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RESEARCH PAPER

Investigating the mechanisms underlying phytoprotection by plant growth-promoting rhizobacteria in *Spartina densiflora* under metal stress

K. Paredes-Páliz[1], R. Rodríguez-Vázquez[1], B. Duarte[2], M. A. Caviedes[1], E. Mateos-Naranjo[3], S. Redondo-Gómez[3], M. I. Caçador[2], I. D. Rodríguez-Llorente[1] & E. Pajuelo[1]

1 Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain
2 MARE - Marine and Environmental Sciences Centre, Faculty of Sciences of the University of Lisbon, Lisbon, Portugal
3 Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

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Correspondence
E. Pajuelo, Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla. c/ Profesor García González, 2, 41012-Sevilla, Spain.
E-mail: epajuelo@us.es

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ABSTRACT

- Pollution of coasts by toxic metals and metalloids is a worldwide problem for which phytoremediation using halophytes and associated microbiomes is becoming relevant. Metal(loid) excess is a constraint for plant establishment and development, and plant growth promoting rhizobacteria (PGPR) mitigate plant stress under these conditions. However, mechanisms underlying this effect remain elusive. The effect of toxic metal (loid)s on activity and gene expression of ROS-scavenging enzymes in roots of the halophyte *Spartina densiflora* grown on real polluted sediments in a greenhouse experiment was investigated.
- Sediments of the metal-polluted joint estuary of Tinto and Odiel rivers and control, unpollutred samples from the Piedras estuary were collected and submitted to ICP-OES. Seeds of *S. densiflora* were collected from the polluted Odiel marshes and grown in polluted and unpolluted sediments. Rhizophere biofilm-forming bacteria were selected based on metal tolerance and inoculated to *S. densiflora* and grown for 4 months. Fresh or frozen harvested plants were used for enzyme assays and gene expression studies, respectively.
- Metal excess induced SOD (five-fold increase), whereas CAT and ascorbate peroxidase displayed minor induction (twofold). A twofold increase of TBARs indicated membrane damage. Our results showed that metal-resistant PGPR (*P. agglomerans* RSO6 and RSO7 and *B. aryabhattai* RSO25) contributed to alleviate metal stress, as deduced from lower levels of all antioxidant enzymes to levels below those of non-exposed plants. The oxidative stress index (OSI) decreased between 50 and 75% upon inoculation.
- The results also evidenced the important role of PAL, involved in secondary metabolism and/or lignin synthesis, as a pathway for metal stress management in this halophyte upon inoculation with appropriate PGPR, since the different inoculation treatments enhanced *PAL* expression between 3.75- and five-fold. Our data confirm, at the molecular level, the role of PGPR in alleviating metal stress in *S. densiflora* and evidence the difficulty of working with halophytes for which little genetic information is available.

INTRODUCTION

Pollution by toxic metals and metalloids is gradually being recognised as serious through increasing industrialisation and disturbance to natural biogeochemical cycles (Wuana & Okieimen 2011). Estuaries and coasts are particularly endangered ecosystems and often act as sinks for pollutants both from the seashore and carried by rivers (Qian *et al.* 2015; Vikas & Dwarakish 2015). In this regard, the Odiel estuary (SW Spain) is one of the most metal-contaminated salt marshes in Europe (Nieto *et al.* 2007; Morillo *et al.* 2008).

Regarding their role in biological systems, metals are classified as essential (micronutrients), *e.g.* Fe, Mn, Cu, Zn, Ni or non-essential, *e.g.* Cd, Pb, As, Hg and Cr (He *et al.* 2005). However, beyond threshold limits all metals disrupt metabolism, increase production of reactive oxygen species (ROS; Hossain *et al.* 2012), damage DNA (Lin *et al.* 2008) and cause membrane lipid peroxidation (Upadhyay & Panda 2010). Many strategies to counterbalance the harmful effects of toxic metals and metalloids have been reported (Peng *et al.* 2009). Among these, phytoremediation is considered a green technology in which plants can take up, detoxify, translocate and accumulate toxic metal(loid)s in the aboveground biomass, which must be harvested for appropriate disposal (Vamerali *et al.* 2010). This technique, known as phytoextraction, is based on the use of hyperaccumulator plants (Krämer 2010). Conversely,

some plants, known as non-hyperaccumulators or excluders, adopt a different strategy by reducing either metal uptake and/or translocation to the shoot, hence preventing their entry into the food chain (Méndez & Maier 2008).

High salt concentrations in salt marshes select for halophytic plants (Shabala 2013; Flowers & Colmer 2015), some of which can also tolerate toxic metal(loid)s (Pedro *et al.* 2015; Sghaier *et al.* 2016) and hence have been proposed for use in metal phytoremediation (Manousaki & Kalogerakis 2011; Van Oosten & Maggio 2015). Among them, *Spartina* species are resistant to multiple stress conditions (Redondo-Gómez 2013; Mateos-Naranjo *et al.* 2015; Ainouche & Gray 2016) and possess high capacity for metal accumulation in roots, as well as metal phytostabilisation in the rhizosphere (Mateos-Naranjo *et al.* 2008a,b, 2011; Redondo-Gómez 2013). Part of this capacity is attributed to the formation of Fe/Mn plaques on the roots, which act as a primary barrier to prevent the entrance of metals (Fresno *et al.* 2016; Yang *et al.* 2016).

