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Isolation of plant-growth-promoting and metal-resistant cultivable bacteria from *Arthrocnemum macrostachyum* in the Odiel marshes with potential use in phytoremediation

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ABSTRACT

Arthrocnemum macrostachyum is a halophyte naturally growing in southwest coasts of Spain that can tolerate and accumulate heavy metals. A total of 48 bacteria (30 endophytes and 18 from the rhizosphere) were isolated from *A. macrostachyum* growing in the Odiel River marshes, an ecosystem with high levels of contamination. All the isolates exhibited plant-growth-promoting (PGP) properties and most of them were multiresistant to heavy metals. Although the presence of heavy metals reduced the capability of the isolates to exhibit PGP properties, several strains were able to maintain their properties or even enhance them in the presence of concrete metals. Two bacterial consortia with the best-performing endophytic or rhizospheric strains were selected for further experiments. Bacterial inoculation accelerated germination of *A. macrostachyum* seeds in both the absence and presence of heavy metals. These results suggest that inoculation of *A. macrostachyum* with the selected bacteria could ameliorate plant establishment and growth in contaminated marshes.

1. Introduction

Soil contamination with heavy metals is a global problem for environment and public health (Vangronsveld et al., 2009), mainly because of their low solubility and bioavailability and their capacity as carcinogenic and mutagenic agent (Davis et al., 2011). Industrial activities taking place near salt marshes have contributed to the introduction of a huge amount of contaminants, including heavy metals, in these environments. Metals discharged in the marshes are introduced into the food chain, affecting all the inhabitants of the ecosystem (Álvarez-Rogel et al., 2004). An example of this sort of contamination is the joint estuary of the Odiel and Tinto rivers in Huelva (SW Spain), one of the most polluted areas in the world, because of the high concentration of metals (mainly As, Cu, Pb, and Zn) found in their sediments (Nelson and Lamothe, 1993; Sainz et al., 2004; Mesa et al., 2015a). For >100 years, intense mining activity took place in this area; recently, the beginning of industrialization of the region in the late 1960s has contributed to this contamination (Nelson and Lamothe, 1993; Davis et al., 2000). This estuary needs restorative intervention urgently. In order to regenerate this ecosystem, phytoremediation, the use of plants to remove or neutralize pollutants, could be an interesting biotechnological strategy, as it has several environmental advantages over traditional physicochemical approaches: it improves soil fertility, favors the ecological development of the ecosystem, and maintains the esthetic of the landscapes (Vangronsveld et al., 2009).

Phytostabilization, the phytoremediation technique aimed to immobilize metals into plant roots and rhizosphere, has been proposed as an appropriate tool to recover soils exposed to continuous mine drainage (Mendez and Maier, 2008; Yang et al., 2014). This technique involves the use of metal-resistant plants that are able to facilitate metal precipitation onto root surfaces and/or accumulation into them (Mendez and Maier, 2008). This is a long-term actuation that requires well-adapted and preferably autochthonous plants that are able to maintain high growth ratios in the presence of contaminants (Mendez and Maier, 2008; Ali et al., 2013). Plant adaptation and growth in an ecosystem is intimately connected with complex soil ecological processes, highly influenced by microorganisms colonizing both the rhizosphere and phyllosphere. These microorganisms provide nutrients to plants and can reduce harmful effects of contaminants in degraded soils (Newman and Reynolds, 2005; Yang et al., 2008). In this way, plant-growth-promoting bacteria (PGPB) associated with plants used for phytoremediation may contribute to metal plant tolerance, growth, and uptake, thus accelerating the remediation process (Tak et al., 2013; Ullah et al., 2015).

PGPB improve plant growth by different mechanisms, including among others atmospheric nitrogen fixation, production of auxins and

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other phytohormones, phosphorus solubilization, production of siderophores, and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase synthesis (Glick, 2012). Through these mechanisms, PGPB increase plant health and robustness, thus facilitating plant adaptation in stressful conditions. Bacteria that assist in the phytoremediation of heavy metals can contribute to this process direct or indirectly (Ullah et al., 2015). Direct mechanisms include enhanced metal solubilization and bioavailability, thus resulting in increased plant metal uptake (Glick, 2010). PGPB produce siderophores, chelating agents that are able to bind metals and enhance their bioavailability (Rajkumar et al., 2009, 2010). Low molecular weight organic acids and biosurfactants produced by PGPB have also the potential to improve metal mobilization (Mucha et al., 2005; Rajkumar et al., 2012). Other bacterial processes that modify plant metal availability are reduction/oxidization, methylation, precipitation, and biosorption (Rajkumar et al., 2012). Concerning indirect processes, bacteria can enhance phytoremediation providing essential nutrients whose uptake is limited under metal stress conditions (Ouzounidou et al., 2006). PGPB can fix atmospheric nitrogen, solubilize phosphorous, and produce siderophores for iron chelation even under metal stress conditions, thus facilitating the uptake of these nutrients (Ullah et al., 2015). Bacterial production of phytohormones under metal stress affects plant hormone levels and benefits plant response in such situations (Glick, 2012). Some PGPB are able to produce ACC deaminase, which hydrolyzes ACC, the precursor of the plant hormone ethylene, whose overproduction under stressful conditions might inhibit plant growth (Glick, 2012).

Arthrocnemum macrostachyum is a promising candidate to be used in phytoremediation, particularly phytostabilization, of metal-contaminated estuaries. This halophytic shrub is distributed by the Mediterranean region and abundant in the Odiel marshes (Redondo-Gómez et al., 2010a), and has shown ability for metal uptake and accumulation in mining wastes and polluted estuaries (Conesa and Schulin, 2010; Madejón et al., 2009). Nevertheless, nothing is known about the bacteria associated with this plant. Thus, the aims of this study were to: (1) isolate, identify, and characterize bacteria associated with *A. macrostachyum* growing in a contaminated estuary; (2) characterize the resistance of these bacteria toward heavy metals; (3) study the presence of PGP properties in both the presence and absence of heavy metals and select the best-performing isolates; and (4) evaluate the effect of the selected bacteria on the germination of *A. macrostachyum* seeds under metal stress conditions.

2. Material and methods

2.1. Collection of samples and chemical analysis

Three plants of *A. macrostachyum* without a senescent appearance, and with similar size and age, were collected together with its rhizospheric soil (between 10 and 20 cm depth, which corresponds to the area of higher roots biomass) in February 2015 from a middlemarsh site in the Odiel marshes (Huelva, Spain) (37° 13′N - 6° 57′O).

Each sample was placed in individual plastic bags using a plastic shovel or gloves, for rhizosediments and plant samples, respectively, according to Almeida et al. (2004), and immediately transported to the laboratory and stored at 4 °C until processing.

Chemical analysis of soil samples and plant tissues was done exactly as described in Mesa et al. (2015b). Concentration of elements was measured by inductively coupled plasma–atomic emission spectroscopy (ICP–AES) (ARLFisons3410, USA). Conductivity was determined using a Crison-522 (Spain) conductivity meter and redox potential and pH with a Crison pH/mVp-506 (Spain) portable meter (Mesa et al., 2015b). Finally, sand, silt, and clay percentages were determined by means of the Bouyoucos hydrometer method (Bouyoucos, 1936).

