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Ecosystem services and plant physiological status during endophyte-assisted phytoremediation of metal contaminated soil

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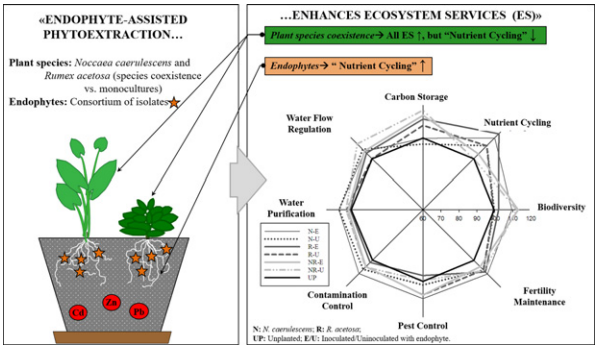
HIGHLIGHTS

- An endophyte-assisted phytoextraction study was performed.
- *N. caerulescens* and *R. acetosa* grew more and extracted more Zn when grown together.
- Inoculation of the endophytes improved plant physiological status.
- Plant growth and endophyte inoculation enhanced ecosystem services.

GRAPHICAL ABSTRACT



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ABSTRACT

Mining sites shelter a characteristic biodiversity with large potential for the phytoremediation of metal contaminated soils. Endophytic plant growth-promoting bacteria were isolated from two metal-(hyper)accumulator plant species growing in a metal contaminated mine soil. After characterizing their plant growth-promoting traits, consortia of putative endophytes were used to carry out an endophyte-assisted phytoextraction experiment using *Noccaea caerulescens* and *Rumex acetosa* (singly and in combination) under controlled conditions. We evaluated the influence of endophyte-inoculated plants on soil physicochemical and microbial properties, as well as plant physiological parameters and metal concentrations. Data interpretation through the grouping of soil properties within a set of ecosystem services was also carried out. When grown together, we observed a 41 and 16% increase in the growth of *N. caerulescens* and *R. acetosa* plants, respectively, as well as higher values of Zn phytoextraction and soil microbial biomass and functional diversity. Inoculation of the consortia of putative endophytes did not lead to higher values of plant metal uptake, but it improved the plants' physiological status, by increasing the content of chlorophylls and carotenoids by up to 28 and 36%, respectively, indicating a reduction in the stress level of plants. Endophyte-inoculation also stimulated soil microbial communities: higher values of acid phosphatase activity (related to the phosphate solubilising traits of the endophytes), bacterial and fungal abundance, and structural diversity. The positive effects of plant growth and endophyte inoculation on soil properties were reflected in an enhancement of some ecosystem services (biodiversity, nutrient cycling, water flow regulation, water purification and contamination control).

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1. Introduction

Phytoextraction, i.e. the use of plants to extract heavy metals from soil, is a promising phytoremediation option for metal contaminated sites, owing to its being aesthetically pleasing and low-labour-intensive, its low cost and environmentally-friendly character (Chen et al., 2010; Epelde et al., 2012), and the possibility of creating value from contaminated land while minimising environmental risk (Robinson et al., 2009). The amount of metal phytoextracted depends on concentration in aboveground plant material and plant biomass (Barrutia et al., 2009). However, the small biomass and slow growth of many (hyper)accumulators, as well as a low soil metal bioavailability, can limit the effectiveness of phytoextraction (Rajkumar et al., 2009). This has brought up the necessity to explore possibilities for stimulating plant growth and metal uptake during phytoextraction.

Mining sites shelter a characteristic biodiversity, adapted to harsh conditions, with large potential for the phytoremediation and phytomanagement of metal contaminated soils (Barrutia et al., 2011). Selection of (pseudo)metallophytes from these areas, with high metal tolerance and the capacity to accumulate them, is crucial for effective phytoextraction (Barrutia et al., 2009). It has been widely reported (Chen et al., 2010; Luo et al., 2011; Xinxian et al., 2011) that plant growth-promoting rhizobacteria and bacterial endophytes have great potential to enhance phytoremediation of metal contaminated sites.

The objective of remediating metal-contaminated soil is to remove metals, or render them harmless, and restore soil functioning (Epelde et al., 2014a). Soil microbial status can be a suitable indicator of the effectiveness of phytoremediation treatments (Epelde et al., 2008). And ecosystem services can be used to assess soil quality (Rutgers et al., 2012; Velasquez et al., 2007) and monitor phytoremediation (Epelde et al., 2014a).

The aim of this study was to assess the effectiveness of endophyte-assisted phytoremediation of metal contaminated soil, with particular emphasis on the recovery of ecosystem services. This was accomplished by: (i) isolating putative bacterial endophytes from two native plant species collected from an abandoned Pb-Zn mine; (ii) identifying strains with plant growth-promoting traits; and (iii) investigating endophyte-assisted phytoextraction of Pb, Zn and Cd. We also propose grouping soil microbial parameters within a set of ecosystem services to facilitate interpretation and provide guidelines for phytoremediation programs.

2. Materials and methods

2.1. Site description and plant sampling

The study started in an abandoned Pb/Zn mine (Coto "Txomin", province of Biscay, Spain, 43°43′N, 3°26′W) that presents very high levels of toxic heavy metals (i.e., Cd, Pb and Zn) in the soil. For a more detailed description and characterization of the mine, see Barrutia et al. (2011). Healthy plants of *Noccaea caerulescens* J. & C. Presl. and *Rumex acetosa* L. were randomly collected from the mine. Plant samples were thoroughly washed, as described in Burges et al. (2016).