To cope with toxic metals and metalloids, plants have evolved multiple strategies, including reduced uptake, chelation, precipitation, compartmentalisation into vacuoles and cell walls, cell wall modification, metal loading to the phloem, synthesis of secondary metabolites, ethylene synthesis and signalling, synthesis of reducing agents such as glutathione, ascorbate, glyoxal, and ROS scavenging enzymes (Hossain *et al.* 2012; Sytar *et al.* 2013). ROS are signalling molecules involved in many essential physiological processes like lignification and programmed cell death, and act as signalling/alarm molecules in regulation of gene expression (Finkel 2011; Bhattacharjee 2012). Exposure of plants to toxic metal(loid)s induces the production of ROS that inhibit most cellular processes (Gill & Tuteja 2010). Thus, the intracellular level of ROS must be tightly regulated through a control system composed of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POD), ascorbate peroxidase (APX) and guaiacol peroxidases (GPX), among others, together with non-enzymatic antioxidants including glutathione (GSH), ascorbic acid (AsA) and tocopherol (Gill & Tuteja 2010; Hernández *et al.* 2015).

Besides this antioxidant system, other general stress pathways are activated on metal exposure. Some genes are considered as markers for metal stress in plants (Ciarmiello *et al.* 2011) *e.g.* genes coding for cysteine synthase, glutathione reductase and glutathione transferase, and enzymes of the glutathione and phytochelatin synthesis pathway are involved in metal detoxification (Yadav 2010). The genes *STO*, *P5CS* and *LEA-II* are related to salt stress (Nagaoka & Takano 2003; Liang *et al.* 2013; Kosová *et al.* 2014), *P450* codes for cytochrome P450 monooxygenase involved in responses to drought and salt stresses (Tamiru *et al.* 2015), *Nramp1* encodes a metal transporter (Thomine *et al.* 2000), genes coding for SOD and PX are ROS scavenging enzymes (Hossain *et al.* 2012), *ETR1* encodes an ethylene receptor (Dey & Vlot 2015; Keunen *et al.* 2016; and *PAL* codes for phenylalanine ammonium lyase, which is the first and key enzyme in the synthesis of secondary metabolites such as terpenes, flavonoids, isoflavonoids and brassinosteroids, and is also involved in lignin synthesis (Gholizadeh & Kohnehrouz 2010).

Rhizosphere bacteria have a decisive influence on plant metal accumulation, as well as in ameliorating plant growth under an array of stress situations (Rajkumar *et al.* 2012; Goswami *et al.* 2016). Plant growth-promoting rhizobacteria (PGPR) colonise the rhizosphere of plants and promote plant growth through mechanisms such as solubilisation of mineral phosphates, biological N_2 fixation, production of siderophores and phytohormones, and can induce systemic resistance in the plant (De-Bashan *et al.* 2012; Pérez-Montaño *et al.* 2014, 2014; Goswami *et al.* 2016). Moreover, some rhizobacteria are involved in the formation of the Fe/Mn plaques, leading to metal phytostabilisation (Lakshmanan *et al.* 2015; Dong *et al.* 2016). In previous studies our group (Paredes-Páliz *et al.* 2016a) isolated rhizobacteria from *S. maritima* naturally growing in the Odiel estuary. Among these, the Gram-negative *Pantoea agglomerans* strains RSO6 and RSO7, and Gram-positive *Bacillus aryabhattai* strain RSO25 were selected based on their PGP properties, high metal tolerance, metal biosorption capacity and the ability to form biofilms (Paredes-Páliz *et al.* 2016a). The effect of plant inoculation with these bacteria has been tested: they improved germination of *S. densiflora* seeds under metal stress 2.5–3.0-fold (Paredes-Páliz *et al.* 2016b) and favoured growth, photosynthetic activity and accumulation of toxic metals and metalloids in roots (phytostabilisation; Paredes-Páliz *et al.* 2017). Thus, it appears that PGPR have a protective effect on plant roots, alleviating stress caused by metal exposure (Goswami *et al.* 2016). However, the mechanism underlying this phytoprotection is not fully established.

The present study aims to analyse the effect of inoculation with metal-tolerant PGPR (Gram-positive *B. aryabhattai* RSO25, Gram-negative *P. agglomerans* RSO6 and RSO7, and consortium containing all three) on several stress parameters of *S. densiflora* grown on sediments polluted by toxic metals and metalloids, at two different levels. We assessed (i) the activity of ROS-scavenging enzymes, and (ii) changes in the expression of the abovementioned genes as markers of metal stress.