2.2. Isolation of cultivable bacteria

Aerial parts and roots were separated and surface-disinfected as follows: the samples were treated with 70% (v/v) ethanol for 1 min by shaking, 15 min with 3% (v/v) sodium hypochlorite by slowly shaking, and then washed four times with sterile distilled water. Three samples of roots and three of aerial parts from each plant (each sample containing 3 fragments of plant material with 2–3 cm length) were crushed separately with a mortar in 1 ml 0.9% sterile saline solution and the generated mixture was directly plated (100 μl per plate) into three Petri dishes with tryptic soy agar (TSA) medium supplemented with 0.3 M NaCl and the three plates supplemented with 0.6 M NaCl. The plates were incubated at 28 °C for 72 h.

In order to isolate rhizospheric bacteria, the soil samples (near the root of the plant) were shaken with 0.9% sterile saline solution for 5 min and the suspension was plated into three TSA 0.3 M NaCl plates and three TSA 0.6 M NaCl plates and incubated at 28 °C for 72 h. Bacteria were isolated according to the different colony morphology, Gram staining, and motility. For motility, single colonies were transferred to 5 ml of liquid TSB (tryptic soybroth) and incubated at 28 °C for 30 min with gentle shaking. A drop of this culture was deposited over a slide, covered with a cover slip and observed using an Olympus CX41 microscope (100× objective).

2.3. DNA extraction, 16S rRNA amplification and Box-PCR fingerprinting analysis

Genomic DNA was extracted using the i-genomic BYF DNA Extraction kit (Intron Biotechnology Ltd., South Korea), according to the manufacturer's instructions. 16S rRNA amplification was performed using 16F27 primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16R1488 primer (5′-CGGTTACCTTGTTAGGACTTCACC-3′) and the following PCR conditions: initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 3 min, and final extension at 72 °C for 5 min. 16S rDNA sequences were compared with those located in the databases using the EzTaxon server (Chun et al., 2007). The 16S rRNA sequences were deposited in the NCBI GenBank.

Box-PCR fingerprinting analysis to assess genetic diversity of isolated bacteria was performed using BOX A1R primer following the conditions described in Mesa et al., 2015b. The gel was photographed and analyzed with CLIQS 1D Pro Software (TotalLab, UK). Dendograms and similarities were determined by calculating Pearson's product moment correlation coefficient (Jobson, 1991).

2.4. Enzymatic activities

Pectinase and cellulase activities were examined according to the method described by Elbeltagy et al. (2000). Amylase activity was performed in starch agar plates incubated for 7 days at 28 °C and revealed with 10 ml of lugol. Lipase and protease activities were observed by the presence of halos around bacteria after incubation in Tween and casein agars, respectively, for 7 days at 28 °C (Prescott, 2002). Concerning DNAsa activity, bacteria were incubated 7 days at 28 °C in DNA agar plates. The plates were then revealed with 1 M HCl. Halos in dark background were observed in bacteria with DNAsa activity. Chitinase activity was performed as described in Mesa et al. (2015a). The plates were supplemented with 0.3 M NaCl.

2.5. Resistance to salt and heavy metals

In order to determinate resistance toward NaCl, bacteria were plated in TSA supplemented with increasing concentrations of NaCl ranging from 0.5 to 4 M (prepared by adding SW30 solution) and incubated at 28 °C for 72 h.

Similarly, resistance toward heavy metals was performed in TSA plates containing 0.3 M NaCl. Plates were made by adding increasing amounts of heavy metals from the following stock solutions: $NaAsO_2$ 0.5 M, $CdCl_2$ 1 M, $CuSO_4$ 1 M, $CoCl_2$ 1 M, $NiCl_2$ 0.5 M, $Pb(NO_3)_2$ 0.5 M, and $ZnSO_4$ 1 M. Final metal concentrations ranged from 0.1 to 60 mM As, 0.1 to 3.5 mM Cd, 0.1 to 3.5 mM Cu, 0.1 to 25 mM Co, 0.1 to 25 mM Ni, 0.1 to 25 mM Pb, and 0.1 to 5 mM Zn. Bacteria were grown at 28 °C for 3–4 days.

The resistance was expressed as the maximum tolerable concentration (MTC) for each element, that is, the maximum concentration of NaCl or metal(loid) that allowed the visible growth of the bacteria.

2.6. PGP properties

All the properties were evaluated in the presence of 0.3 M NaCl. For nitrogen fixation, bacteria were incubated in a minimum medium without a nitrogen source (Doebereiner, 1995) for 72 h at 28 °C. Siderophores were produced in CAS (chrome azurol S) agar plates (Schwyn and Neilands, 1987). The plates were incubated for 7 days at 28 °C in darkness. Bacteria that produced siderophores showed an orange halo. Phosphate solubilization was determined by the presence of a halo around the bacteria in NBRIP plates (Nautiyal, 1999) after 7 days at 28 °C. The production of indoleacetic acid (IAA) was evaluated in a nutrient broth media (Biolife, Italy) supplemented with L-tryptophan (100 mg/l) and incubated at 28 °C for 72 h with shaking. Then, Salkowski reagent (Gordon and Weber, 1951) was added and optical density was measured at 535 nm using a spectrophotometer (Lambda25; PerkinElmer, USA). The ACC deaminase activity was performed as described by Penrose and Glick (2003). Briefly, the isolated strains were incubated in a DF salts minimal medium with $(NH_4)_2SO_2$ as the source of nitrogen for 24 h at 28 °C by shaking. Afterward, an aliquot was transferred to a DF salts minimal medium with 3 mM ACC and incubated for another 24 h at the same temperature. Finally, biofilm formation was assayed following the method described by del Castillo et al. (2012), starting from a bacterial culture in TSB supplemented with 0.3 M NaCl.

2.6.1. PGP properties in the presence of heavy metals

PGP properties, except ACC deaminase and biofilm formation, were also studied in the presence of $NaAsO_2$ 1 mM, $CuSO_4$ 1 mM, $CoCl_2$ 1 mM, $NiCl_2$ 1 mM, $Pb(NO_3)_2$ 1 mM, $ZnSO_4$ 1 mM, or $CdCl_2$ 0.25 mM. Nitrogen fixation could not be studied in the presence of Pb, as the stock solution contained nitrogen and it was not possible to measure the production of IAA due to the precipitation of Pb with the Salkowski reagent.

2.7. Selection of bacterial consortia

Considering bacterial resistance toward NaCl and metals, the presence of enzymatic activities and PGP properties, and the effect of heavy metals over these properties, two bacterial consortia with the best-performing endophytic or rhizospheric strains were selected for further experiments.

2.8. Inoculation of A. macrostachyum seeds

2.8.1. Preparation of bacterial inoculum

Strains selected for inoculation of seeds were incubated separately in TSB medium supplemented with 0.3 M NaCl for 24 h at 28 °C and continuous shaking. After incubation, cultures containing 10^8 cells/ml were centrifuged in 50-ml Falcon tubes at 8000 rpm for 10 min. Pellets were washed with 0.9% saline solution, centrifuged for 10 min in the same conditions and then resuspended in 5 ml of 0.9% saline solution. Finally, the cultures were mixed in a 50-ml tube.

