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Pseudometallophytes colonising Pb/Zn mine tailings: A description of the plant–microorganism–rhizosphere soil system and isolation of metal-tolerant bacteria

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ABSTRACT

The plant-microorganism-soil system of three pseudometallophytes (*Betula celtiberica*, *Cytisus scoparius* and *Festuca rubra*) growing in a Pb/Zn mine was characterised. Plant metal accumulation, soil metal fractions (rhizosphere and non-vegetated) and bacterial densities were determined. Total Cd, Pb and Zn in non-vegetated soils was up to 50, 3000 and 20,000 mg kg^{-1} dry weight, respectively. The residual fraction dominated non-vegetated soils, whereas plant-available fractions became important in rhizosphere soils. All plant species effectively excluded metals from the shoot. *F. rubra* presented a shoot:root transport factor of $\leq$0.2 and this population could be useful in future phytostabilisation trials. Culturable bacterial densities and diversity were low (predominantly *Actinobacteria*). Rhizosphere soils hosted higher total and metal-tolerant bacterial densities. Seventy-four metal-tolerant rhizobacteria were isolated, and characterised genotypically (BOX-PCR, 16S rDNA) and phenotypically [Cd/Zn tolerance, biosurfactant production and plant growth promoting (PGP) traits]. Several isolates resisted high concentrations of Cd and Zn, and only a few presented PGP traits. Fourteen isolates were evaluated for promoting plant growth of two species (*Salix viminalis* and *Festuca pratensis*). Thirteen inoculants enhanced growth of *F. pratensis*, while only three enhanced growth of *S. viminalis*. Growth enhancement could not always be related to isolate PGP traits. In conclusion, some isolates show potential application in phytostabilisation or phytoextraction techniques.

1. Introduction

Mining activities are known to be among the principal causes of soil contamination. The mine-spoils and tailings generated by this industry generally present hostile environments for plant growth, due to low nutrient availability, low organic matter content, high acidity and often elevated trace metal content [1,2]. Despite these unfavourable conditions, plant metallophytes have evolved biological mechanisms permitting them to resist and tolerate toxic concentrations of metals, and colonise this type of substrate [3]. Many studies have focused on these plants due to their potential use in the rehabilitation of metal-contaminated land or, more recently, due to their possible application in phytoremediation [4–6]. Phytoextraction aims to remove trace metals from the soil through their uptake and accumulation by plants; whereas phytostabilisation aims to establish a vegetation cover and promote in situ inactivation of metals. Metal-tolerant populations of common plant species (pseudometallophytes) are able to resist higher concentrations of metals compared with members of the same species growing on uncontaminated soils. On the other hand, true metallophytes have evolved over time on substrates derived from weathered mineral deposits [3]. Metallicolous populations of temperate grasses, such as *Agrostis capillaris*, *Agrostis gigantea* and *Festuca rubra*, have shown a good ability to colonize Pb-, Zn- and Cu-contaminated soils and have been successfully applied in phytostabilisation [7].

Metalliferous soils do not only provide sources of interesting flora but also of metal-tolerant microorganisms [2,8–10]. Like plants, these microorganisms have adapted to the extreme conditions and can aid the establishment and proliferation of colonising plant species [11,12]. The plant growth-promoting bacteria (PGPB) include phosphate and potassium solubilisers, the free living N_2-fixing bacteria, rhizobia etc. A growing number of studies suggest that the phytostabilisation process can be more effective after inoculating plants with PGPB, due to an enhanced plant metal tolerance, growth and survival [12–14]. A successful vegetative cover on

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multi-metal-contaminated mine tailings was achieved after inoculating native plant species with PGPB in combination with soil amendments [12]. The use of inoculants also reduced the requirement for amendments and associated economic costs. A substantial improvement in the growth of *Albizia lebbeck* was observed on gypsum mine soils due to inoculation with *Bradyrhizobium* sp. [15]. Inoculating *Lupinus luteus* with a bacterial consortium of metal resistant PGP rhizobacteria improved plant growth, and reduced plant metal accumulation, in a multi-metal-contaminated soil [16].

Plant-associated microorganisms can also influence the trace metal mobility and availability to plants through the release of chelating agents, acidification, phosphate solubilisation or redox changes. A microbial-mediated reduction in metal availability is particularly interesting from a phytostabilisation point of view. Pishchik et al. [17] suggested that Cd-tolerant plant growth-promoting rhizobacteria (PGPR) (*Arthrobacter mysorens* 7, *Flavobacterium* sp. L30, *Klebsiella mobilis* CIAM 880) migrated from the rhizoplane to the rhizosphere where they bound soluble Cd in biologically unavailable complexed forms. Metabolic processes of both plants and microorganisms can strongly affect trace metal behaviour in the rhizosphere [18]. Exploiting interactions between (pseudo)metallophytes and their associated microflora in the rhizosphere could assist in the further development of phytoremediation strategies. To do this, further studies characterising plants and their associated microorganisms in metalliferous sites are necessary.

The overall objective of this study was to characterise the plant-microorganism-soil system associated with dominant pseudometallophytes colonizing a Pb/Zn-mining area. As part of this objective we (a) evaluated the bioavailability and chemical fractionation of Cd, Pb, and Zn in the rhizosphere, and the accumulation of these metals by plants; (b) obtained a collection of metal-tolerant rhizobacterial isolates with potential application in phytoremediation; and (c) assessed the effects of selected rhizobacteria on growth of two plant species (*Festuca pratensis* Huds. and *Salix viminalis* L.) commonly used in phytoremediation strategies.

2. Materials and methods

2.1. Study site and sampling

This study was carried out in the abandoned Pb–Zn mine of Rubiales in the Lugo province of NW Spain (UTM 29T 660781/4726800). Metal deposits are rich in sulphides, principally in the form of sphalerite (ZnS) and galena (PbS) in a 7 to 1 ratio [19]. The mine operated from 1977 until the early 1990s, and during the 1980s the average annual production was 95000 t of zinc concentrates (with 61% Zn, 0.12% Cd and 0.16% Hg) and 15000 t of lead concentrates (with a 70% Pb content) [19]. The mine tailings cover a surface area of 30 ha, and large areas were re-planted with birch at the time of the mine closure. The surrounding area is characterised by the presence of sand stone, quartzite and slate, which alternate with limestone and dolomite [19]. The most frequent natural soils in the area are Alumi-umbric Leptosols and Regosols [20]. The climate of the region is Oceanic, with a mean annual precipitation of 2000 mm and annual temperature of 8–9 °C [21].

Plant and soil samples were taken in April 2010 at one of the most contaminated points of the mine tailings, where the vegetation cover is low with frequent bare areas [22]. The spontaneous vegetation at this point is predominantly *Cytisus scoparius* and *Betula celtiberica* and some gramineae. Five individuals of *C. scoparius* (L.) Link and *B. celtiberica* Rothm. & Vasc., and seven individuals of *F. rubra* L. were sampled. All three plants had spontaneously grown at the site, and in the case of *C. scoparius* and *B. celtiberica* only young plants (approximately <10 years) were sampled. Due to a severely stunted growth (<50 cm height), it was possible to collect the whole plant including the root ball. The rhizosphere soil was defined as the soil attached to roots after gentle crushing of the root ball and shaking the root system. In addition, surface soil samples (0–10 cm) were collected from bare patches where no plants were found growing (non-vegetated soil).