MATERIAL AND METHODS

Soil samples and collection of seeds

Sediments of the joint estuary of Tinto and Odiel rivers were collected in December 2015 in the Odiel marshes (37°15′N, 6°58′W, SW Spain) at a depth of 10 cm. As a control, samples of sediments from an unpolluted estuary (Piedras estuary, situated near the Tinto-Odiel estuary, but not affected by metal pollution; 37°13′N, 7°10′W) were collected. Characterisation of soil properties and determination of metals (As, Cu, Pb and Zn) in sediments with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) was performed according to Cambrollé *et al.* (2008).

Ripe spikes of *S. densiflora* were collected in the Odiel marshes from 30 different clumps chosen randomly. Caryopses were stripped from the spikes and those with seeds were selected and stored in the dark at 4 °C until use.

Plant growth conditions and inoculation treatments

Spartina densiflora plants were grown on metal-polluted and unpolluted sediments mixed with perlite (sediment:perlite 3:1). Seeds were surface-sterilised in sodium hypochlorite (5%, v/v) for 5 min and exhaustively washed with sterile water. Fifty seeds per treatment were sown 1-cm deep in individual plastic

pots filled with previously sterilised sediment. Pots were maintained at 2–25 °C; 40–60% relative humidity and natural daylight from 250–1000 μmol m^{-2} s^{-1}. Watering was done twice a week.

Bacteria used in this work were previously isolated from the rhizosphere of *Spartina* from the Odiel marshes (Paredes-Páliz *et al.* 2016a). Gram-negative *P. agglomerans* strains RSO6 and RSO7 and Gram-positive *B. aryabhattai* strain RSO25 were selected among 25 initial isolates, based on high tolerance to toxic metals and metalloids, high metal biosorption capacity, plant growth-promoting (PGP) properties and the ability to form biofilms (Paredes-Páliz *et al.* 2016a). Inoculation treatments were as follows: (i) non-inoculated; (ii) inoculated with Gram-negative *P. agglomerans* strains RSO6 and RSO7; (iii) inoculated with Gram-positive *B. aryabhattai* strain RSO25; (iv) inoculated with a consortium of all three bacteria. The three bacterial strains were cultivated separately in 100 ml tryptic soy broth (TSB) at 28 °C and continuous shaking (200 rpm) for 24–48 h. Cultures were centrifuged at 8.000 × *g* and pellets suspended in sterile distilled water. Absorbance at 600 nm was measured using a PerkinElmer Lambda 25 UV/Vis spectrophotometer (Waltham, MA, USA) and adjusted to 1.5 with sterile distilled water. Plants were inoculated at the beginning of the experiment and once a week during the experimental period (4 months). The pots received 1 ml of the bacterial inoculant (either 1 ml of one strain or 1 ml of the consortium, all with OD 600 nm of 1.5).

After 4 months, plants were harvested and half quickly frozen in liquid N_2 and stored at −80 °C for gene expression studies, or used fresh for enzyme assays.

Determination of antioxidant enzymes

Enzyme extraction was performed at 4 °C. Five hundred mg fresh roots were ground with mortar and pestle in liquid N_2 and thawed in 8 ml 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. All the enzyme assays were made at room temperature.

Catalase (CAT) activity was measured by monitoring consumption of H_2O_2 at 240 nm ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$) using a PerkinElmer Lambda 25 UV/Vis spectrophotometer (PerkinElmer; Duarte *et al.* 2015). The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.6, 0.1 mM Na-EDTA and 100 mM H_2O_2. The reaction started with addition of 100 μl root extract. Ascorbate peroxidase (APX) was analysed according to Duarte *et al.* (2015) in 50 mM sodium phosphate buffer, pH 7.0, 12 mM H_2O_2 and 0.25 mM L-ascorbate. The reaction started with addition of 100 μl enzyme extract. Activity was measured as the decrease in absorbance at 290 nm due to ascorbate oxidation, and calculated using the molar extinction coefficient of $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. Superoxide dismutase (SOD) activity was evaluated by monitoring the reduction of pyrogallol at 325 nm (Duarte *et al.* 2015). The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.6, 0.1 mM Na-EDTA and 3 mM pyrogallol. The reaction was started with the addition of 10 μl enzyme extract. For guaiacol peroxidase (GPX), the reaction mixture consisted of 50 mM sodium phosphate buffer, pH 7.0, 2 mM H_2O_2 and 20 mM guaiacol. The reaction started with addition of 100 μl enzyme extract. Activity was measured by monitoring absorbance at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). Control assays were made in the absence of substrate in order to evaluate auto-oxidation of the substrate (Duarte *et al.* 2015).

Protein content was determined according to Bradford (1976) using bovine serum albumin (fraction V, Sigma) as standard.

Determination of thiobarbituric acid reactive substances (TBARs)

Root samples were homogenised in 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) at a ratio of 100:1 (w/v) (Duarte *et al.* 2015). The homogenate was extracted at 95 °C for 30 min, and the reaction immediately interrupted by chilling in ice then centrifuged at 3000 × *g* for 5 min at 4 °C. Absorbances at 532 nm and 600 nm were measured with a PerkinElmer Lambda 25 UV/Vis spectrophotometer. The concentration of TBARS was calculated from the value at 532 nm (after subtracting absorbance at 600 nm due to turbidity) using the molar extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$.