2.8.2. Seed germination

A. macrostachyum seeds were collected in March 2015 from Odiel marshes (Huelva, Spain) (37° 13′N–6° 57′O). A total of 600 seeds were surface-disinfected with 10% sodium hypochlorite for 10 min and then washed six times with sterile distillate water. Of them, 200 seeds were preinoculated with the selected endophytic bacteria by submerging them in 15 ml of culture (containing 5 ml of each strain) during 1 h and shaking. The same procedure was performed with 200 seeds which were preinoculated with the best rhizospheric bacteria. Finally, 200 seeds were treated with 0.9% saline solution in the same conditions and kept as control. The seeds were then plated in 9% agar plates (25 seeds per plate and four plates per condition) supplemented with a mixture of metals, containing 25 μM of each As, Cu, Pb, and Zn. Seeds were germinated for 20 days in the following conditions: 10 h at 20 °C, 50% humidity, 100% illumination (400–700 nm, 35 $\mu mol\ m^{-2}\ s^{-2}$) 2) and 14 h at 5 °C, 50% humidity, and 0% illumination. Seed germination was observed every day for 3 weeks to record the germination kinetics.

3. Results

3.1. Isolation and identification of cultivable bacteria from A. macrostachyum rhizosphere and tissues

Physicochemical properties of soil samples from Odiel marsh are listed in Table 1. Concentration of metal(loids)s in tissues of *A. macrostachyum* and in rhizospheric soil collected in the estuary of the Odiel River was also measured (Table 1). Soil data were similar to those recently published by Mesa et al. (2015a).

According to morphology of colonies and Gram staining, 48 different cultivable isolates were obtained from the aerial part, roots, and rhizosphere of *A. macrostachyum* growing in the Odiel river marshes (Table 1). Eleven cultivable bacterial strains were endophytes isolated from the phyllosphere (aerial part of the plants; EAod) and 19 from the roots of the same plants (EAR), while 18 isolates were recovered from their rhizospheres (RA). Almost 50% of the species isolated from the rhizosphere were also found in the roots as endophytes.

Gram-negative rods represented 75% of the isolates, 21% were Gram-positive rods, and 4% Gram-positive cocci. Noteworthy, most Gram-positive bacteria were isolated from the rhizosphere of the plants. 16S rDNA sequences pointed to genera *Bacillus*, *Pseudoalteromonas*, *Vibrio*, *Staphylococcus*, *Kushneria*, and *Halomonasas* are the most abundant (Table 2), but genus *Kushneria* was only present in the phyllosphere. Partial 16S rDNA sequences for some isolates and almost complete sequences of strains selected for further experiments involving seeds and plants were obtained (Table 2).

Genetic diversity by Box-PCR was also studied (Fig. 1). Only strains EAod3 and EAod 4, related to *Kushneria marisflavi* species, showed the same band profile.

Although 16S rDNA sequences and Box-PCR profiles suggested that several isolates belonged to the same species, they could be considered different strains, as they showed different properties and characteristics throughout this study.

No further characterization of strains RA16 (Table 1), RA2, RA13, and EAR2 (data not shown) was carried out, as preliminary 16S rDNA sequences revealed identity with the pathogen *Bacillus anthracis*.

3.2. Characterization of strains isolated from A. macrostachyum

3.2.1. Screening for enzymatic activities and motility

For characterization of the isolated bacteria, the presence of several enzymatic activities, including protease, lipase, amylase, DNAse, cellulose, pectinase, and chitinase, was studied (Supplementary Table 1 and Fig. 2). Most of the strains presented DNAse and in lower extent protease, while lipase and amylase appeared in approximately 50% of them. Small differences in the occurrence of these activities could be observed between endophytic and rhizospheric bacteria. On the contrary,

Table 1
Physicochemical properties of soil and total arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) concentrations in soil and in roots and shoots of *Arthrocnemum macrostachyum* from Odiel marsh.

Physico-chemical properties							
Soil	Texture (%)		Conductivity (mS cm^{-1})		Redox potential (mV)		pH
Odiel	71/20/9		11.2 ± 0.4		135 ± 13		6.8 ± 0.2
Heavy metal concentration (mg/kg)							
	As (mg kg^{-1})	Cd (mg kg^{-1})	Co (mg kg^{-1})	Cu (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
Soil	253.8 ± 5.1	2.4 ± 0.1	9.5 ± 0.2	856.1 ± 17.1	18.0 ± 0.4	319.6 ± 6.4	1516.3 ± 30.3
Roots	52.8 ± 1.1	0.3 ± 0.0	1.5 ± 0.0	117.9 ± 2.4	16.8 ± 0.3	29.5 ± 0.6	137.8 ± 2.8
Shoots	7.2 ± 0.5	<0.1	<0.5	32.1 ± 0.6	1.9 ± 0.0	5.0 ± 0.1	61.1 ± 1.2

Values are mean ± S.E. of five replicates. Texture (silt/clay/sand percentage).

the presence of cellulose, pectinase, and chitinase activities was less frequent among the isolates. Cellulase activity appeared more frequently in endophytic than in rhizospheric bacteria and, conversely, higher number of strains showed pectinase activity in the rhizosphere. Finally, the number of bacteria with chitinase in either the rhizosphere or inside the plants was similar (Fig. 2).

Concerning motility, 83% of endophytes were mobile, while only 33% of rhizospheric bacteria did.

Table 2
Closest species to the strains isolated from *Arthrocnemum macrostachyum* based on the 16S rRNA partial sequence. In bold: strains selected for further experiments.

Strain	Accession no.	Related species	Sequenced fragment (bp)	Identity (%)
EAod1	KU320854	*Halomonas songnenensis*	1186	75.76
EAod2	KU320855	*Halomonas arcis*	1402	96.43
EAod3	KU320856	***Kushneria marisflavi***	1467	98.63
EAod4	KU320857	*Kushneria marisflavi*	1413	98.08
EAod5	KU320858	*Halomonas arcis*	1404	95.50
EAod6	KU320859	*Halomonas songnenensis*	1151	85.39
EAod7	KU320860	*Kushneria marisflavi*	1418	97.23
EAod8	KU320861	*Kushneria indalinina*	1423	98.58
EAod9	KU320862	*Vibrio sagamiensis*	1410	95.14
EAod10	KU320863	***Micrococcus aloeverae***	1421	97.97
EAod11	KU320864	*Halomonas arcis*	1403	97.50
EAR1	KU320865	*Vibrio kanaloae*	1253	96.93
EAR2	KU320866	*Bacillus thuringiensis*	1110	98.10
EAR3	KU320867	*Pseudoalteromonas undina*	1327	96.64
EAR4	KU320868	*Pseudoalteromonas prydzensis*	1187	98.12
EAR5	KU320869	*Pseudoalteromonas agarivorans*	1158	88.16
EAR6	KU320870	*Pseudoalteromonas tetraodonis*	1030	83.67
EAR7	KU320871	*Pseudoalteromonas tetraodonis*	1136	84.24
EAR8	KU320872	***Bacillus vietnamensis***	1374	97.80
EAR9	KU320873	*Halomonas zincidurans*	1364	94.49
EAR10	KU320874	*Alteromonas halophila*	1367	94.72
EAR11	KU320875	*Pseudoalteromonas prydzensis*	1131	96.55
EAR12	KU320876	*Pseudoalteromonas agarivorans*	947	98.40
EAR13	KU320877	*Pseudoalteromonas agarivorans*	1076	98.23
EAR14	KU320878	*Halomonas zincidurans*	1085	97.51
EAR15	KU320879	*Bacillus hwajinpoensis*	1385	97.07
EAR16	KU320880	*Pseudoalteromonas tetraodonis*	877	83.47
EAR17	KU320881	*Pseudoalteromonas espejiana*	1337	95.14
EAR18	KU320882	***Halomonas zincidurans***	1262	96.59
EAR19	KU320883	*Marinobacter nanhaiticus*	1358	97.54
RA1	KU588386	***Vibrio kanaloae***	1425	96.54
RA2	KU588387	*Bacillus thuringiensis*	1324	97.86
RA3	KU588388	*Acinetobacter ursingii*	912	86.70
RA4	KU588389	*Pseudoalteromonas undina*	1365	98.14
RA5	KU588390	*Pseudoalteromonas prydzensis*	805	95.30
RA6	KU588391	*Pseudoalteromonas tetraodonis*	1129	98.05
RA7	KU588392	*Bacillus vietnamensis*	976	88.82
RA8	KU588393	***Pseudoalteromonas distinct***	1366	98.76
RA9	KU588394	*Bacillus hwajinpoensis*	1148	94.12
RA10	KU588395	*Pseudoalteromonas tetraodonis*	957	95.09
RA11	KU588396	*Halomonas alkaliantarctica*	628	78.12
RA12	KU588397	*Bacillus timonensis*	1235	91.84
RA13	KU588398	*Bacillus cereus*	1260	97.21
RA14	KU588399	*Bacillus enclensis*	1122	79.87
RA15	KU588400	***Pseudoalteromonas prydzensis***	1389	97.55
RA16	KU588401	*Bacillus anthracis*	488	96.61
RA17	KU588402	*Pseudoalteromonas issachenkonii*	997	89.90
RA18	KU588386	***Staphylococcus warneri***	1428	97.53