2.2. Isolation, characterization and identification of endophytic bacteria

Putative endophyte bacteria were isolated from roots, stems and leaves of the collected plants (Surette et al., 2003). Plant tissues were surface disinfected by immersing in bleach (5% available chlorine) for 3 min, 3% hydrogen peroxide solution for 3 min, and rinsing three times with sterile distilled water. Roots, stems and leaves (0.5 g FW) were then macerated in 10 ml 3× Ringer's solution (Surette et al., 2003), using a mortar and pestle, adding quartz sand to improve wall disruption, and incubated at room temperature in an orbital shaker for 1 h. Tissue extracts were serially diluted to 10^{-3} with 3× Ringer's solution. Aliquots of 100 μl of each dilution were plated in duplicate on tryptic soy agar (TSA) and Luria Bertani's (LB) agar plates supplemented with 100 mg l^{-1} cycloheximide. Plates were incubated at 28 °C for 72 h. Thirty-one putative endophyte bacterial strains were isolated based on colony morphotype (colour, size and shape). Culture stocks were prepared and stored as described by Burges et al. (2016).

All endophytic bacterial strains were tested for their 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Penrose and Glick, 2003). ACC deaminase activity has been suggested as a major mechanism that bacterial endophytes use to promote plant growth, because it ameliorates plant stress by blocking ethylene production (Glick, 2014; Hardoim et al., 2008). In consequence, only those endophytic bacterial strains containing ACC deaminase activity were tested for the other plant growth-promoting traits studied here: indole-3-acetic acid (IAA) production (Becerra-Castro et al., 2011), siderophore production (Schwyn and Neilands, 1987) and phosphate solubilising activity (Nautiyal, 1999). For the determination of indole-3-acetic acid production, putative endophytes were incubated in liquid medium supplemented with 0.5 mg ml^{-1} L-tryptophan for 5 days, and subsequently quantified spectrophotometrically after incubating the cell suspension's supernatant with Salkowski's reagent for 25 min. Metal and salt tolerance were determined according to Long et al. (2011) and Rashid et al. (2012), respectively, as described in Burges et al. (2016), and a phenotypic characterization was carried out using GEN III MicroPlates™ (Biolog Inc., Hayward, USA)]. Taxonomic identification was determined as detailed in Burges et al. (2016).

2.3. Soil characterization and experimental design

The experiment was carried out in pots containing heavy metal contaminated soil from the abandoned mine. Soil was collected from the top layer (0–20 cm) and immediately transported to our laboratory where visible roots were removed. The mine soil was sieved to <4 mm and thoroughly mixed using a cement mixer. Soil sub-samples were air-dried and sieved to <2 mm prior to physicochemical characterization (MAPA, 1994). The soil was characterized as a sandy-loam with the following properties: pH = 6.8; OM (%) = 4.9; total N (%) = 0.21; extractable P (mg kg^{-1} dry weight-DW soil) = 2.2; and extractable K^+ (mg kg^{-1} DW soil) = 54. Total Cd, Pb and Zn was determined by atomic absorption (Spectra AA-250 plus, Varian, Australia) following aqua regia digestion (McGrath and Cunliffe, 1985). Soil concentrations were 12.9 mg Cd, 6345 mg Pb and 18,284 mg Zn per kg (DW) soil.

Two species of metallophytes were used: (i) the hyperaccumulator *N. caerulescens* and (ii) the accumulator *R. acetosa*. Seeds of *N. caerulescens* (Hernández-Allica et al., 2006) and *R. acetosa* (Barrutia et al., 2009) were collected from the abandoned mine and germinated for 1 month on a mixture of perlite and vermiculite (1:3; v/v, moistened with deionized H_2O) under the following growth chamber conditions: 14/10 h light/dark cycle, 20/16 °C day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 μmol photon m^{-2} s^{-1}. Subsequently, plants were transplanted to a mixture of perlite and vermiculite (2:3; v/v), moistened with a nutrient solution [1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM K_2HPO_4, 0.1 mM KCl, 2 mM MES-HCl buffer pH 6, 1 mM KOH, 10 μM H_3BO_3, 0.2 μM $Na_2MoO_4 \cdot 2H_2O$, 1.8 μM $MnSO_4 \cdot 4H_2O$, 0.3 μM $CuSO_4 \cdot 5H_2O$, 0.5 μM $NiSO_4 \cdot 6H_2O$, 100 μM Fe-EDDHA] supplemented with 1 μM $ZnSO_4 \cdot 7H_2O$ or 5 μM $ZnSO_4 \cdot 7H_2O$ for *N. caerulescens*, and allowed to grow for 1 month. Equally developed plants were selected for the experiment.

Endophytic bacterial strains that showed the best performance for any of the plant growth-promoting traits described above were used to form the consortia for inoculation (Table 1): isolates NR1, NL1 and NL2 from *N. caerulescens* for inoculation of *N. caerulescens*; and isolates RR1, RR3 and RL2 from *R. acetosa* for inoculation of *R. acetosa*. The consortia of endophytes (putative) were obtained by growing the isolates separately, as described in Burges et al. (2016), and mixing equal volumes of individual bacterial cultures. For inoculation, *N. caerulescens* and *R. acetosa* plants were soaked for 2 h in the corresponding bacterial consortium following Chen et al. (2010). Four plants were transferred to