Evaluation of global stress: oxidative stress index (OSI)

The OSI was recently proposed (Pérez-Palacios *et al.* 2017) as a parameter to estimate overall oxidative stress, and is calculated as an average of values of the different antioxidant enzymes divided by their basal levels in the absence of metal (loid)s, as follows:

$$OSI = \frac{CATx/CAT0 + APXx/APX0 + GPXx/GPX0 + SODx/SOD0 + TBARSx/TBARS}{5}$$

where CATx, APXx, GPXx, SODx and TBARSx are values of these enzyme activities in the presence of metal(loid)s and, CAT0, APX0, GPX0, SOD0 and TBARS0 are the values of the same activities in control conditions, *i.e.* in the absence of metal(loid)s.

The level of OSI in control conditions is 1. Values above 1 correspond to plants with higher oxidative stress than the control, with increasing values in parallel with stress, whereas values below 1 indicate lower oxidative stress than in control plants.

Isolation of plant RNA and qRT-PCR of stress-related genes

Total RNA (two independent extractions for each sample) was extracted from 100 mg root tissues of *S. densiflora* grown under the different metal/inoculation treatments, using a Plant RNA Purification kit (Canvax, Cordoba, Spain) following the

instructions of the manufacturer with RNAase-free material and solutions. To ensure that there was no residual DNA in RNA preparations, they were additionally treated with the Turbo DNA-Free Kit (ThermoFisher, USA). Control PCR of housekeeping genes (see below) was performed in order confirm no amplification in DNA-free RNA samples. Immediately after extraction, all samples were subjected to cDNA synthesis using the QuantiTect Reverse Transcription kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA samples were stored at −20 °C for up 2 weeks.

The qRT-PCRs were performed using SensiFAST™ SYBR® NO-ROX kit (BIOLINE, France) and an ECO thermocycler (Illumina, San Diego, CA, USA) following the supplier's instructions. Pairs of primers spanning a DNA fragment between 80–170 bp were designed for the most conserved regions of the genes available in the database using Clustalw Omega (EMBL-EBI, Heidelberg, Germany). The characteristics of the primers (length, G + C content, Tm, probability of self-dimers and heterodimer formation, hairpin structures, etc.) were tested with OligoAnalyzer 3.1 (Integrated DNA Technologies, Skokie, IL, USA). Table S1 shows the primer pairs used for each gene and *Tm* used in PCR amplification reactions. The amplification reactions were: initial denaturation at 95 °C for 2 min, 40 cycles at 95 °C for 5 s, 55–65 °C (depending on gene, see Table S1) for 10 s, 72 °C for 15 s, and a final step at 95 °C 15 s, 55 °C for 15 s, 95 °C for 15 s.

The housekeeping genes *His1* and *EF1* were used to normalise results from different samples. Expression signals were quantified and normalised using Eco™ software version 4.1.2.0 (Illumina). The expression fold was calculated according to Livak & Schmittgen (2001):

$$\Delta\mathbf{Cq} = \mathbf{AVECq}_{(\mathrm{TargetAssay})} - \mathbf{AVECq}_{(\mathrm{ReferenceAssay})}$$

$$\Delta\Delta\mathbf{Cq} = \Delta\mathbf{Cq}_{(\mathrm{TestSample})} - \Delta\mathbf{Cq}_{(\mathrm{ReferenceSample})}$$

$$\mathrm{RQ} = 2^{-\Delta\Delta\mathrm{Cq}}$$

Statistical analysis

Results are means ± SD of nine determinations in the case of enzyme activity and TBARS (three independent biological samples × three replicates) and six determinations (two independent biological samples × three replicates) for qRt-PCR experiments. Differences among treatments were assessed by one-way ANOVA followed by a Least Significant Difference (LSD) test. Significant differences at $P < 0.05$ and $P < 0.01$ are indicated by one or two asterisks, respectively.

RESULTS

Physicochemical properties and concentrations of metals in sediments

The physicochemical properties of Odiel and Piedras sediments (Table 1) were very different in texture; sand was more abundant in the Piedras estuary, whereas silt was preponderant in the Odiel estuary. The pH was near neutral in both estuaries, although slightly more acidic in the Odiel estuary, which might contribute to higher mobilisation of metals. Regarding organic matter and N content, values were similar. Major differences were found in conductivity and redox potential; salinity was higher in Odiel than Piedras (according to a more internal position of the Piedras sampling site with regard to the coast) whereas a higher positive redox potential revealed more oxidising conditions in Odiel sediment as compared to the Piedras sediment.

In terms of concentration of toxic metals and metalloids, the most relevant (Table 1) were As, Cu, Pb and Zn, together with Fe, due to solubilisation of this last element from the Iberian Pyrite Belt (IPB; Nieto *et al.* 2007). Levels of As, Cu and Zn surpassed those of thresholds in regional environmental regulations (Junta de Andalucía 1999) two- to three-fold. Pb concentration, although high, did not reach the level of intervention for Natural Parks and Forest Areas. Cd was not a relevant pollutant in this estuary. The Piedras estuary, whose sediments were used as control, was not affected by metal pollution.