3.2.2. Resistance to salt and heavy metals

One important task for the use of these bacteria in heavy metal-contaminated soils is the resistance to those metals. Most of bacteria were multiresistant to several heavy metals (Supplementary Table 2), growing some of them at metal concentrations as high as 56 mM As (EAod10), 23 mM Ni (EAod7), 21 mM Co (EAR1 and RA1), 20 mM Pb (EAod3, 4 and 10), 4.5 mM Zn (EAR11), or 3 mM Cd (RA3). In general, the highest values of metal resistance for a particular metal were found in endophytic strains. The abundance of strains with resistance toward the different metals among the three groups of isolates is shown in Fig. 3A.

Odiel River marshes are mainly contaminated with As, Cu, Pb, and Zn (Table 2). Approximately 50% of the rhizospheric bacteria were able to grow at As concentrations >10 mM arsenite, while only 10% of the endophytes did (Fig. 4). On the contrary, 38% of root endophytes did not grow in the minimal concentration of As assayed (0.1 mM) (Fig. 4 and Supplementary Table 2).

All the strains isolated from plant roots and rhizosphere grew in a Cu concentration range of 2–3 mM (Fig. 4). Nevertheless, 64% of the endophytes isolated from the phyllosphere did not grow at 0.1 mM Cu and 36% of them were able to grow at a maximum of 1 mM (Fig. 4 and Supplementary Table 2).

The behavior of the isolates against Pb was peculiar. Endophytes of the phyllosphere were resistant to high concentrations of Pb, ranging from 11 to 20 mM (Fig. 4) and these values were always higher than those observed in strains isolated from either the roots or rhizosphere (Supplementary Table 2). Most of the rhizospheric bacteria and root endophytes showed high resistance toward Pb, with an average concentration of 8 mM (Fig. 4 and Supplementary Table 2).

The general resistance toward Zn ranged from 0.3 to 4.5 mM (Supplementary Table 2). Endophytes isolated from the phyllosphere and two-thirds of root endophytes grew at Zn concentrations <2 mM, while one-third of root endophytes grew at concentrations between 3 and 4.5 mM Zn. Most of rhizospheric bacteria grew at concentrations between 3 and 4 mM Zn (Fig. 4 and Supplementary Table 2).

Concerning other metals tested, the highest values of resistance toward Cd and Hg (two metals with high toxicity) were present among rhizospheric bacteria and root endophytes. Several strains showed resistance values ranging from 1.3 to 3 mM Cd and 0.3 to 0.8 mM Hg. The highest differences among bacterial groups could be observed for Hg, as no endophyte of the phyllosphere grew at the minimal concentration assayed (0.1 mM) (Fig. 3 and Supplementary Table 2). Finally, approximately 70% of isolated bacteria grew in the presence of concentrations >2 mM Ni or Co (Fig. 3), without significant differences between endophytes and rhizospheric strains. However, the highest

