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Bioaugmentation with bacteria selected from the microbiome enhances *Arthrocnemum macrostachyum* metal accumulation and tolerance

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ABSTRACT

A glasshouse experiment was designed to investigate the role of bacterial *consortia* isolated from the endosphere (CE) and rhizosphere (CR) of *Arthrocnemum macrostachyum* on its metal uptake capacity and tolerance in plants grown in metal polluted sediments. *A. macrostachyum* plants were randomly assigned to three bioaugmentation treatments (CE, CR and without inoculation) during 120 days. Bioaugmentation with both bacterial *consortia* enhanced *A. macrostachyum* capacity to accumulate ions in its roots, while shoot ions concentration only increased with CE treatment. Furthermore bioaugmentation ameliorated the phytotoxicity levels, which was reflected in an increment of plant growth of 59 and 113% for shoots and 52 and 98% for roots with CE and CR treatments, respectively. This effect was supported by bacteria beneficial effect on photochemical apparatus and the modulation of its oxidative stress machinery. These findings indicated that bacteria selected from the microbiome can be claimed to improve *A. macrostachyum* metal remediation efficiency.

1. Introduction

Phytoremediation is a promising clean-up solution to act upon of the organic and inorganic pollutants in a wide variety of contaminated sites, by extracting, degrading or immobilizing them through the use of plants (Marques et al., 2011; Weis and Weis, 2004). Plants used for metal remediation should be chosen on the basis of their potential to uptake, accumulate and transport large amount of metals, as well as taking into account its ability to maintain high growth rates under metal excess (Marques et al., 2011). Sometimes phytoremediation efficiency is compromised by growth inhibition as a consequence of the increment of metal phytotoxicity levels in plant tissues (Khan et al., 2015). Recently a number of scientists have begun to explore the possibility of using the microbial populations which colonizing the rhizosphere (Mesa et al., 2015b; Pajuelo et al., 2014) or the endosphere (Doty, 2008; Mesa et al., 2015a; Newman and Reynolds, 2005; Rajkumar et al., 2009) in order to make this technology more efficacious. Among these bacterial populations the interest has been particularly focused plant growth promoting bacteria (PGPB), which have demonstrated the ability to promote plant growth under stress conditions (Ahemad and Kibret, 2014; de Bashan et al., 2012; Glick, 2010; Mesa et al., 2015a, 2015b; Mateos-Naranjo et al., 2015; Navarro-Torre et al., 2016a; Weyens et al., 2009). However the effect of microbial inoculation on plant metal uptake does not follow a clear pattern, being highly dependent of sediment and contaminant characteristics, as well as of plant-microbe partnerships (Phieler et al., 2014; Sessitsch et al., 2013). Therefore, this study was designed to contribute to fill these gaps of knowledge.

Arthrocnemum macrostachyum (Moric) C. Koch is a common halophytic shrub in SW Iberian Peninsula, which is distributed in coastal areas through Mediterranean basin to the Middle East and Asia (Redondo-Gómez et al., 2010a). In the marshes of the south-west of Spain develops populations distributed from the middle to high elevations in the tidal range, which are occasionally subject to tidal inundations and seasonal hypersalinity (Redondo-Gómez et al., 2010a). This species represent suitable model plant to study plant-bacterial interactions and specifically in terms of metal phytoremediation efficiency, since this species has demonstrated a great capacity for accumulating metals in its tissues (Redondo-Gomez et al., 2010b), being considered a suitable candidate for remediation of metal polluted coastal sediments (Conesa and Schulin, 2010; Redondo-Gomez et al., 2010b). In addition, Navarro-Torre et al. (2016b) recently isolated different bacterial strains from the rhizosphere and endosphere of *A. macrostachyum* plants from the Odiel estuary, which exhibited several plant growth promoting (PGP) properties, even in the presence of heavy metals. Among all the isolates, the strains *Vibrio kanaloae* RA1, *Pseudoalteromonas distinct* RA8, *Pseudoalteromonas prydzensis* RA15 and *Staphylococcus warneri* RA18 isolated from the rhizosphere of *A. macrostachyum* and the strains

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Kushneria marisflavi EAod3, *Micrococcus aloeverae* EAod10, *Bacillus vietnamensis* EAR8 and *Halomonas zincidurans* EAR18 isolated from the endosphere, were proposed as the best candidates to design two bacterial *consortia* aimed to inoculate *A. macrostachyum* plants to improve its metal remediation potential (Navarro-Torre et al., 2016b). Thus, in the present study, a continuation of this previous one, we hypothesized that these selected bacterial *consortia* could play an important role on metal uptake, accumulation and transport, as well as on the mitigation of phytotoxicity effects arising from metal accumulation in plant tissues.

The aim of this study was to investigate the role of bacteria isolated from the microbiome of *A. macrostachyum* in its metal uptake capacity and tolerance to metal excess.

2. Material and methods

2.1. Plant and sediment source

Seeds of *A. macrostachyum* and samples of the first 15 cm of sediment around the plants were collected in March 2015 from the Odiel marshes (37°15′N, 6°58′W; SW Spain) and subsequently transported to the laboratory. Then, seeds were disinfected with 10% sodium hypochlorite for 10 min and washed six times with sterile distillated water. These seeds were plated in 9% agar plates and placed in a growth chamber (AGP-700-HR ESP; Radiber, Barcelona, Spain) with a regime of 10 h of light (20 °C, 50% HR and 35 μmol m^{-2} s^{-1}, 400–700 nm) and 14 h dark (5 °C and 50% HR) for 20 days. After, germinated seedlings were transferred to individual pots (9 cm high × 11 cm diameter, n = 30) filled with perlite. Seedlings were maintained in a greenhouse with the following conditions: temperature between 21 and 25 °C, 40–60% relative humidity, natural daylight of 250 as minimum and 1000 μmol m^{-2} s^{-1} as maximum light and supplemented with 20% Hoagland solution (Hoagland and Arnon, 1938).