Level of ROS-scavenging enzymes in roots exposed to heavy metals and effects of bacterial inoculation

Toxic metals and metalloids led to an increase in all ROS-scavenging enzymes in plant roots (Fig. 1A). CAT and APX activity increased 18–25% but GPX activity was not significantly affected. Overall, SOD activity increased five-fold upon metal exposure.

Inoculation with Gram-negative *P. agglomerans* RSO6 and RSO7 led to 60% reduction of CAT activity with regard to the non-inoculated control (Fig. 2A), whereas inoculation with Gram-positive *B. aryabhattai* RSO25 reduced CAT activity 82%. The consortium did not decrease CAT activity. For APX activity (Fig. 2B), inoculation with *Pantoea* strains led to a reduction of 75%, whereas inoculation with *Bacillus* strain decreased APX activity 40% (although no significant in our conditions). Moreover, inoculation with the consortium did not lead to significant reductions of APX activity. With regard to GPX activity (Fig. 2C), treatment with *B. aryabhattai* RSO25 led to the largest reduction in activity (90% reduction), whereas inoculation with *P. agglomerans* RSO6 and RSO7 produced an 83% reduction and the consortium had a minor, although significant effect (13% reduction in relation to non-inoculated control). For SOD activity (Fig. 2D), inoculation with the consortium led to the largest reduction (80%), whereas inoculation with *Pantoea* strains and *Bacillus* led to 55% and 70% reductions, respectively.

Evaluation of TBARS as markers of membrane injury

The TBARS, in particular malondialdehyde (MDA), are considered markers of membrane injury due to lipid peroxidation upon metal exposure (Upadhyay & Panda 2010). The presence of toxic metals and metalloids in sediments led to a significant increase (30%) in levels of TBARs, indicating membrane damage (Fig. 3A). However, the different inoculation treatments partially attenuated the deleterious effect of metal exposure on membranes. Treatment with *P. agglomerans* RSO6 and RSO7 produced the largest fall in TBARS (84% reduction), while inoculation with *B. aryabhattai* RSO25 led to a 60% reduction,

Table 1. Physicochemical properties and total content of relevant metals and plant nutrients in soils from Odiel and Piedras salt marshes. Values are mean ± SE of five replicates. (*) Texture (silt/clay/sand percentage). (**) Threshold for metal concentration in soils of Natural Parks and Forest Areas according to Regional Regulation (Junta de Andalucía. Consejería de Medioambiente 1999).

Physico-chemical properties						
Soil	Texture (*) (%)	Redox potential (mV)	Conductivity (mS cm^{-1})	pH	Organic matter (%)	Nitrogen (%)
Odiel	60/16/24	262 ± 10	15.62 ± 0.5	6.88 ± 0.1	13.5 ± 0.2	0.27 ± 0.1
Piedras	20/14/66	150 ± 15	1.2 ± 0.4	7.6 ± 0.3	11.4 ± 0.5	0.23 ± 0.1

Heavy metals concentration in soil					
Soil	As (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})	Pb (mg kg^{-1})	Cd (mg kg^{-1})
Odiel	339.8 ± 12.0	2522.7 ± 65.3	1318.4 ± 26.8	406.7 ± 29.4	4.02 ± 0.13
Piedras	6.5 ± 0.1	78 ± 0.2	19 ± 0.17	16 ± 0.3	0.22 ± 0.08
Level for intervention (**)	>100	>1000	>500	>1000	>15

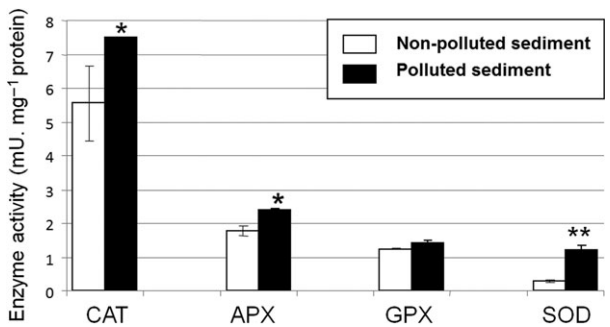

Fig. 1. Enzyme activity of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) in roots of plants grown on polluted and control sediments. Data are means of nine determinations (three biological samples x three replicates). SD indicated by bars. $*P < 0.05$, $**P < 0.01$.

and inoculation with the consortium led to minor but significant reductions (20% in relation to the non-inoculated control; Fig. 3B).

Mitigation of plant stress upon inoculation: evaluation with the OSI

The OSI is a parameter recently introduced to obtain an overall estimation of oxidative stress (Pérez-Palacios *et al.* 2017). The highest values of OSI (Fig. 4) were recorded in non-inoculated plants grown on metal-polluted sediments, for which the OSI was approximately twice that of control plants. Inoculation with *P. agglomerans* RSO6 and RSO7 or *B. aryabhattai* RSO25 led to reductions of 65–75% in the OSI, with slight differences between inoculants, whereas plants inoculated with the consortium had similar levels to plants grown on non-polluted sediments (Fig. 4).