2.2. Soil and plant analyses

Soil analyses were carried out on the air-dried, <2 mm fraction of non-vegetated and rhizosphere soil samples. Soil pH was measured in H_2O and 0.1 M KCl using a 1:2.5 soil:solution ratio. Total C and N were analysed by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI), and dissolved organic C (DOC) was measured in a 1:5 soil/H_2O extract (1 h shaking) using a TOC-5000 total carbon analyser (Model FLOWSYS, SYSFEA, Italy). The carbonate fraction was determined following the Schleiber method [23]. Exchangeable cations were extracted with 1 M NH_4Cl. Sodium and K were determined by emission spectrophotometry; Ca and Mg by atomic absorption spectroscopy (AAS; PerkinElmer 2380, Norwalk, CT). Soils (0.5 g) were digested in a 3:1 mixture of concentrated HNO_3:HCl in Teflon PFA vessels in a microwave accelerated reaction system (MarsXpress;CEM Corp., USA) and total concentrations of metals were analysed by AAS. Water-soluble Cd, Pb and Zn concentrations were analysed by AAS with graphite furnace (PerkinElmer 4110 ZL) in soil extracts after 30 min shaking using a 1:2.5 soil:H_2O ratio. A metal fractionation scheme was carried out following a modified BCR protocol [24]. First, soils were shaken at room temperature with 1 M NH_4Cl for 16 h. This extracts the water-soluble and exchangeable metal fraction (exchangeable). Second, the resulting residue was shaken at room temperature with 0.11 M CH_3COOH for 16 h. This step extracts the carbonate-bound metal fraction (carbonate). Third, the residue was shaken for 16 h at room temperature with 0.10 M NH_2OH. HCl adjusted to pH 2.0 with high purity HNO_3. This extracts mainly iron and manganese oxide bound forms (reducible). Fourth, the residue was digested with 30% H_2O_2, taken to dryness on a water bath heated to 85 °C, and shaken with 1 M NH_4OAc adjusted to pH 2.0 with HOAc for 16 h. This step extracts primarily organically bound and sulphide metals (oxidisable fraction). Finally, the residual fraction (silicate-bound metals) was digested as above, and the concentration of Cd, Pb and Zn were analysed in the filtered supernatants of each extraction by AAS as above. All metal concentrations were expressed in $\mu g\,kg^{-1}$ or $mg\,kg^{-1}$ dry weight (DW) soil.

Shoots and roots of plants were separated, washed with pressurised tap water (and 0.05 M $CaCl_2$ in the case of roots) followed by deionised water, oven-dried at 45 °C and ground. Plant tissues (0.1 g) were digested in a 2:1 mixture of concentrated HNO_3:HCl on a hot plate at 160 °C, and the concentration of Cd, Pb and Zn were measured as above and expressed in $mg\,kg^{-1}$DW plant material. Shoot metal content, in the case of *B. celtiberica* individuals, refers to leaf concentrations. The transport factor (TF) was calculated as the ratio of metal concentrations in the shoots to that in the roots.

2.3. Determination of soil culturable bacterial densities and isolation of rhizobacterial strains

Four grams of fresh rhizosphere or non-vegetated soil were suspended in 16 ml sterile sodium hexametaphosphate solution (1%) and shaken for 30 min in an end-over-end shaker. Soil suspensions were diluted in 10-fold series and plated in duplicate onto modified 284 agar medium as described previously [25]. Densities of culturable metal-tolerant bacteria were determined in 284 medium supplemented with either, 0.1 mM or 1 mM of Cd (as $CdSO_4.8/3H_2O$) or, with 1 mM or 3 mM of Zn (as $Zn(SO_4)_2{\cdot}7H_2O$). After 7 days (28 °C), colony forming units were counted and

calculated per gram soil (CFUs g^{-1} soil). The rhizosphere effect was calculated as the ratio of the number of microorganisms in the rhizosphere over the number of microorganisms in the non-vegetated soil (R/NV). Distinct metal-tolerant morphotypes (1–5 colonies) associated with each plant species were sub-cultured at least three times and cryo preserved at −70 °C in culture medium supplemented with 15% (v/v) glycerol.

2.4. Phenotypic characterisation of rhizobacterial isolates

Rhizobacterial strains were screened for metal resistance using 284 agar medium supplemented with Cd (0, 0.5, 1.0, 2.0, 4.0, 5.0, 6.0 mM Cd) or Zn (0, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0 mM Zn). The maximal tolerable concentration (MTC) of Cd/Zn was recorded as the highest metal concentration in which the isolate grew. Rhizobacterial strains were also screened for the ability to produce biosurfactants, and for various PGP characteristics: phosphate solubilisation capacity, siderophore production and indoleacetic acid (IAA) production as described previously [25].

2.5. Genotypic characterisation of rhizobacterial strains

DNA was extracted from all purified bacterial strains as described previously [25]. Extracted DNA was amplified by PCR using the BOX A1R as described previously [25]. PCR products were separated by electrophoresis in agarose gels (1.8%) and ethidium bromide-stained gel images were analysed using the Gel Compar-Bionumerics programme (v5.1, Applied Maths, Belgium). Isolates were grouped according to their BOX-PCR profiles at a similarity level of 92%.

Amplification targeting the 16S rDNA gene was carried out as described previously on those strains showing distinct BOX-PCR profiles [25]. PCR products were purified and partially sequenced (approximately 600 bases) using the primer 16S-27F. Sequence data were checked using the Chromas v.1.45 software (Technelysium Pty. Ltd., Australia) and assessed for similarity with sequences of type strains in the Ribosomal Database Project [26]. Sequences are available in the EMBL database (www.ebi.ac.uk) under accession numbers HE585527–HE585572.

2.6. Influence of rhizobacteria on growth and metal uptake of F. pratensis and S. viminalis

A selection of the rhizobacterial strains were used in a pot experiment to evaluate their influence on plant growth and metal uptake by *F. pratensis* (metal-tolerant population; [27]) and *S. viminalis* (metal-tolerant population; unpublished data). The rhizobacterial strains were selected according to their MTC values and phenotypic traits (Table 3).

