by sulfate-reducing bacteria were enriched in proteins. The EPS produced by *B. thuringiensis* could be efficient with regard to increasing the percentage of stable aggregates only in the acidic tailing due to its suitable conditions of acidity.

In the neutral tailing, the improvement of structural stability produced by the inoculation with *B. thuringiensis* could be related to the increase in rhizosphere microbial activity observed in this soil. Indeed, in absence of plant other native bacterial species of *Bacillus* was tested and had no effect on soil aggregate stability in both mine tailings (Carrasco et al., 2009). Because, in both tailings, the inoculation of the metal-tolerant bacterium took place under famine conditions, the stimulation of microbial activity could have been mediated by the survival of the plants in the neutral tailing.

4.2. Influence of the treatments on plant growth

Only the organic amendment stimulated the growth of *P. miliaceum* plants, in both tailings. The effectiveness of fermented sugar beet could be not only due to an improvement in the soil structural stability, but also to the available nutrient supply in soil. During the course of *A. niger* fermentation, the rock phosphate solubilises, increasing the level of bioavailable P in the sugar beet (Vassilev et al., 1995). We have previously showed that the addition of this organic amendment increased the total N, available P and extractable K contents of the soil (Alguacil et al., 2003), favouring the plant growth. In contrast, root-induced changes in the rhizosphere soil may increase the solubility of metals, making them more available for plant uptake and/or leaching (Roulier et al., 2008). Organic amendments can decrease the plant-availability of heavy metals in soil by adsorption and by forming stable complexes with their organic matter (Shuman, 1999). Thus, the increased bioavailability of metals mediated by roots may be reduced following the addition of organic amendments. Recently, O'Dell et al. (2007) demonstrated that the application of yard waste compost improved the growth of *Bromus carinatus* on the acidic Cu–Zn minespoil primarily by reducing substrate bioavailable Cu and Zn concentrations. This depends upon the particular metal and soil type involved, and also upon the characteristics of the organic matter, particularly with respect to its degree of humification and its effect on soil pH (Walker et al., 2003). The addition of organic amendment increased the pH of both tailings which, in turn, decreased the solubility of some metals and stimulated plant growth. Previously, Medina et al. (2006) recorded that *Aspergillus*-treated sugar beet waste was effective for decreasing Zn accumulation in plants of *T. repens*. In this study, the effect of this type of residue on heavy metal fractionation in soil was pH-dependent. The addition of organic amendment was more effective in the acidic tailing than in the neutral tailing to decreasing heavy metal bioavailability, as indicated by soluble concentrations of Zn, Cu, Cd and Ni. In the acidic tailing, the organic amendment could have alleviated the potential toxicity of the heavy metals, decreasing their solubility in soil. In fact, the organic amendment was more effective for stimulating the growth of the *P. miliaceum* plants in the acidic soil than in the neutral soil. Taking into account that these tailings present a large spatial variability in pH, the effectiveness of this methodology for promoting the plant growth will depend on soil pH and on its ability for reducing the bioavailability of metals.

5. Conclusions

We can conclude that the microbially-treated sugar beet in combination with an autochthonous, metal-adapted bacterium proved to be an effective strategy for stabilising the structure of rhizosphere soil of *P. miliaceum* and for mitigating the risks of pollution dispersion in degraded semiarid mine tailings, although its effectiveness depended on environmental pH. The aggregate-stabilising effect of microbial polysaccharides and rhizosphere microorganism activity, stimulated by the organic amendment, was less relevant in the acidic soil than in the neutral soil. The mechanisms responsible for the improvement in structural stability promoted by bacterial inoculation are still unknown but the proliferation of the same bacterium and/or of other microorganisms in the rhizosphere soil seems to have been involved, at least in the neutral soil.

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