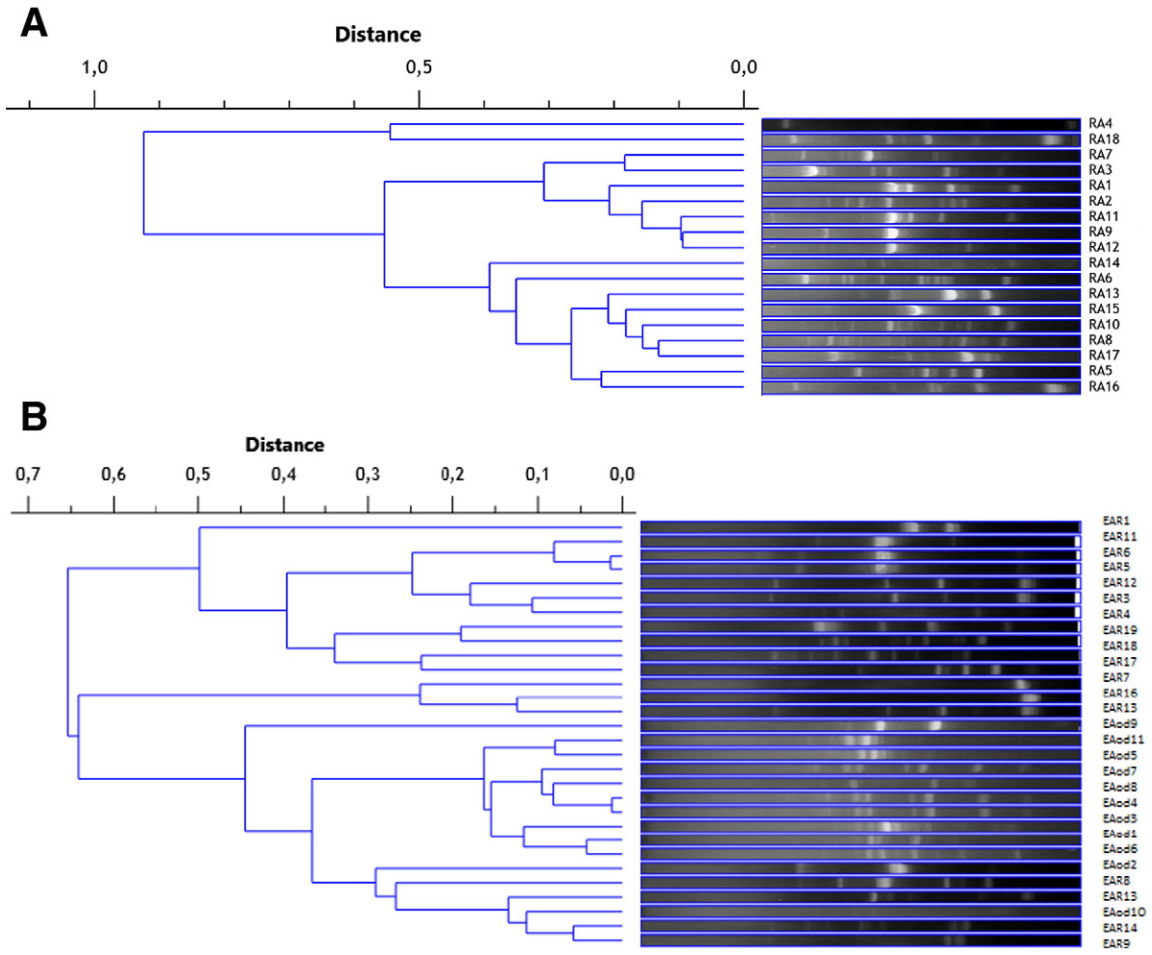

Fig. 1. Box-PCR patterns of the rhizospheric (A) and the endophytic (B) bacteria isolates from *A. macrostachyum* in the Odiel River estuary compared using dendogram. Scales at the top of the dendograms represent similarity.

values of resistance toward Ni appeared in endophytes isolated from the phyllosphere of the plant (Supplementary Table 2).

Regarding salt resistance, most of bacteria grew at elevated NaCl concentrations and could be considered halotolerant (Supplementary Table 2). In addition, 36% of bacteria did not grow in the absence of salt; hence, they are considered halophilic bacteria (Supplementary Table 2). Endophytes from the phyllosphere showed the highest values of resistance toward NaCl, while no significant difference could be observed for this characteristic between rhizospheric bacteria and root endophytes (Fig. 3B).

3.2.3. Screening for in vitro PGP properties

The presence of PGP properties in the isolates is a crucial aspect for their use as inoculants for phytoremediation purposes. All of them showed at least one PGP property (Fig. 5).

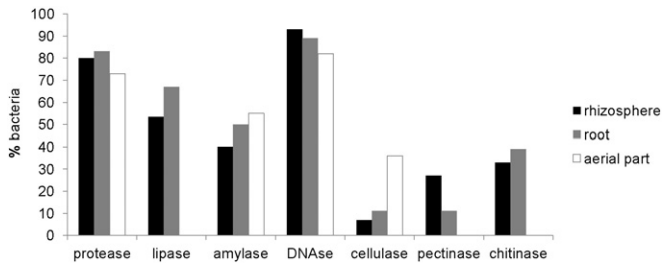

Fig. 2. Enzymatic activities present in isolated strains. Percentage of each group of isolates showing the property is compared.

The majority proportion (86%) of the strains produced siderophores, the most frequent PGP property among the isolates (Fig. 5). Semiquantitative test revealed that strains RA1, EAR1, and EAR8 were able to

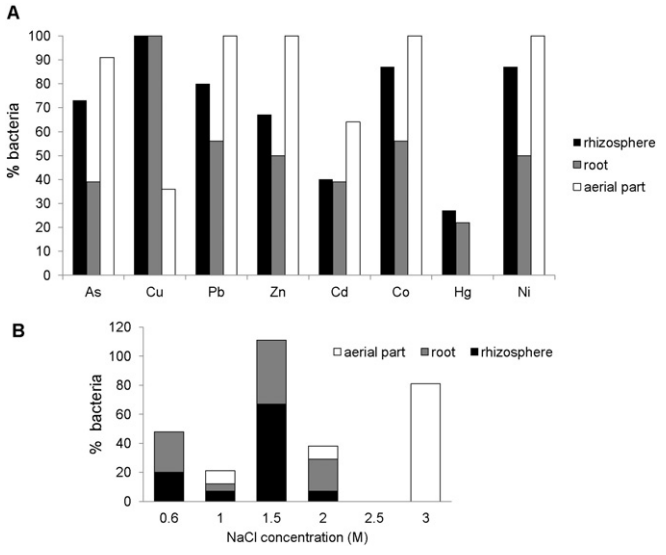

Fig. 3. Resistance of the isolated strains toward heavy metals (A) and NaCl (B). Percentage of bacteria able to grow at concentrations ≥1 mM As, Cu, Pb, Zn, Co, or Ni and ≥0.5 mM Cd or Hg were considered as resistant in (A). Percentage of bacteria able to grow at each MTC is represented in (B).

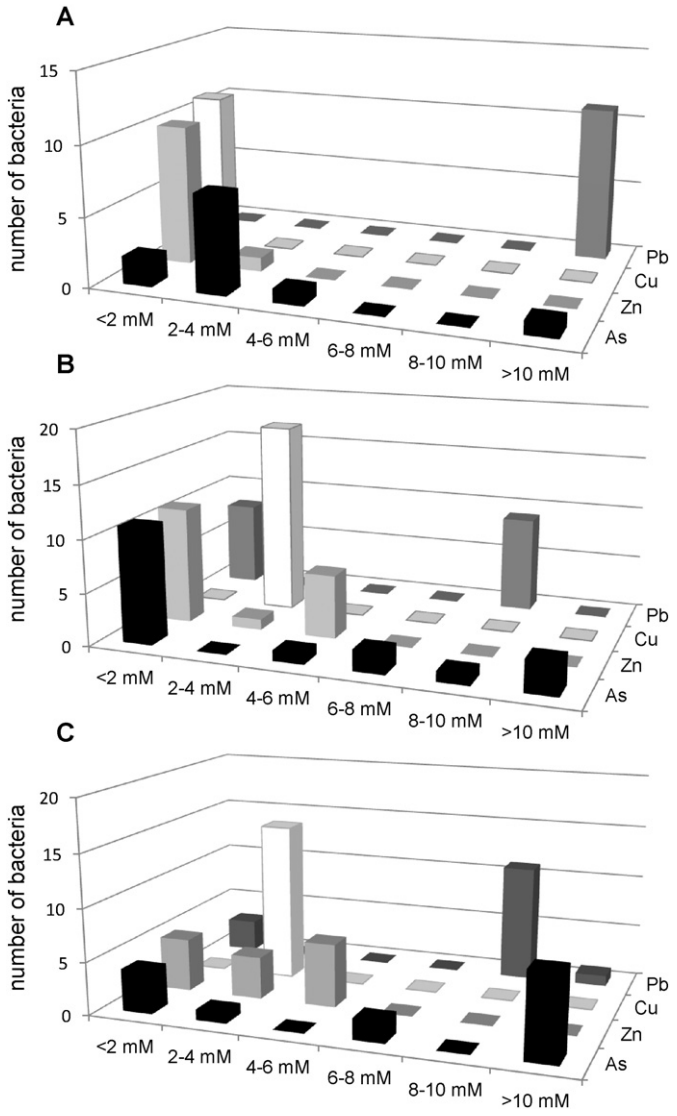

Fig. 4. Number of endophytes isolated from aerial part (A), roots (B), and the number of rhizospheric isolates (C) grouped according to As, Cu, Pb, or Zn resistance range.

produce significant amounts of siderophores than the rest of strains (Supplementary Table 3). Production of IAA and nitrogen fixation were also quite frequent among isolates (Fig. 5). For IAA, only values >1 mg l^{-1} were considered (Supplementary Table 3). EAod10 and EAod2 produced the highest amounts of IAA, 9.05 and 5.35 mg l^{-1}, respectively. A proportion of 20% of the isolates solubilized phosphate (Fig. 5). EAod3 and EAod8 showed the highest phosphate-solubilizing capability (Supplementary Table 3). Only 15% of the isolated bacteria were able to form biofilms (Supplementary Table 3). Finally, the presence of ACC deaminase activity in the isolates was also studied, but it was not present in any strain.