Two-week-old rooted cuttings of *S. viminalis* and seeds of *F. pratensis* were potted in perlite:quartz sand (2:1 v/v). After germination, *F. pratensis* were thinned out to four plants per pot. Plants were watered with half-strength Hoagland solution for two weeks until bacterial inoculation. Fresh cultures of bacterial strains were grown in 869 agar medium [28] for 3 days, harvested by centrifugation (6000 rpm, 15 min) and resuspended in 10 mM $MgSO_4$ to an OD_{660} of 1.0 (about 10^7 cells ml^{-1}). Each plant pot was inoculated with 9 ml of bacterial suspension. The same amount of sterile 10 mM $MgSO_4$ was added to non-inoculated pots. Seven replicates of each plant species were prepared for each inoculation treatment. After inoculation, plants were watered with half-strength Hoagland solution supplemented with 2 μM Cd and 100 μM Zn for seven weeks. Shoots and roots were then separated, washed in deionised water (0.05 M $CaCl_2$ followed by rinsing in the case of roots), oven-dried at 45 °C, weighed and ground. Plants were grown in an environmentally controlled growth chamber (16/8 h light/darkness, day/night temperature 26/20 °C, PPFD 190 μmol m^{-2} s^{-1}) and solutions replenished every 2 days. Oven-dried leaves were digested in a 2:1 HNO_3:HCl mixture and P, K, Ca, Mg, Fe, Cd, Cu, Mn and Zn measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed in mg kg^{-1} dry weight plant material.

2.7. Statistical analyses

Differences in soil physicochemical parameters, plant metal content and microbial densities were determined using analyses of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference test. Significant effects of bacterial strains on biomass production, nutrient and metal content in the plant inoculation experiment were determined using ANOVA followed by the "post-hoc" Dunnet test.

3. Results

3.1. Soil physicochemical properties, metal content and fractionation, and plant metal contents

Non-vegetated soils presented alkaline pH (pH 8.3) with an important carbonate fraction (10.4%), and low N content (<0.1%) (Table 1). Cation exchange capacity (CEC) was low (<10 $cmol_c$ kg^{-1}) and dominated by Ca. Rhizosphere soils presented higher CEC (up to 1.5-fold) and a higher DOC (up to 8-fold) compared to non-vegetated soil, but a similar total C content (1.5–2.0%). DOC increased in the order *F. rubra* < *C. scoparius* < *B. celtiberica*. On the contrary, pH values were lower in rhizosphere soils (pH 7.5–8.0) (Table 1). Total Cd, Pb and Zn in non-vegetated soil were close to 50 mg kg^{-1}, 3000 mg kg^{-1} and 20,000 mg kg^{-1}, respectively (Fig. 1a). In contrast, metal concentrations in the rhizosphere were consistently lower (1.5–2-fold), decreasing in the order *B. celtiberica* > *C. scoparius* ≥ *F. rubra* (Fig. 1a). Concentrations of water-soluble metals followed the same order as total concentrations but tended to be higher in the rhizosphere compared to non-vegetated soil (2.5–19-fold higher) (Fig. 1b).

The residual fraction was dominant in non-vegetated soils, representing more than 60% of total Cd and Zn, and almost 50% of total Pb (Fig. 1c). Of the non-residual fractions, Cd was detected in the exchangeable pool or oxidisable fraction (10 and 17%, respectively). Carbonate-bound and reducible fractions represented in this case a minor percentage of the total concentration (4 and 5%, respectively; Fig. 1c). After the residual fraction, the carbonate and oxidisable pools were the most dominant for Pb and Zn (22 and 25% for Pb, and 12 and 18% for Zn, respectively). Despite representing less than 1% of the total metal content, exchangeable Pb and Zn were important in terms of absolute values (20 mg kg^{-1} Pb and 257 mg kg^{-1} Zn).

In rhizosphere soils, the residual fraction was not always the dominant pool and the more labile metal fractions generally represented a higher % than in non-vegetated soil (Fig. 1c). Metal fractionation was similar between the rhizosphere of the three species. Cd was principally in an exchangeable form, representing 30% of total Cd in the rhizosphere of *F. rubra* and *C. scoparius*. In the rhizosphere of *B. celtiberica*, Cd was associated with the exchange complex or carbonate-bound (15% of total Cd in both cases). Zn was mainly associated with carbonates (22–29% of total Zn), while Pb was associated with both carbonates and the oxidisable fraction (representing 24–32% and 29–32% of total Pb, respectively; Fig. 1c).

Shoot concentrations of Cd and Pb did not differ significantly between species, ranging from 5.8 to 6.9 mg kg^{-1} Cd and 69 to 139 mg kg^{-1} Pb (Fig. 2). In the case of Zn, leaves of *B. celtiberica*

Table 1
Physicochemical properties (mean ± SE) of non-vegetated (n = 5) and rhizosphere soils (n = 5–7).

	Non-vegetated soil	Rhizosphere soils		
		F. rubra	*C. scoparius*	*B. celtiberica*
pH_{H_2O}	8.3 ± 0.1c	8.0 ± 0.1b	7.9 ± 0.1b	7.5 ± 0.0 a
pH_{KCl}	7.6 ± 0.1b	7.7 ± 0.1b	7.6 ± 0.1b	7.2 ± 0.1 a
Total organic C (mg l^{-1})	6.3 ± 0.6a	23.6 ± 4.2b	41.8 ± 11.9c	51.9 ± 6.0 c
Carbonates (%)	10.4 ± 0.1	n.d.	n.d.	n.d.
C (%)	1.9 ± 0.3a	1.9 ± 0.1a	1.5 ± 0.4a	2.0 ± 0.0 a
N (%)	<D.L.	<D.L.	<D.L.	<D.L.
Exchangeable cations ($cmol_c$ kg^{-1} DW)				
Ca^{2+}	9.1 ± 0.1	12.3 ± 0.7	12.4 ± 2.1	10.1 ± 0.3
Mg^{2+}	0.3 ± 0.01	0.4 ± 0.03	0.9 ± 0.3	0.4 ± 0.04
Na^{+}	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.04	0.2 ± 0.03
K^{+}	0.01 ± 0.0	0.06 ± 0.01	0.2 ± 0.05	0.17 ± 0.03
CEC	9.4 ± 0.2a	12.9 ± 0.7b	13.7 ± 2.4b	10.9 ± 0.3 ab
Ca/Mg	33 ± 1	30 ± 2	16 ± 3	23 ± 3

Within each row, different letters indicate significant differences at $p < 0.05$. "n.d", not determined.

accumulated significantly more Zn than either *C. scoparius* or *F. rubra* (1935 mg kg^{-1} compared to 1205 mg kg^{-1} and 1037 mg kg^{-1}). However, all three species excluded Cd, Pb and Zn from their shoot tissues (Fig. 2). TF values were consistently less than 1, especially in the case of Pb (≤0.2). This excluder characteristic was particularly evident in *F. rubra*, which presented the highest root concentrations (34 mg kg^{-1} Cd, 1308 mg kg^{-1} Pb and 5485 mg kg^{-1} Zn) and lowest shoot:root ratios (≤0.2) for all 3 metals. In contrast, the highest TF values were observed in *B. celtiberica* [Cd (0.4) and Zn (0.8)].

3.2. Abundance of culturable bacteria, phylogenetic affiliation and phenotypic traits

In non-vegetated soil, a mean microbial density of 3.2 ± 0.1 log CFUs g^{-1} soil was determined. Densities of Cd- and Zn-tolerant bacteria (cultivated in medium with 0.1 mM Cd or 1.0 mM Zn) were lower and represented 22% and 10% of the total culturable abundance, respectively. This percentage was reduced to less than 5% of the total population when the metal concentration was increased to 1.0 mM Cd or 3.0 mM Zn (Table 2).

