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Bacterial inoculants for enhanced seed germination of *Spartina densiflora*: Implications for restoration of metal polluted areas

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ABSTRACT

The design of effective phytoremediation programs is severely hindered by poor seed germination on metal polluted soils. The possibility that inoculation with plant growth promoting rhizobacteria (PGPR) could help overcoming this problem is hypothesized. Our aim was investigating the role of PGPR in *Spartina densiflora* seed germination on sediments with different physicochemical characteristics and metal pollution degrees. Gram negative *Pantoea agglomerans* RSO6 and RSO7, and gram positive *Bacillus aryabhattai* RSO25, together with the *consortium* of the three strains, were used for independent inoculation experiments. The presence of metals (As, Cu, Pb and Zn) in sediments reduced seed germination by 80%. Inoculation with *Bacillus aryabhattai* RSO25 or *Pantoea agglomerans* RSO6 and RSO7 enhanced up to 2.5 fold the germination rate of *S. densiflora* in polluted sediments regarding non-inoculated controls. Moreover, the germination process was accelerated and the germination period was extended. The *consortium* did not achieve further improvements in seed germination.

1. Introduction

Environmental pollution by heavy metals and trace elements is one of the most serious problems worldwide, increasing in parallel with human technology development (Wuana and Okieimen, 2011). Deleterious effects of metal pollution are especially significant in estuarine systems, such as salt marshes, since the widespread and diverse usage from industry activity has elevated metal concentrations in sediments and water above permitted levels in many estuaries and marine environments worldwide (Xia et al., 2011; Gao et al., 2015; Soliman et al., 2015).

Literature is full of strategies designed to counterbalance the harmful effects of heavy metals, including physical, chemical and biological methods that immobilize or remove metals from the environment (Bentley et al., 2005; Peng et al., 2009). But recently phytoremediation, the use of plants to act upon the pollutants, by extracting, degrading or immobilizing them, is gaining *momentum*, due to its cost-effectiveness, long-term applicability and ecological compatibility (Tangahu et al., 2011; Laghlimi et al., 2015). Among plant species with phytoremediation potential, the halophyte cordgrass *Spartina densiflora* possesses a high capacity for accumulating heavy metals in its tissues, particularly in roots (Mateos-Naranjo et al., 2008a, 2008b, 2015a), as well as for phytostabilizing them in its rhizosphere (Cambrollé et al., 2008; Redondo-Gómez et al., 2011; Redondo-Gómez, 2013).

As an adjunct to make phytoremediation more efficacious, a number of scientists have begun to explore the possibility of using soil bacteria together with plants, in order to alleviate plant stress in the presence of pollutants (Glick, 2010; De-Bashan et al., 2012; Phieler et al., 2013; Pajuelo et al., 2014; Nadeem et al., 2015). It has recently been found that the bioaugmentation of *S. densiflora* adult plants with a selected bacterial *consortium* isolated from its rhizosphere can be claimed to enhance plant growth response and tolerance to the physicochemical properties of marshes soils (Andrades-Moreno et al., 2014; Mateos-Naranjo et al., 2015b). However, little is known about the response of bacteria-plant interactions in terms of seed germination (Andrades-Moreno et al., 2014; Faisal, 2013; Ndeddy and Babalola, 2016), despite that *S. densiflora* is characterized by its prolific seed production (Mateos-Naranjo et al., 2008c).

Seed germination is strongly inhibited by the presence of excess metals in soils (Sethy and Ghosh, 2013). Several processes are involved, with differences depending on the metal, including, inhibition of enzyme activities (Sfaxi-Bousbih et al., 2010; Singh et al., 2011), disruption of metabolism (Zhang et al., 2009), increased ROS production and activation of antioxidant system (Sharma and Dietz, 2009; Hossain et al., 2012), damage to DNA (Lin et al., 2008), DNA methylation (Rancelis et al., 2012), damage to membranes and electrolyte leakage (Rahoui et al., 2010), etc. In this particular, the negative effect of heavy metals on seed germination is a handicap for phytoremediation projects, since the germination and initial establishment of the plants on metal polluted soils are severely hindered (Singh et al., 2007; Mateos-Naranjo et al.,

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2011; Ndeddy and Babalola, 2016). Thus we hypothesize that inoculation with PGPR could facilitate the germination of *S. densiflora* in sediments with different physicochemical properties, being this information essential in order to improve the design of restoration programs. The aim of this study was to investigate the role of bacteria isolated from the rhizosphere of *Spartina maritima* in the germination of *S. densiflora* seeds on sediments with contrasting physicochemical properties and different degrees of metal pollution. Comparison between inoculants based on gram positive and gram negative bacteria is also approached.

2. Materials and methods

2.1. Seed and sediment source

Ripe spikes of *S. densiflora* were collected in December 2014 in the Odiel marshes (37°15′N, 6°58′W; SW Spain) from 30 different clumps chosen randomly located in a well-drained gently sloping intertidal lagoon (mean sea level + 1.85 m relative to SHZ). Caryopses were stripped from the spikes and those with seeds were selected and stored in the dark for 4 months at 4 °C until the beginning of the experiment.