3.3. Effect of heavy metals on in vitro PGP properties

In order to determine how the presence of heavy metals could affect the PGP properties showed by the different isolates, they were assayed on plates supplemented with the most abundant heavy metals in Odiel marshes soils and also with Cd.

In general, there was a decrease in the number of bacteria with PGP properties in the presence of heavy metals. Nevertheless, the number of bacteria that produced IAA or solubilized phosphate increased in the presence of As or Cd, respectively (Fig. 6).

There was a reduction in the number of bacteria that produced siderophores in the presence of metals, particularly in the presence of Cd (Fig. 6). Nevertheless, strain RA18 was able to produce siderophores only in the presence of heavy metals (Supplementary Table 4).

The number of isolates producing IAA decreased, except in the presence of As (Fig. 6). In that case, not only the number of producers, but also the amount of IAA produced increased (Supplementary Table 5). A major proportion of 73% of rhizospheric bacteria produced >2 mg l^{-1} and almost 50% of endophytes >3 mg l^{-1}. Bacteria such as EAod10 produced almost fourfold the amount detected in the absence of As and EAR11 >20 folds (10.27 *vs.* 0.47 mg l^{-1}). Despite the decrease in the amount of bacteria produced in the presence of the other metals, some bacteria produced high values of IAA (Supplementary Table 5). IAA production was particularly affected by Cu.

The PGP property most sensitive to the presence of metals was nitrogen fixation. In fact, only RA1 fixed nitrogen in the presence of all metals and EAod10 in the presence of As (Supplementary Table 5).

Finally, the number of bacteria that solubilized phosphate increased in the presence of Cd (Fig. 6). Strains such as RA8, RA9, RA15, and endophytes EAR4 and EAR7, unable to solubilize phosphate in the absence of Cd, showed this property in the presence of this metal (Supplementary Table 4).

3.4. Effect of the selected consortia on seed germination in the presence and absence of heavy metals

Among endophytes, *Kushneria* sp. EAod3, *Micrococcus* sp. EAod10, *Bacillus* sp. EAR8, and *Halomonas* sp. EAR18 were selected considering their characteristics. A second *consortium* composed of the rhizospheric isolates *Vibrio* sp. RA1, *Pseudoalteromonas* sp. RA8 and RA15, and *Staphylococcus* sp. RA18 was also designed.

A. macrostachyum seeds were inoculated separately with both selected *consortia* in the presence and absence of heavy metals. Although germination kinetics was monitored for 21 days, no change in the number of germinated seeds was observed after 1 week (Fig. 7). In the absence of heavy metals, no significant difference in germination kinetics was observed between noninoculated seeds and seeds inoculated with the *consortium* of endophytes (Fig. 7A). Nevertheless, germination was accelerated in seeds inoculated with the rhizospheric *consortium*, because it began between days 2 and 3, with >80% of germinated seeds in 4 days and close to 100% in 5 days, while noninoculated seeds showed only 20% of germinated seeds in 4 days and needed 1 week to reach 100% of germination (Fig. 7A). On the contrary, germination kinetics of seeds inoculated with either the rhizospheric or endophytic *consortium* was very similar in the presence of small concentrations of heavy metals (Fig. 7B). In that case, both groups of inoculants accelerated germination when compared with noninoculated seeds, with 70% *versus* 20% of germinated seeds in 4 days, or 85% *versus* 55% in 5 days (Fig. 7B). After 1 week, 97% of inoculated seeds germinated and close to 90% of noninoculated seeds did.

4. Discussion

A. macrostachyum is a halophyte naturally growing in the southwestern coasts of Spain potentially useful for phytoremediation purposes, because it can tolerate and accumulate heavy metals (Conesa and Schulin, 2010; Madejón et al., 2009; Redondo-Gómez et al., 2010b). The ecological role of mycorrhizas in the rhizosphere of *A. macrostachyum* in polluted Mediterranean salt marshes has been described (Carrasco et al., 2006), but nothing is known about cultivable bacteria associated to this plant. In this study, 48 different bacterial strains were isolated from *A. macrostachyum* microbiome growing in a highly contaminated estuary. A proportion of 40% of the rhizospheric isolates were assigned to *Bacillus* genus. Identification of *Bacillus* strains in plant rhizosphere is frequent in studies describing bacteria diversity of soils (Garbeva et al., 2003). Furthermore, the genus *Bacillus* is one of

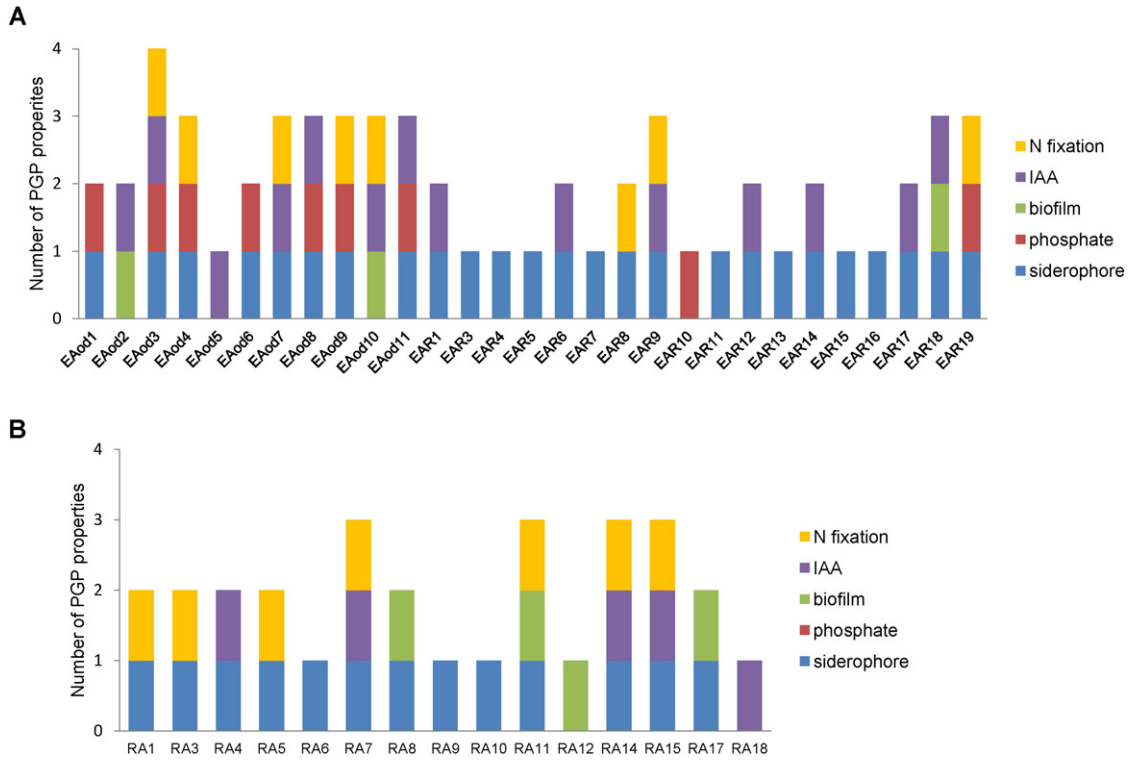

Fig. 5. The presence of PGP properties in endophytic (A) and rhizospheric (B) strains. For IAA production, only those strains able to produce ≥1 mg l^{-1} have been considered.