Microbial densities were higher in the rhizosphere of all the three plant species compared to non-vegetated soil (in many cases by one or two logarithmic units; Table 2). This was the case for both the total (R/NV ratios of 5–40) and metal-tolerant populations (R/NV ratios of 1–1265). *C. scoparius* hosted the highest bacterial densities in the rhizosphere. Abundance of bacteria cultivated in medium supplemented with 0.1 mM Cd tended to be higher than the total population in Cd-free medium. Bacterial densities were reduced at the higher Cd concentration (1.0 mM) or at both concentrations of Zn (1.0 and 3.0 mM) (Table 2). However, this toxic effect of metals was less evident in the plant rhizosphere than in non-vegetated soil. At 1.0 mM Cd, rhizobacterial densities represented 14, 19 and 25% of the total population in *F. rubra*, *C. scoparius* and *B. celtiberica* respectively, compared to 4% in non-vegetated soil. At 1.0 mM Zn, rhizobacterial densities represented 61, 39 and 34% of the total population in *F. rubra*, *C. scoparius* and *B. celtiberica* respectively, compared to 10% in non-vegetated soil. This percentage was reduced to <7% at 3.0 mM Zn in all three species, reaching values similar to those recorded in non-vegetated soil.

A total of 74 metal-tolerant rhizobacterial strains were isolated. According to their BOX-PCR profiles, isolates were allocated into 46 distinct groups (Table 3). Thirty-seven of these were represented by only one isolate. BOX-PCR groups containing more than one bacterial strain were generally composed of strains isolated from the same host plant (e.g. all 6 isolates from group B02 were from *F. rubra*, while in B26 all four isolates were from *C. scoparius*). In contrast, some groups (e.g. B15 and B30) included strains with the same BOX profile but isolated from different plant species i.e. these strains are associated with more than one plant species. Rhizobacterial strains were primarily affiliated with the Phylum *Actinobacteria*. At the genus level, the majority belonged to the genera *Streptomyces* (61%), *Tsukamurella* (18%) or *Pseudomonas* (18%). In terms of Cd and Zn tolerance, the isolate collection can be divided into two main groups. In the first group (Box groups 1–24) isolates showed a lower metal tolerance, with MTC values of <0.5–2 mM for Cd, and <0.5–2.5 mM for Zn. In the second group, (Box groups 25–46) MTC values increased to 4–5 mM for Cd, and 2.5–25 mM for Zn. Isolates belonging to the *Pseudomonas* genera were all included in the first group with lower MTC values. On the contrary, all of the isolates belonging to the *Tsukamurella* genera were included in the second group with higher MTC values. Rhizobacterial isolates classified as *Streptomyces* were interspersed among both groups.

Thirty eight percent of the isolates presented at least one PGP trait and/or the ability to produce biosurfactants. Twenty percent of the isolates produced biosurfactants. Fifteen percent were able to solubilise inorganic phosphate, mainly identified as members of the genera *Pseudomonas*. Only 6 strains were siderophore-producers and none were isolated from *F. rubra*. Only two strains were IAA-producers (P87, *Massilia niastensis*, 98.0% similarity; P30, *Rhodococcus erythropolis*, 99.2% similarity).

Table 2
Microbial densities (mean ± SE) of total and metal-tolerant culturable bacteria (log CFUs g^{-1} soil) in non-vegetated (n = 5) and rhizosphere soils (n = 5–7).

	Non-vegetated soil	Rhizosphere soils		
		F. rubra	*C. scoparius*	*B. celtiberica*
284	3.2 ± 0.1a	3.9 ± 0.6ab	4.8 ± 0.5b	4.2 ± 0.4 ab
284 + Cd 0.1 mM	2.4 ± 0.2a	4.4 ± 0.5b	5.5 ± 0.2b	4.6 ± 0.4 b
284 + Cd 1 mM	1.4 ± 0.5a	3.2 ± 0.7b	4.5 ± 0.4b	3.6 ± 0.3b
284 + Zn 1 mM	2.1 ± 0.2a	3.8 ± 0.3a	4.1 ± 0.8a	2.8 ± 1.1a
284 + Zn 3 mM	1.6 ± 0.1a	2.6 ± 0.9a	1.8 ± 1.0a	1.5 ± 0.9a

Within each row, different letters indicate significant differences at $p < 0.05$.

Table 3
BOX-PCR fingerprints, partial 16S rDNA sequence identification, and phenotypic characteristics of bacterial isolates from the rhizosphere of *F. rubra* (F), *C. scoparius* (C), and *B. celtiberica* (B). Isolates used in the plant inoculation experiment are highlighted in bold.