In addition, samples of the first 10 cm of sediment were collected from two different natural areas at Odiel and Piedras marshes (Gulf of Cadiz, SW Spain), and subsequently transported to the laboratory for their physic-chemical characterization. Measurements of sediment texture, conductivity, pH, redox potential, sediment metal concentrations (n = 5) were determined by using the method employed by Mateos-Naranjo et al. (2011). The physicochemical properties of the sediments are given in Table 1. Moreover, in order to eliminate any bacteria present, sediments were sterilized three times at 121 °C for 30 min, separated by 24 h, and stored in sterile plastic bags until the beginning of the germination experiment.

2.2. Bacterial inocula and seed germination experiment

Bacteria used in this work were isolated from the rhizosphere of *S. maritima* grown in the Odiel marshes and identified as the gram negative *Pantoea agglomerans* strains RSO6 and RSO7 and the gram positive *Bacillus aryabhattai* strain RSO25 (Paredes-Páliz et al., in revision). They were selected due to their high resistance to several metals and metal biosorption, together with plant growth promoting (PGP) properties and the capacity for biofilms' formation (Paredes-Páliz et al., in revision).

In May 2015 a glasshouse experiment was performed to assess the effect of individual bacterial and a bacterial *consortium* on seed germination of *S. densiflora*. The three bacterial strains were cultivated separately in 100 ml of Tryptic Soy Broth (TSB) medium at 28 °C and continuous shaking (200 rpm) for 24–48 h depending on the bacterium. Cultures were centrifuged at 8.000 ×g and pellets were suspended in sterile distilled water for the inoculation process. The absorbance of the cultures at 600 nm was determined and adjusted to 1.5 for all of them with sterile distilled water. Moreover, in order to guarantee that there were not antagonistic effects between the strains in the *consortium*, an antagonist test was done as follows: cultures of the three strains with equal absorbance at 600 nm were mixed. The mix was diluted up to 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. One hundred microliters of the diluted samples was plated on TSA medium and plates were incubated at 28 ° C for 24 h (n = 3). Our results indicated that there were not antagonistic effects between the components of the *consortium* (data not shown).

Seeds used for experiment were previously surface-sterilized by vigorous shaking in sodium hypochlorite solution (5%, v/v) for 5 min and then washed with sterilized water. Five 10-seed replicates were sown 1 cm deep in individual plastic pots of 100 ml filled with sterile homogenates of either, previously collected sediments (Odiel and Piedras) mixed with perlite in order to increase the hydraulic conductivity (proportion 3:1).

Pots were kept at controlled 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 flux and were randomly assigned to four inoculation treatments (without inoculation, inoculation with strain RSO25, with strains RSO6 and RSO7 together and with the *consortium* integrated by the strains RSO6, RSO7 and RSO25) in combination with the two collected sediments (n = 50, one seed per pot and 50 pots per sediment and inoculation treatment). Inoculation process was done at the beginning of the experiment and was repeated once a week during the experiment period. 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.

Pots were daily inspected for 50 days and seed germination considered after cotyledon appearance. Seven germination characteristic parameters were determined at the end of the experiment: final germination percentage, number of days to first germination (FGD), number of days to final germination (LGD), mean time to germination (MTG), coefficient of velocity of germination (CVG) and mean daily germination (MDG) (Curado et al., 2010; Mateos-Naranjo et al., 2011; Jahanian et al., 2012).

2.3. Statistical analysis

Statistical analyses were done using 'Statistica' v. 6.0 (Statsoft Inc.). Comparisons between means of germination parameters in relation with the different sediments and inoculation treatments were tested using two-way ANOVA (F-test) analyses. Data were first tested for normality with the Kolmogorov–Smirnov test and for homogeneity of variance with the Brown–Forsythe test. Significant test results were followed by Tukey tests for identification of important contrasts.

Table 1
Physicochemical properties and concentration of arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn) and iron (Fe) of sediments from Piedras and Odiel marshes.

Sediment	Physico-chemical properties		Metal (mg Kg^{-1})	
Piedras	Texture (%)	20/14/66	As	6.5 ± 0.1
	pH	7.6 ± 0.3	Cd	0.2 ± 0.1
	Redox potential (mV)	150 ± 15	Cu	19.0 ± 0.2
	Conductivity (mS cm^{-1})	1.2 ± 0.4	Pb	16.0 ± 0.3
	Organic matter (%)	11.4 ± 0.5	Zn	78.0 ± 0.2
	Nitrogen (%)	0.2 ± 0.1	Fe	12,669 ± 229
Odiel	Texture (%)	60/16/24	As	339.8 ± 12.0
	pH	6.9 ± 0.1	Cd	4.0 ± 0.13
	Redox potential (mV)	262 ± 10	Cu	1318.4 ± 26.8
	Conductivity (mS cm^{-1})	15.6 ± 0.5	Pb	406.7 ± 29.4
	Organic matter (%)	13.5 ± 0.2	Zn	2522.7 ± 65.3
	Nitrogen (%)	0.3 ± 0.1	Fe	71026 ± 343

Data are means ± SE of five replicates. Texture (silt/clay/sand percentage).

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