the most predominant in the rhizosphere of plants growing in metal-contaminated soils (Abou-Shanab et al., 2007), including estuaries (Mesa et al., 2015b), because of its capacity to resist metals and NaCl (Nies and Silver, 2007). Colonization of endophytes into the plant takes place mainly through the secondary roots (Dong et al., 2003; Lamb et al., 1996), which explains why 39% of rhizospheric bacteria were also found as root endophytes. Concerning endophytes isolated from the phyllosphere, most of the strains belonged to halotolerant and halophilic genera, such as *Halomonas* and *Kushneria*. It is important to note the low percentage of identity with previously described species of some isolates, suggesting a significant unclassified diversity of cultivable bacteria associated to *A. macrostachyum* growing in this

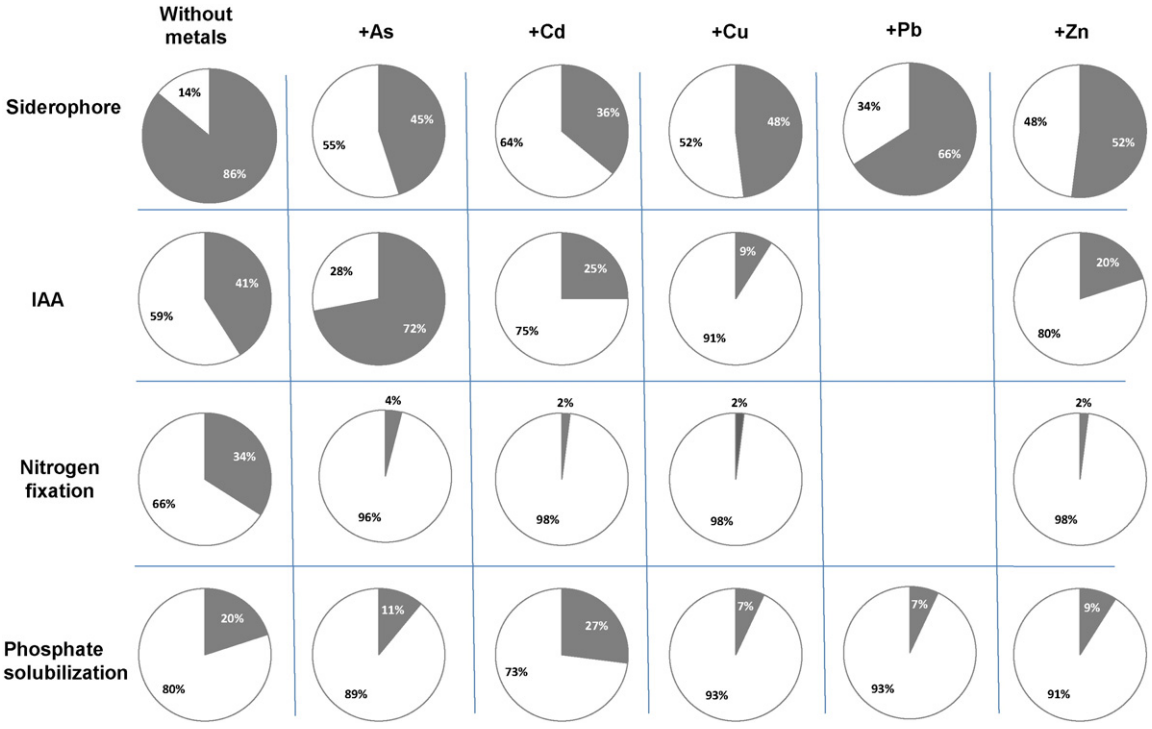

Fig. 6. Effect of heavy metals on PGP properties. Percentage of isolates with the different PGP properties in the absence and presence of metals. Media were supplemented with 1 mM As, Cu, Pb or Zn, or 0.25 mM Cd.

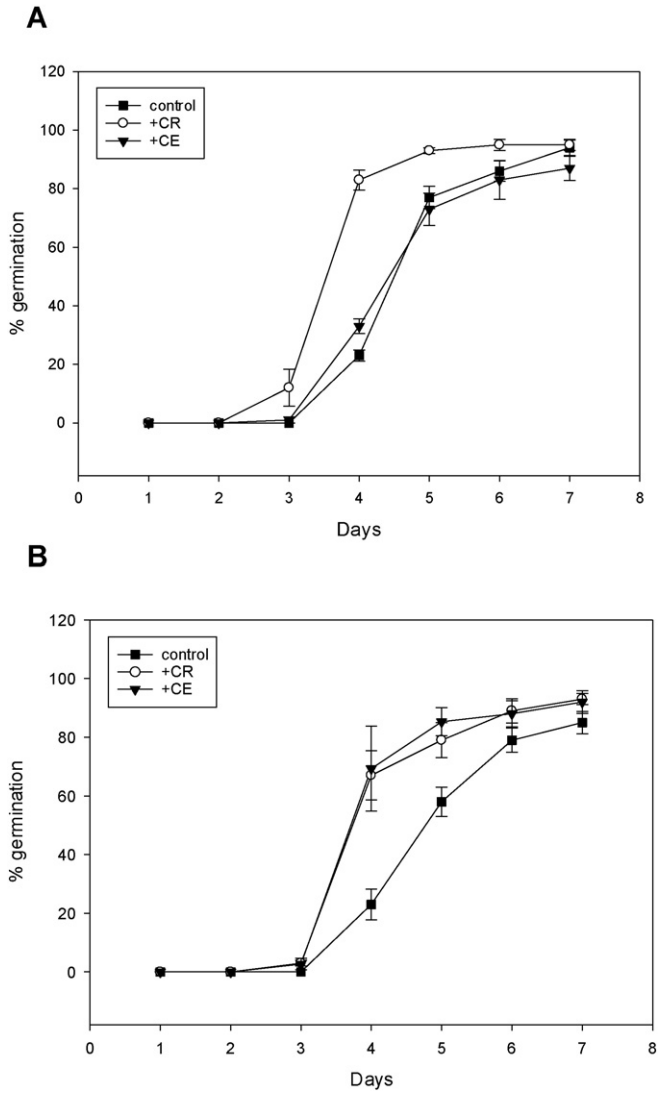

Fig. 7. *A. macrostachyum* seeds accumulative germination kinetics in the absence (A) and presence (B) of heavy metals. Control: noninoculated seeds; +CR: seeds inoculated with the rhizospheric *consortium*; +CE: seeds inoculated with the endophytic *consortium*. Results are means ± SD of four independent plates with 25 seeds per plate.

contaminated ecosystem. Although in some cases this low identity was probably due to partial sequencing with one single (forward) primer, the identity of some isolates was low despite the fact that the length of the sequence was almost complete (>1400 bp). Ongoing taxonomic studies of some of these strains will determine if they constitute new species.