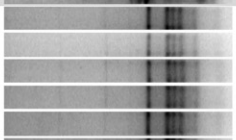
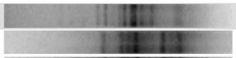
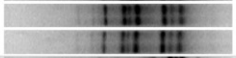
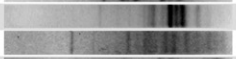
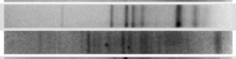
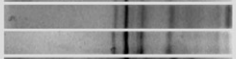
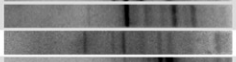
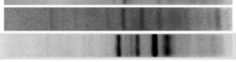
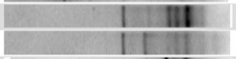
Most similar type strain (% similarity)	Group	Box Profile	Isolate	Host	Cd	Zn	PO_4	Surf	Sid	IAA
Massilia niastensis EU808005 (98.0)	B01		**P87**	F	0.5	1	-	-	-	+
Pseudomonas costantinii AF374472 (99.7)	B02		P36	F	0.5	<0.5	+	+	-	-
			P39	F	0.5	0.5	-	+	-	-
			P28	F	0.5	<0.5	+	+	-	-
			P29	F	0.5	0.5	+	+	-	-
			P31	F	0.5	2.5	+	+	-	-
			P33	F	0.5	0.5	+	+	-	-
Pseudomonas costantinii AF374472 (99.7)	B03		P34	F	0.5	1	+	+	-	-
Pseudomonas lini AY035996 (98.9)	B04		P24	F	<0.5	0.5	+	+	-	-
Pseudomonas koreensis AF468452 (98.8)	B05		**P35**	F	0.5	0.5	+	-	-	-
Pseudomonas lurida AJ581999 (98.3)	B06		**P42**	B	1	5	+	-	-	-
			P41	B	0.5	0.5	+	-	+	-
			P48	B	1	2.5	-	-	-	-
Pseudomonas reinekei AM293565 (99.0)	B07		P18	C	1	0.5	-	-	+	-
Acinetobacter ursingii AJ275038 (99.8)	B08		P23	F	2	2.5	-	-	-	-
Streptomyces canus AY999775 (100)	B09		P84	F	<0.5	2.5	-	-	-	-
Streptomyces umbrinus AB184305 (96.2)	B10		**P65**	C	1	2.5	-	-	+	-
Streptomyces canus AY999775 (99.3)	B11		P66	C	1	2.5	-	-	-	-
Streptomyces alboniger AY845349 (97.2)	B12		P86	F	<0.5	1	+	-	-	-
Pseudomonas rhizosphaerae AY152673 (98.5)	B13		P38	F	0.5	1	+	+	-	-
Streptomyces canus AY999775 (99.2)	B14		P83	F	1	5	+	-	-	-
Streptomyces canus AY999775 (99.4)	B15		P80	F	1	1		-	-	-
			P45	B	1	2.5	-	-	-	-
			P73	C	0.5	2.5	-	-	-	-
			P68	C	1	2.5	-	-	-	-
			P69	C	1	2.5	-	-	-	-
			P67	C	0.5	2.5	-	-	-	-
			P40	B	2	2.5	-	-	-	-
Streptomyces canus AY999775 (99.0)	B16		P70	C	1	2.5	-	-	-	-
Streptomyces canus AY999775 (99.7)	B17		P49	B	0.5	1	-	+	-	-
Streptomyces canus AY999775 (99.6)	B18		P63	C	0.5	2.5	-	-	+	-
Streptomyces canus AY999775 (99.2)	B19		P89	F	0.5	2.5		-	-	-
Streptomyces canus AY999775 (98.2)	B20		P85	F	<0.5	2.5	+	-	-	-
			P82	F	1	2.5		-	-	-
Streptomyces canus AY999775 (99.6)	B21		P81	F	1	2.5	-	-	-	-
Streptomyces phaeochromogenes AB184738 (99.5)	B22		P37	F	2	5	-	-	-	-
Streptomyces floridae AB184656 (99.7)	B23		**P12**	C	1	25	-	-	-	-
			P13	C	2	25	-	-	-	-
Streptomyces floridae AB184656 (100)	B24		P50	C	2	25	-	+	-	-
Streptomyces coelescens AF503496 (97.7)	B25		P79	F	1	2.5	-	-	-	-
			P76	F	4	5	-	-	-	-
Streptomyces afghaniensis AB184847 (100)	B26		P07	C	5	5	-	-	-	-
			P08	C	1	2.5	-	-	-	-
			P09	C	5	5	-	-	-	-
			P10	C	5	5	-	-	-	-
Streptomyces arenae AJ399485 (97.9)	B27		P46	B	4	5	-	-	-	-
Streptomyces arenae AJ399485 (99.0)	B28		P43	B	4	5	-	-	-	-
Streptomyces arenae AJ399485 (97.6)	B29		P55	B	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.1)	B30		P51	B	4	5	-	-	-	-
			P56	B	4	25	-	-	+	-
			P25	F	4	25	-	-	-	-
			P88	F	6	15	-	-	-	-
Streptomyces coelescens AF503496 (97.7)	B31		P32	F	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.6)	B32		P53	B	4	25	-	-	-	-
Streptomyces canus AY999775 (99.5)	B33		P15	C	4	5	-	-	-	-
Streptomyces coelescens AF503496 (98.0)	B34		P52	F	4	5	-	-	-	-
Streptomyces coelescens AF503496 (99.0)	B35		**P71**	C	4	10	-	+	-	-
Streptomyces coelescens AF503496 (97.0)	B36		**P54**	B	4	25	-	-	-	-
Streptomyces coelescens AF503496 (98.7)	B37		**P26**	F	4	25	-	-	-	-
Streptomyces coelescens AF503496 (98.2)	B38		**P64**	C	5	5	-	-	+	-
Rhodococcus erythropolis X79289 (99.2)	B39		**P30**	F	4	2.5	-	-	-	+
Tsukamurella tyrosinosolvens AY238514 (99.9)	B40		P14	C	4	10	-	-	-	-
Tsukamurella spumae Z37150 (99.9)	B41		P17	C	4	10	-	-	-	-

Table 3
(*Continued*).

Tsukamurella spumae Z37150 (99.6)	B42	P11	C	4	10	-	-	-	-
Tsukamurella paurometabola AF283280 (98.0)	B43	P16	C	5	25	-	-	-	-
		P27	F	5	25	-	-	-	-
		P74	F	4	10	-	+	-	-
		P75	F	5	25	-	+	-	-
Tsukamurella strandjordii AF283283 (100)	B44	P78	F	4	15	-	+	-	-
		P90	F	4	15	-	-	-	-
		P20	F	5	25	-			-
		P21	F	5	25	-	-	-	-
Tsukamurella strandjordii AF283283 (99.8)	B45	P19	F	5	25	-	-	-	-
Tsukamurella strandjordii AF283283 (100)	B46	P22	F	6	25		-	-	-

Cd, MTC mM Cd; Zn, MTC mM Zn; PO_4, phosphate solubilisation capacity; Surf, biosurfactant producer; Sid, siderophore producer; IAA, indoleacetic acid producer.

3.3. *Re-inoculation of metal-tolerant* F. pratensis *and* S. viminalis

Fourteen strains were selected (seven from each MTC group) for a re-inoculation experiment with *F. pratensis* and *S. viminalis* plants (Table 3). In both plant species, those strains which had a positive (or negative) effect on shoot biomass had a similar effect on root biomass (R^2 0.87; $p < 0.01$). In general, inoculating *F. pratensis* plants with the rhizobacterial strains improved plant growth (Fig. 3a). Strains with a positive effect increased shoot biomass by 1.3–1.9-fold, and root biomass by 1.3–2.6-fold, compared to non-inoculated plants. Plants inoculated with the strain P87