Evaluation of stress-related genes in *S. densiflora*: effect of inoculation on gene expression

In order to unravel molecular mechanism underlying the protection of plant roots by PGPR, expression levels of several stress-related genes were investigated. The genes were selected based on their involvement in metal(loid) stress management in different plants and represent different pathways of stress alleviation (Hossain *et al.* 2012; Lafuente *et al.* 2015; Keunen *et al.* 2016). Initially, 12 genes were selected (Table S1) and two genes encoding the translation elongation factor (*EF-1α*) and Histone1 (*his1*) were used as housekeeping genes. Only three of the 12 genes investigated could be amplified, *i.e. ETR-1* encoding an ethylene receptor (band of 116 bp), *PAL* encoding phenylalanine ammonium lyase (band of 108 bp) and the *GR* coding for glutathione reductase (band of 121 bp; Table S1). PCR products were sequenced in order to confirm the identity of the genes. The rest of the genes did not give any amplification signals, the sizes of the products were not these expected, or showed no sequence homology with the expected genes, so were discarded.

The level of gene expression upon inoculation was examined with qRT-PCR (Fig. 5). Expression levels between 0.5 and 2.0 compared with the control (plants grown on non-polluted sediments) were not considered statistically significant, since the difference corresponds to less than one PCR cycle (Livak & Schmittgen 2001). Neither *GR* nor *ETR* showed significant differences in samples grown on Odiel or Piedras estuaries. Moreover, the inoculation treatments had no remarkable effect on their expression levels. Conversely, there were significant differences in *PAL*. The presence of toxic metals and metalloids did not induce the *PAL* gene *per se* (Fig. 5), and there were no significant differences in expression of *PAL* in exposed *versus* non-exposed plants without inoculation. However, markedly different behaviour was found upon inoculation: all three inoculation treatments enhanced *PAL* expression; maximum expression was achieved upon inoculation with *B. aryabhattai* RSO25 (five-fold increase), whereas inoculation with *P. agglomerans* or the consortium led to 3.75- and 3.93-fold enhancement, respectively.

DISCUSSION

The presence of metals triggers high oxidative stress in plants leading to synthesis of non-enzymatic small molecules and metabolites (ascorbate, glutathione, polyphenols) and enzymes (CAT, SOD, APX, GPX, among others) to counterbalance the production of ROS (Hossain *et al.* 2012). These have been found in *S. densiflora*, with highest activity in SOD (five-fold), indicating the major contribution of this enzyme to ROS-scavenging in roots under metal exposure. CAT, APX and GPX