Most of the isolates were multiresistant to heavy metals. A high percentage of strains was resistant toward elevated amounts of As, Cu, Pb, and Zn, the most relevant contaminants in the Odiel marshes. The resistance toward Cu was lower among endophytes of phyllosphere than among the other two groups of isolates. It is noteworthy that *A. macrostachyum* accumulates Cu in roots. On the contrary, although many strains grew in the presence of high concentrations of Pb, the highest levels of resistance were observed in endophytes of phyllosphere. Previous reports indicated that *A. macrostachyum* accumulate high amounts of Pb (100–960 mg per kg) in the aerial part (Conesa and Schulin, 2010; Mendez and Maier, 2008; Álvarez-Rogel et al., 2004; García et al., 2003) suggesting that endophytes could help the plant in the uptake of this metal. It has also been noted that this plant accumulates Cd in the roots (Redondo-Gómez et al., 2010b), so not surprisingly the strains with the highest resistance toward this metal were found in the roots and rhizosphere of the plant. Concerning salt tolerance, most of the strains were either halotolerant or halophilic, showing the highest levels of tolerance the endophytes isolated from the phyllosphere, where *A. macrostachyum* is able to accumulate high concentrations of NaCl (Redondo-Gómez et al., 2010a).

The presence of enzymatic activities in the isolated bacteria is an important attribute for their use as inoculants. These enzymes are important, because they contribute to bacterial colonization (in the case of endophytes) and to degrade plant residues and acquire plant nutrients (Wang and Dai, 2010). Cellulase, pectinase, and chitinase activities, the most relevant enzymatic activities, were present in both the rhizospheric and endophytic bacteria. Chitinase activity, found in root and rhizosphere isolates, could increment the resistance of the plant toward fungus (Quecine et al., 2012; Trotel-Aziz et al., 2008). On the contrary, cellulase activity, mainly found in endophytes, could allow cell wall degradation and bacterial colonization. Motility, a characteristic present in most of endophytes, is responsible for the spreading of these bacteria through the plant (Compant et al., 2010).

The roots of plants attract beneficial microorganisms, particularly under stress (Bais et al., 2006; Dzantor, 2007). At least one PGP property could be observed in each strain. Most of the isolates produced siderophores, which could affect heavy metal uptake by the plants (Gururani et al., 2012). In addition, it could also act as a biocontrol mechanism (Gaiero et al., 2013). Another property widely distributed among the isolated bacteria was IAA production, an essential hormone in root development (Lambrech et al., 2000). Phosphate is frequently found in the soil in insoluble form, so bacteria able to solubilize phosphate make it available to the plant (Hameeda et al., 2008). Several strains showed this property. The capability to fix nitrogen, present in almost half of bacteria, may provide this element to the plant (Bhattacharyya and Jha, 2012). Finally, biofilm formation could help during bacterial attachment to the root and also to remove heavy metals, as extracellular polymeric substances involved in biofilm formation are able to immobilize and concentrate metals into their structure (Das et al., 2012). Few isolates were able to form biofilms. In general, the amount of bacteria with PGP properties decreased in the presence of heavy metals. Interestingly, some strains were able to retain their PGP properties. However, in some cases, IAA production increased in the presence of As and the number of isolates that solubilized phosphate was higher in the presence of Cd than in the absence of the metal. The effect of Cu over the production of PGP properties was also studied by Mesa et al. (2015b) using strains isolated from the rhizosphere of *Spartina maritima* in polluted marshes, reaching similar conclusions, that is, heavy metals clearly affect some PGP properties in an unpredictable way. These *in vitro* data could help predict the behavior of the bacteria in polluted soils.

With the prospect of future research, the best-performing strains were selected among the isolated bacteria to form two bacterial *consortia*. The endophytes *Kushneria* sp. EAod3, *Micrococcus* sp. EAod10, *Bacillus* sp. EAR8, and *Halomonas* sp. EAR18 were chosen. EAod10 was the best strain, because it not only presented several PGP properties and maintained them in the presence of heavy metals (nitrogen fixation, production of IAA, and biofilm formation), but also was able to solubilize phosphate and produce siderophores with metals in the media. In addition, it showed the highest MTC values for most of metals. EAod3 produced siderophores and solubilized phosphate in both the presence and absence of heavy metals, complementing the PGP properties that were not present in EAod10 in the absence of metals. This strain also produced IAA in the presence of some metals (including Cu) and had high MTC values for most of metals. Concerning root endophytes, EAR8 fixed nitrogen, solubilized phosphate in the presence of Pb, and produced the highest amount of siderophores, as well as grew at high concentrations of heavy metals. Meanwhile, EAR18 was able to form biofilm and produce IAA in the presence and absence of heavy metals. The second *consortium* contained the rhizospheric bacteria *Vibrio* sp. RA1, *Pseudoalteromonas* sp. RA8 and RA15, and *Staphylococcus* sp.

RA18. These strains produced siderophores with and without heavy metals (except RA18, which only produced it in the presence of heavy metals), RA1 being the best producer. They also produced IAA in the presence of heavy metals, while RA15 and RA18 produced the hormone in their absence. In addition, RA15 and RA18 solubilized phosphate with heavy metals in the culture media. RA1 provided the nitrogen fixation capacity to the *consortium* and RA8 the ability to form biofilms. Finally, these strains grew at high concentrations of heavy metals.

Bacterial inoculation accelerated *A. macrostachyum* seed germination in the absence and particularly in the presence of heavy metals (As, Cu, Pb, and Zn), reaching significantly higher germination rates in fewer days. Both *consortia* accelerated seed germination in the presence of small concentrations of metals, while in the absence of metals, acceleration was only relevant when seeds were inoculated with the rhizospheric *consortium*. The beneficial effect of the rhizospheric *consortium* on germination in absence of metals could be explained by the presence of enzymatic activities, such as pectinase and chitinase, which are important to break the cell wall and could contribute to improve the hydration of the seeds. In the presence of metals, IAA produced by both *consortia* may act as antagonist of ethylene produced by seeds in metal stress conditions (Burd et al., 2000), thus contributing to improve germination. These results clearly suggest that selected bacteria could be useful as inoculants to ameliorate *A. macrostachyum* establishment in heavy metal-contaminated marshes when using seeds to propagate the plant.

Our global aim is to reduce and alleviate the effects of metal contamination in marsh soils using microbe-assisted phytoremediation techniques involving autochthonous plants. In a previous work, inoculation of the halophyte *S. maritima* in contaminated marsh soil with a rhizospheric bacterial *consortium* increased plant growth and metal uptake in plant roots, thus improving the phytostabilization potential of the plant (Mesa et al., 2015c). On the contrary, endophytic cultivable bacteria improved *S. maritima* growth but not metal accumulation in plants that grew in the same soil (Mesa et al., 2015a). It looks clear that the behavior of each *consortium* needs to be evaluated *in planta*, so the bacterial *consortia* described in this study will be used to inoculate *A. macrostachyum* plants in contaminated marsh soil to evaluate their ability to promote plant growth and/or metal uptake. The combined use of *S. maritima*, a fast-growing grass that colonizes the lower marsh and *A. macrostachyum*, a shrub able to establish deep root networks into middle marsh soils, assisted by their respective selected PGPB, could be an appropriate strategy to implement marsh restoration programs aimed to recover contaminated coasts of southwest Spain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.06.070.

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