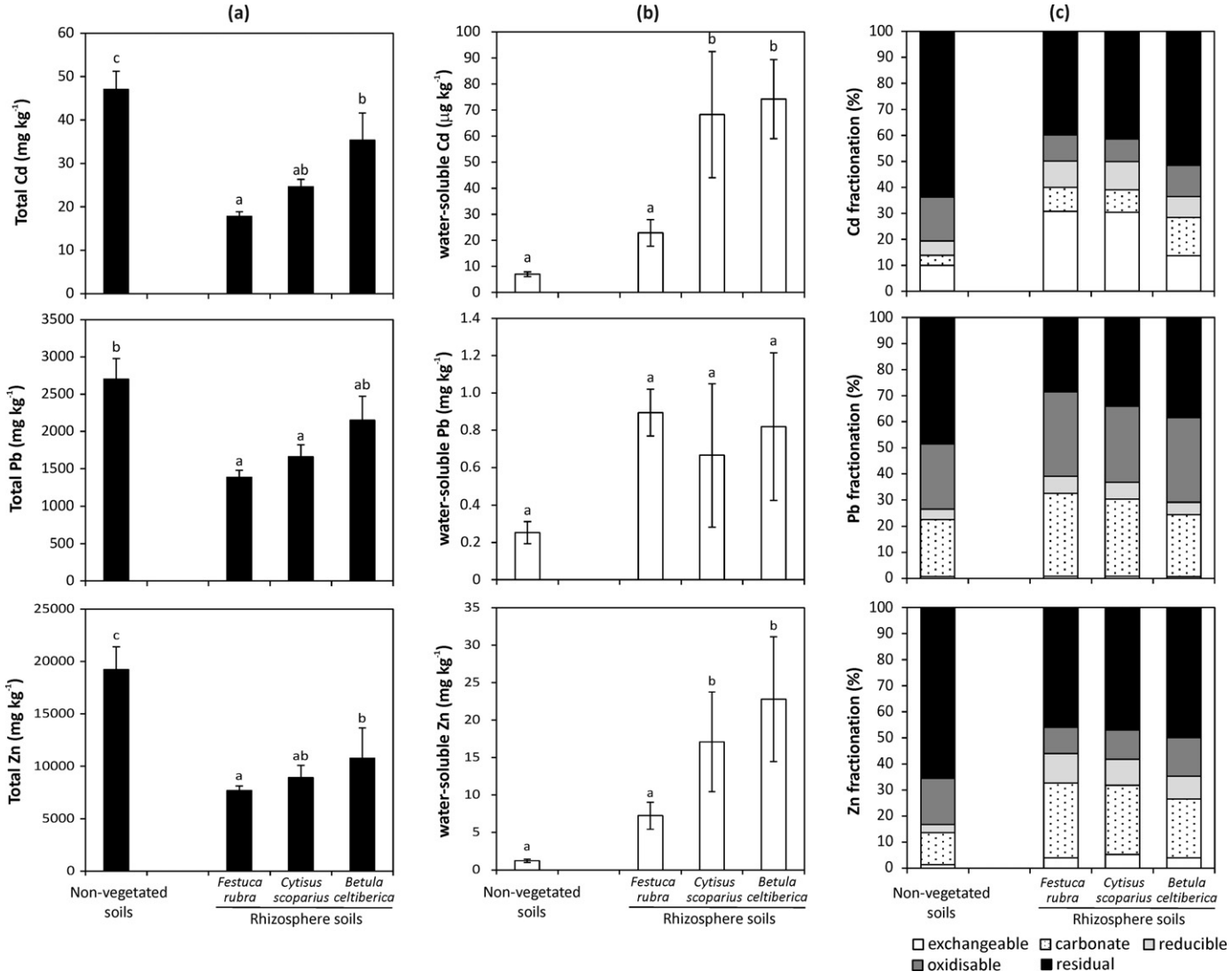


Fig. 1. (a) Total concentrations (mean ± SE), (b) water-soluble concentrations (mean ± SE), and (c) chemical fractionation (percentage distribution) of Cd, Pb, and Zn in non-vegetated ($n = 5$) and rhizosphere soils ($n = 5$–7). Metal concentrations are expressed on the basis of soil dry weight. Different letters indicate significant differences ($p < 0.05$). LOQ: Cd, Pb, Zn, 5.0 mg kg^{-1} (total concentration). LOQ: Cd, 1.0 μg kg^{-1}; Pb, 0.0012 mg kg^{-1}; Zn, 0.012 mg kg^{-1} (water-soluble concentrations). LOQ: Cd, Pb, Zn, 1.0 mg kg^{-1} (chemical fractionation).

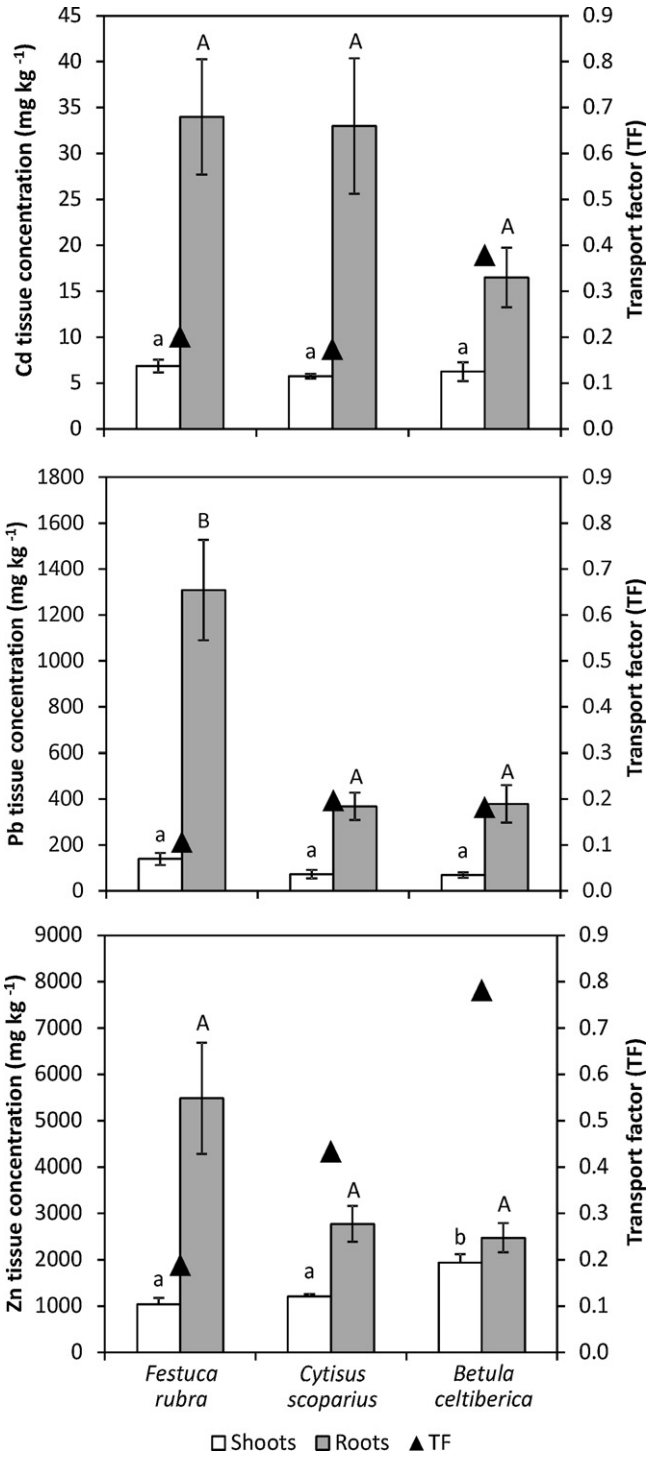


Fig. 2. Metal concentrations (mean ± SE) in shoot and root tissues of plants and shoot:root metal concentration ratio. Metal concentrations are expressed on the basis of tissue dry weight. Different letters indicate significant differences ($p < 0.05$). LOQ: Cd, 1.0 mg kg^{-1}; Pb, Zn 5.0 mg kg^{-1}.

(*M. niastensis*; 98.0% similarity) showed the highest shoot dry weight, whereas those inoculated with strains P12 or P42 (*Streptomyces floridae*; 99.7% similarity; and *Pseudomonas lurida*; 98.3% similarity, respectively) showed the highest root biomass. Strain P54 (*Streptomyces coelescens*; 97.0% similarity) was the only strain which negatively affected growth of *F. pratensis* (both shoot and root biomass; Fig. 3b). Bacterial inoculants did not significantly influence leaf concentrations of macronutrients, such as Ca, Mg, and K: values ranging from 54.1 to 68.9 g kg^{-1} K, 5.8 to 7.2 g kg^{-1} Ca and 5.1 to 6.1 g kg^{-1} Mg (Fig. S1). On the other hand, inoculants tended to reduce leaf Fe content (all inoculants except P30), and in some cases P (P12, P26, P42, P64, P75, P87) and Mn (P42 and P87) (although not significantly). Cd leaf concentrations varied from 7.0 to 10.2 mg kg^{-1} and Zn from 241 to 359 mg kg^{-1}. In general, plants tended to accumulate less Cd and Zn in shoots compared to non-inoculated plants.