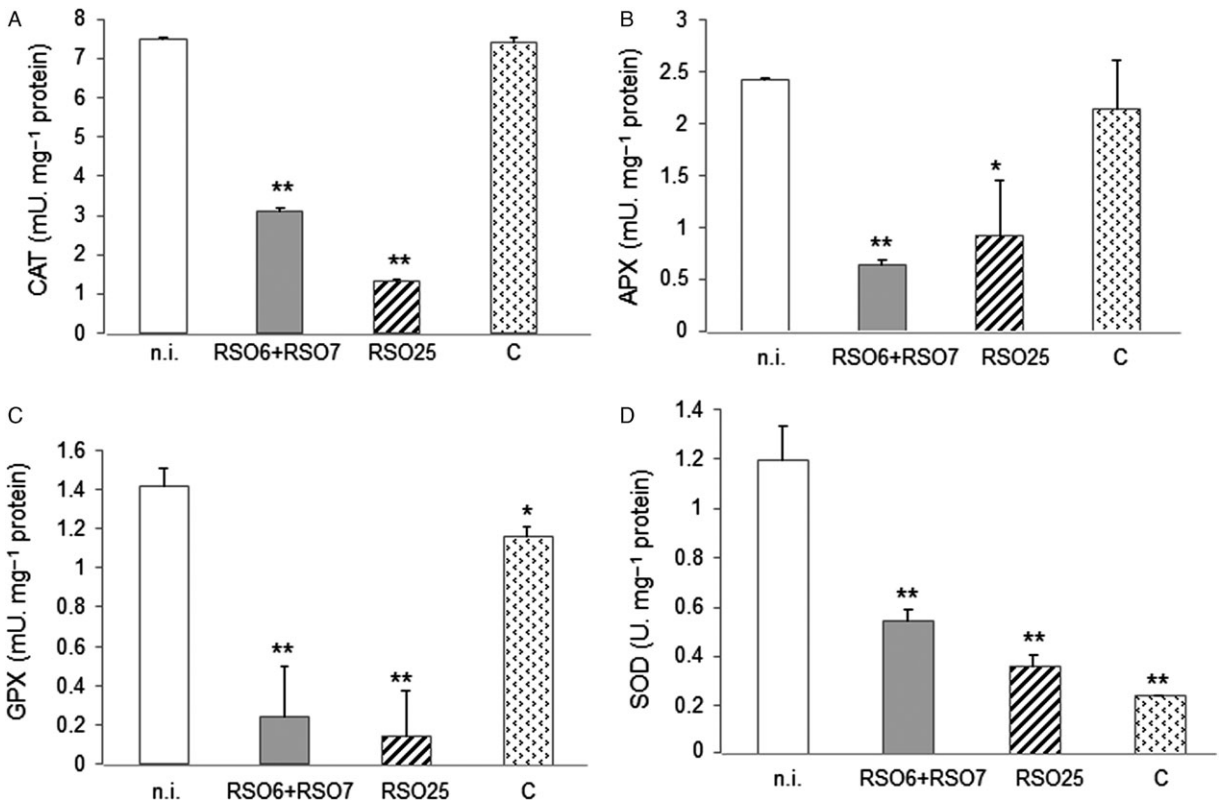

Fig. 2. Enzyme activity of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) in roots of plants grown on polluted sediments and subjected to different inoculation treatments. Data are means of nine determinations (three biological samples x three replicates). SD indicated by bars. $*P < 0.05$, $**P < 0.01$.

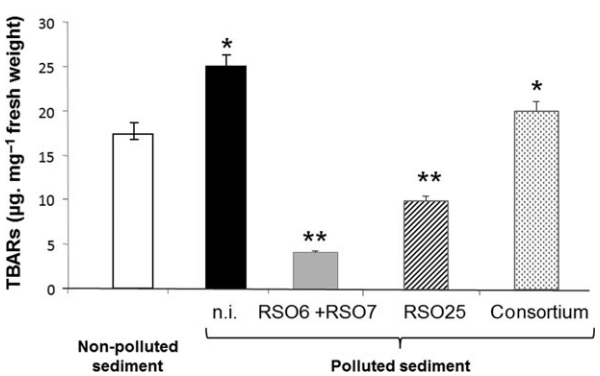

Fig. 3. Content of thiobarbituric acid reactive substances (TBARs) in roots of *S. densiflora*. A. Comparison of TBARs in roots of plants grown on polluted and unpolluted sediment. B. Effect of different inoculation treatments on content of TBARs. Data are means of nine determinations (three biological samples x three replicates). SD indicated by bars. $*P < 0.05$, $**P < 0.01$.

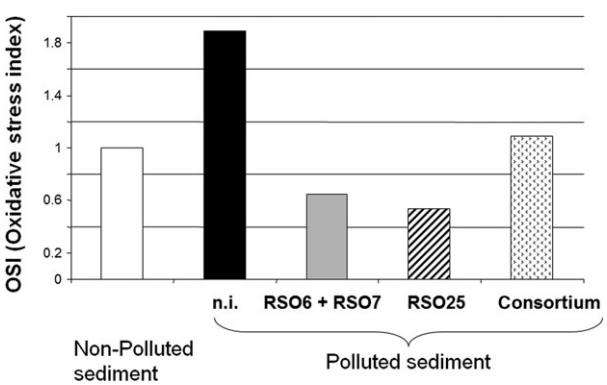

Fig. 4. Oxidative stress index (OSI) in roots of plants grown on polluted and unpolluted sediment and subjected to different inoculation treatments. OSI is proposed as a global parameter to evaluate oxidative stress caused by metals (see Material and Methods for definition).

displayed much lower enhancement compared to SOD (25%, 10% and 15%, respectively).

Recent literature shows examples of the use of metal-resistant PGPR to ameliorate plant growth in the presence of metals (Ashraf *et al.* 2017). Rhizosphere bacteria isolated from *S. densiflora* naturally grown in the Odiel salt marshes showed both resistance to toxic meta(loid)s and PGP properties (Andrades-Moreno *et al.* 2014; Mesa *et al.* 2015a,b). Moreover, previous reports indicated that bio-augmentation of *S. densiflora* with a consortium of PGPR enhanced growth and tolerance to physicochemical properties of salt marshes sediments (Mateos-Naranjo *et al.* 2015), increasing root metal accumulation (Mesa *et al.* 2015b) and improving seed germination (Paredes-Páliz *et al.* 2016b). The *P. agglomerans* strains RSO6 and RSO7 are resistant to several metal(loid)s simultaneously (multi-resistance). They can tolerate As (2–4 mM), Cu (8 mM), Zn (10 mM) and Pb (6–7 mM; Paredes-Páliz *et al.* 2016a).