On the other hand, sediment texture, conductivity, pH, redox potential and total sediment metal concentrations (n = 5) were measured using the method described by Mateos-Naranjo et al. (2011) to determine the physicochemical characteristic of the collected sediment. The physicochemical properties of the sediments are given in Table 1.

2.2. Inoculation and plant assays in polluted soils

Two bacterial *consortia* were selected for this work on basis on their PGP properties according with the recent work by Navarro-Torre et al. (2016b). They represented the best-performing strains among all the isolates characterized in that study. Thus we employed a *consortium* with bacteria isolated from the rhizosphere (CR) of *A. macrostachyum* (integrated by the strains *Vibrio kanaloae* RA1, *Pseudoalteromonas distinct* RA8, *Pseudoalteromonas prydzensis* RA15 and *Staphylococcus warneri* RA18) and a *consortium* with four endophytic strains (CE), two isolated from plant phyllosphere (*Kushneria marisflavi* EAod3 and *Micrococcus aloeverae* EAod10) and two from roots of *A. macrostachyum* (*Bacillus vietnamensis* EAR8 and *Halomonas zincidurans* EAR18). Each strain was cultivated separately in 50 mL of TSB (Tryptic Soy Broth) medium supplemented with 0.3 M NaCl at 28 °C during 24 h in continuous shaking to reach 10^8 cells/mL. Then, cultures were centrifuged for 10 min at 8000 rpm and pellets were washed with 50 mL of 0.9% saline solution, centrifuging at the same conditions. Pellets were re-suspended in 5 mL of 0.9% saline solution and mixed in 50-mL Falcon tubes, forming the CR and CE *consortia* respectively. Finally, both *consortia* were mixed separately with 250 mL of tap water for plant inoculation.

In October 2015, after four months of *A. macrostachyum* plant growth under glasshouse conditions described above, the perlite of *A. macrostachyum* plants was washed off and plants were transferred to individual 250 mL plastic pots (one plant per pot and each pot was placed in an individual shallow tray) filled with a homogenate of Odiel sediment. Pots were randomly assigned to three inoculation treatments (non-inoculated control plants, plants inoculated with CR *consortium* and plants inoculated with CE *consortium*) and maintained in the same glasshouse for 4 months (n = 30; three treatments with 10 pots in each one). During the experiment, plants were inoculated with the *consortia* once a week in the first month and, then, twice a month. Non-inoculated control plants were treated with the same volume of tap water. Also at the beginning of the experiment 1 L of tap water was placed in each of the trays down to a depth of 1 cm, and water levels were monitored and controlled during the experiment.

Metal tissues concentration, growth, physiological and biochemical measurements (including relative growth rate, dry mass, relative water content, gas exchange, chlorophyll fluorescence and antioxidant enzyme analysis) were made in February 2016 when the plants appeared to have stable growth rates, in order to assess the bacterial inoculation effect on plant metal uptake capacity and tolerance.

Table 1
Physicochemical properties and total arsenic (As), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) concentrations in sediments from Odiel marsh.

Physicochemical properties					
Texture (%)	Conductivity (mS cm^{-1})		Redox potential (mV)		pH
61/14/25	15.9 ± 0.9		138 ± 1.0		7.2 ± 0.0
Metal concentration (mg kg^{-1})					
As	Cr	Cu	Ni	Pb	Zn
253.8 ± 5.1	46.7 ± 0.5	856.1 ± 17.1	18.0 ± 0.4	319.6 ± 6.4	1516.3 ± 30.3

Values are mean ± S.E. (n = 5). Texture (silt/clay/sand percentage).

2.3. Ions concentration in plant tissues

At the end of the experiment, shoots and roots of each plant were carefully washed with distilled water before analysis. Then dry samples of the seven plant replicates were ground, and total As, Cr, Cu, Ni, Pb and Zn concentrations were measured as previously described by Mateos-Naranjo et al. (2008) by inductively coupled plasma (ICP) spectroscopy.

2.4. Growth analysis

Plants were harvested at the beginning of the experiment to obtain an estimate initial biomass just before sowing in contaminated sediment (n = 3) and after four months of treatment (n = 7) to get the final dry mass. Plants were separated in roots and shoots and dried at 60 °C for 48 h until constant weight before weighing. The relative growth rate (RGR) of entire plants was calculated by means of the formula:

$$RGR = (\ \ln Bf - \ln B_i)\, D^{-1} \left(g\, g^{-1}\, day^{-1}\right)$$

where Bf = final dry mass, B_i = initial dry mass (an average of the three plants from each treatment dried at the beginning of the experiment) and D = duration of experiment (days).

2.5. Gas exchange and chlorophyll fluorescence analysis

Instantaneous gas exchange measurements were taken on random primary branches of each plant in the three treatments using an infrared gas analyser (IRGA) in an open system (LI-6400XT, LI-COR Inc., Neb., USA) equipped with a light leaf chamber (Li-6400-02B, Li-Cor Inc.) to obtain net photosynthetic rate (A_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i). Measurements (n = 7) were performed between 10:00 and 14:00 h and under a photosynthetic photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1} (with 15% blue light to maximize stomatal aperture), vapour pressure deficit of 2.0–3.0 kPa, air temperature of around 25 °C and an ambient CO_2 concentration (C_a) of 400 μmol mol^{-1}air and before to record each measurement,

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