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In the case of *S. viminalis*, the majority of strains reduced growth compared to non-inoculated plants (Fig. 3b). This was observed for 9 bacterial strains (P12, P26, P29, P41, P54, P56, P65, P71). On the other hand, strains P30 (*R. erythropolis*; 99.2% similarity), P35 (*Pseudomonas koreensis*; 98.8% similarity), P42 (*Pseudomonas lurida*; 98.3% similarity), P64 (*Streptomyces coelescens*; 98.2% similarity) and P87 (*M. niastensis*; 98.0% similarity) enhanced growth of *S. viminalis* (only significant for P87). As for *F. pratensis*, the bacterial inoculants did not have a significant effect on the nutrient content of *S. viminalis* (leaf tissues). However, they tended to increase Ca, Mg, Mn and in some cases P contents (Fig. S2). Cd and Zn bioaccumulation tended to be similar or slightly lower than in non-inoculated plants. Cd leaf concentrations varied from 9.0 to 15.8 mg kg^{-1} and Zn from 217 to 324 mg kg^{-1}.

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4. Discussion

In accordance with the presence of sulphides (principally galena and sphalerite) the sampling site was highly polluted with Pb, Zn and Cd. Total metal contents around this site have previously shown a high level of heterogeneity [22], a characteristic which is also typical of many mine tailings [1,9,29]. Diez Lázaro [22] found values ranging from 2 to 95 mg kg^{-1} for Cd, 46 to 6100 mg kg^{-1} for Pb, and 340 to 52,000 mg kg^{-1} for Zn. The concentrations of Cd, Pb and Zn, exceeded official threshold values established by the Galician government [30]. Although these thresholds are based on total concentrations, in the case of Cd even the more plant-available fractions exceeded permitted values. In addition, since a considerable amount of Pb and Zn are associated with carbonates these two metals are also potentially bioavailable.

Total metal concentrations in rhizosphere soils were always lower than in non-vegetated soil. Obviously, this does not indicate that the plants are able to reduce total metal concentrations, but rather that they are capable of colonising "less" contaminated spots whenever possible. Of the three species, *B. celtiberica* appears to be the most tolerant. Nonetheless, all three species are capable of growing in highly contaminated soils (20–40 mg kg^{-1} of Cd, 1500–2500 mg kg^{-1} of Pb and 7000–10,000 mg kg^{-1} of Zn). Although all three species behaved as excluder plants, the shoot metal concentrations exceeded phytotoxic concentration thresholds proposed by Kabata-Pendias and Pendias [31] (5–30 mg kg^{-1} Cd; 30–300 mg kg^{-1} Pb and 100–400 mg kg^{-1} Zn). In fact, the plants showed visible signs of toxicity in the field, such as an extremely stunted growth. Shoot metal concentrations were in the same range as those obtained by other authors in native vegetation of Pb–Zn mine spoils [6,32–34]. Metal accumulation did not differ between plant species, except for Zn which was more accumulated in the leaves of *B. celtiberica*. In agreement, water-soluble concentrations of this element were also highest in the rhizosphere of this species. The relative increase in plant-available metal pools in the rhizosphere is presumably an indirect consequence of root exudation of organic compounds, the reduction in soil pH and dissolution of mineral phases (e.g. carbonates). The genus *Betula* includes several

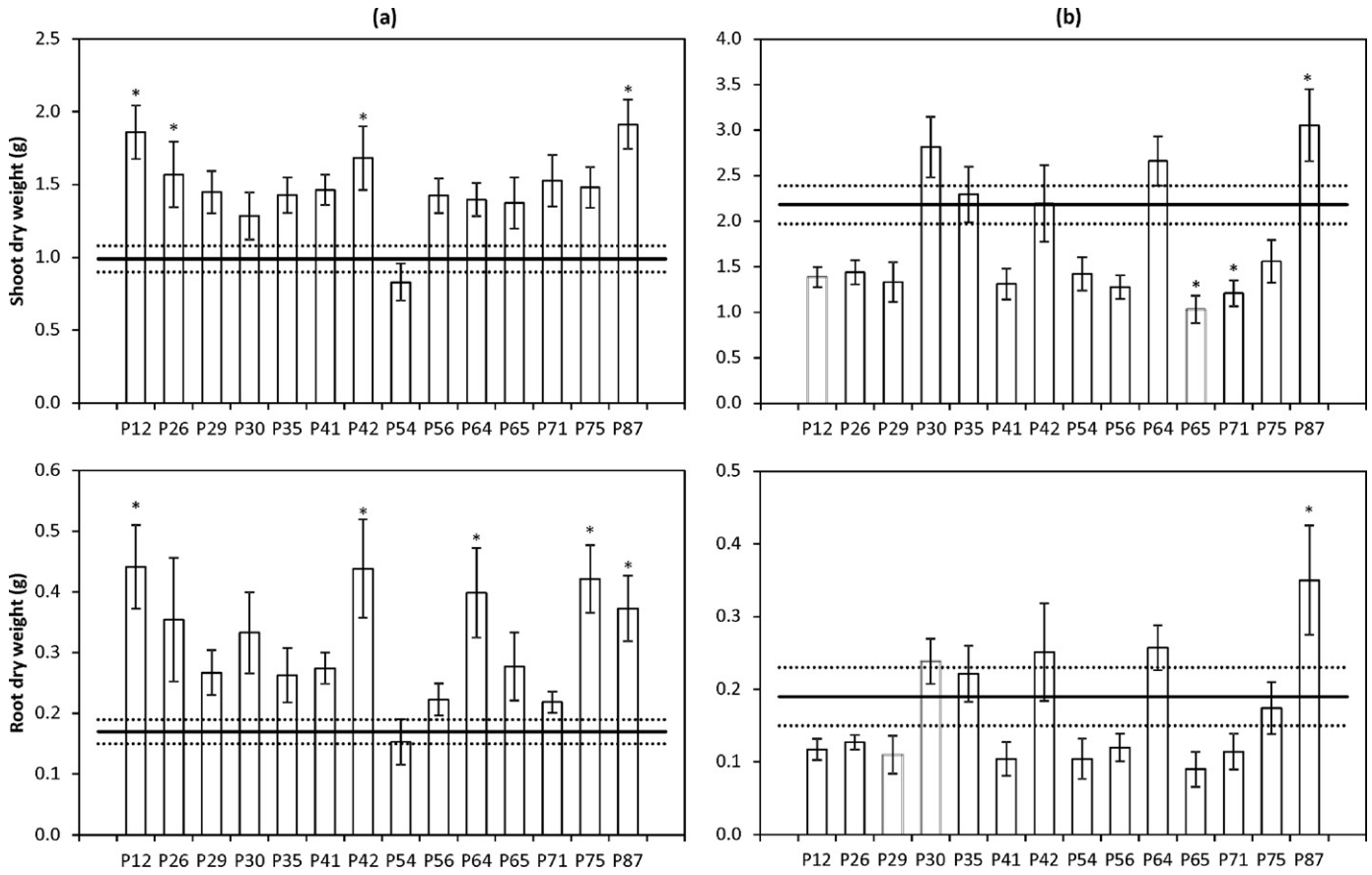

Fig. 3. Shoot and root dry weight (mean ± SE) of (a) *F. pratensis* and (b) *S. viminalis*. Values of non-inoculated controls are indicated by a continuous line (±SE (broken lines)). Asterisks indicate significant differences from the control ($p < 0.05$).

pioneer species which are often found on soils contaminated with trace metals [35]. In contrast, water-soluble metal concentrations were not significantly increased in the rhizosphere of *F. rubra*, and the TF values of this species suggest it to be efficient at excluding metals from its above ground biomass. This population could be a promising candidate for application in phytostabilisation trials but should be combined with appropriate soil amendments and/or bacterial inoculants.

Numerous studies have shown a reduction in the density, metabolic activity and diversity of microbial communities after long-term exposure to trace metals [36–39]. Toxic concentrations of metals typically induce a shift in species composition and the selection of metal-tolerant microorganisms. The density of culturable bacteria observed in the non-vegetated spots at this sampling site was extremely low and, as expected, the rhizosphere always harboured higher bacterial densities. This rhizosphere effect is probably due to the higher concentration of DOC in rhizosphere soil compared to non-vegetated soil. Moreover, all three plant species hosted higher metal-tolerant populations, and these rhizobacteria tolerated higher concentrations of bioavailable metals. Some isolates resisted up to 6 mM Cd and 25 mM Zn, concentrations which are similar to those tolerated by rhizobacteria and endophytes associated with *Salix caprea* trees from a Zn/Pb mining site in Austria [40]. Although there are contradictions, several studies have also found Gram-positive bacteria, and in particular the *Actinobacteria*, to dominate culturable bacterial collections from trace metal-contaminated soils [11,41,42]. Over 90% of the studied culturable bacteria in Pb–Zn mine-soils were affiliated with the *Arthrobacter* genus [11]. In the present study only metal-tolerant strains were sub-cultured for identification which is likely to have led to an underestimation of bacterial diversity. Moreover, since the culturable microbial community represents <1% of the actual diversity further studies using culture-independent approaches would be useful.

For the re-inoculation experiment plant species were selected to represent either phytoextraction or phytostabilisation scenarios. Metal-accumulating *Salix* species have been shown to be ideal extractor plants due to their high biomass production and extensive root system, whereas numerous *Festuca* species have been successfully implemented in long-term field trials of phytostabilisation [7,40]. Here, the effects of the bacterial strains were dependent on the plant species. One of the most contrasting effects was in the case of strain P12 which improved shoot biomass of *F. pratensis* but had the opposite effect on *S. viminalis*. Grandlic et al. [12] found the effect of bacterial strains on the biomass of *Atriplex lentiformis* and *Buchloe dactyloides* was both plant- and substrate-dependent. Nonetheless, five of the tested strains (P30, P35, P42, P64 and P87) had a positive effect on the growth of both plant species. For phytostabilisation, inoculating a metal-excluding population of gramineae with these rhizobacteria could achieve a healthy vegetation cover in a shorter period of time. On the other hand, in the case of phytoextraction a simple increase in biomass production leads to an increase in the overall metal yield. Strains P87, P30 and P64 did not induce a higher accumulation of Cd or Zn in leaves of *S. viminalis* but the microbial-induced increase in biomass production results in an increase in the total metal phytoextracted. Total Cd and Zn extracted by *S. viminalis* (metal accumulated in leaves × leaf biomass production) tended to be higher after inoculating with P87 (mean phytoextracted Cd increased from 59 to 69 μg, and Zn from 1426 to 1768 μg). These results suggest that some of the plant-associated bacteria isolated from this site could be exploited for improving plant growth and performance in metal-contaminated

soils. However, further studies are necessary to ascertain their effect on plant growth, nutrient status and metal accumulation in real-life metal-contaminated soils. Furthermore, the ability of these strains to modify soil metal mobility needs to be evaluated. Some of the PGP traits tested here could influence metal bioavailability in soils. Siderophores can complex a variety of heavy metal ions, and biologically produced surfactants have recently been shown to enhance metal removal from contaminated soils [43]. The ability to produce biosurfactants was the most common trait observed in the isolate collection. Rhizosphere bacteria associated with *S. caprea* have been shown to mobilise or immobilise metals, influencing plant metal accumulation or exclusion [40,44].

Only a few isolates were able to produce siderophores, and none of these were isolated from *F. rubra*. This is perhaps not surprising since gramineae are known producers of phytosiderophores and recruiting siderophore-producing bacteria in their rhizosphere may not be advantageous [45]. However, this trait could be valuable in this type of mine-soil due to limited Fe availability at alkaline pH values. The production of IAA is also known to significantly improve plant growth and biomass production. Many authors have attributed bacterial-induced increases in plant growth in the presence of metals due to the production of this phytohormone [46,47]. Only two strains were capable of producing IAA (P30 and P87), and both enhanced biomass production in *F. pratensis* and *S. viminalis*. However, microbial-induced improvements in plant growth could not always be related to the isolate PGP characteristics. For example, both isolates P56 and P64 are siderophore-producers, and both belong to the genus *Streptomyces*, but they caused contrasting effects in *S. viminalis* growth. Similarly, isolates P41 and P42 are P-solubilisers, but again induced contradicting effects on the growth of this species. It is evident that mechanisms other than those studied here must be involved in this growth enhancement. The environmental conditions to which an inoculant is exposed will influence whether or not certain PGP traits are activated. The screening method used here allows for a rapid selection of interesting strains, however in a hydroponic system (with an adequate nutrient supply) mechanisms of P or Fe acquisition may not be induced. These traits are more likely to be induced in a plant-microbe-soil system, and particularly in mine-soils with nutrient deficiency and toxic concentrations of metals. In such a system the tendencies towards biomass enhancement or the improvement in plant nutrient status which were observed here may be more pronounced.

5. Conclusions

The pseudometallophytes studied were identified as metal excluders: of the three species, *B. celtiberica* tolerated the highest soil metal concentrations and *F. rubra* was the most efficient metal excluder. An increase in labile metal concentrations in the rhizosphere was associated with a higher metal-tolerant bacterial population. We obtained a collection of rhizobacterial isolates, and several of these strains could be potentially useful for improving plant growth and establishment in trace metal-contaminated soils. This plant growth promotion could lead to a healthy vegetation cover in the phytostabilisation of heavily contaminated soils, or alternatively may be an important parameter in improving the metal extraction capacities of plants in the phytoextraction of metal-contaminated soils.

Acknowledgments

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