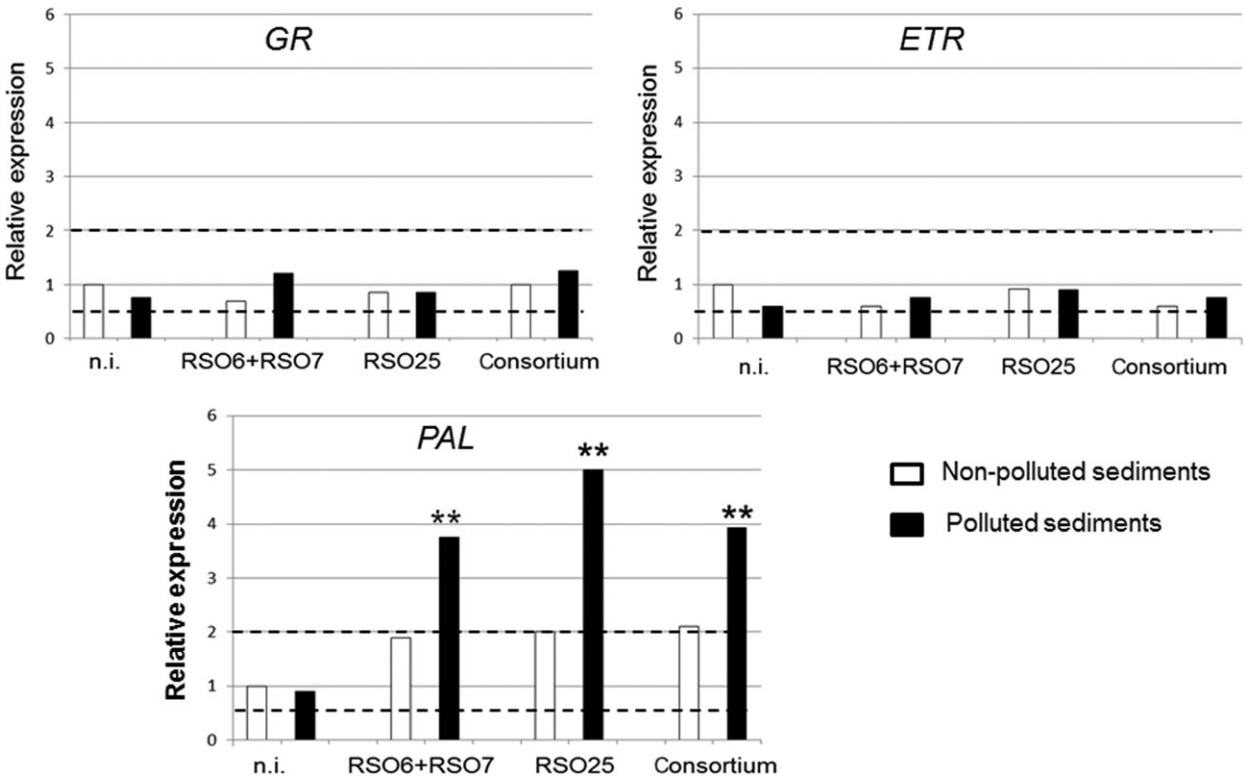

Fig. 5. Expression of glutathione reductase *(GR*), ethylene receptor (*ETR)* and phenylalanine ammonia lyase (*PAL*) in roots of plants grown on polluted and unpolluted sediment and subjected to different inoculation treatments. For each gene, expression level is relative to that of control plants, considered as 1. Expression levels were calculated using the geometric mean of *EF1-α* and *His1* as housekeeping genes. Dashed lines indicate expression between +2 and −2, considered as not significantly different in qRT-PCR.

B. aryabhattai RSO25 also showed elevated tolerance to metals and metalloids, and RSO7 has high metal biosorption capacity and is able to bind up to 26,000 μg Pb g^{-1} dry weight (Paredes-Páliz *et al.* 2016a). It is known that different microorganisms (Gram-positive, Gram-negative and cyanobacteria, yeasts and fungi) with different cell walls display distinct metal biosorption capacities depending on the chemical groups on the surface (Das *et al.* 2008). Furthermore, *Pantoea* strains have the ability to form biofilms (Paredes-Páliz *et al.* 2016a). Bacteria may attenuate plant stress by adsorbing metal onto the bacterial surface thus decreasing metal availability in the vicinity of the root (Das *et al.* 2012; Paredes-Páliz *et al.* 2016a).

The three selected strains had good PGP properties (N_2 fixation, phosphate solubilisation and siderophores and auxin production), some of which were retained in the presence of metals (Paredes-Páliz *et al.* 2016a). Metal-resistant bacteria can reduce plant stress upon metal exposure (Goswami *et al.* 2016) by: formation of biofilms on roots; amelioration of plant nutrient acquisition (through bacterial N_2 fixation, phosphate solubilisation); reduction of plant stress through ACC deaminase activity; and secretion of siderophores and phytohormones (De-Bashan *et al.* 2012; Pieterse *et al.* 2014). In addition, secretion of auxins by PGPR can also affect ethylene signalling in plants and hence stress management (Spaepen *et al.* 2011). The strains used in the present work have already been proved to promote plant growth under metal stress (Paredes-Páliz *et al.* 2017). Different behaviour among Gram-negative and Gram-positive strains was reported, inoculation with *Pantoea* strains decreased metal accumulation, while inoculation with a *Bacillus* strain enhanced metal accumulation, particularly in roots (Paredes-Páliz *et al.* 2017).

Here, inoculation with rhizosphere bacteria led to reductions in activity of all ROS-scavenging enzymes, the degree of what depended on the particular enzyme and/or inoculation treatment. In general, inoculation with *Pantoea* or *Bacillus* strains achieved better results than with the consortium. Antagonistic effects between the bacterial strains *in vitro* were previously ruled out (Paredes-Páliz *et al.* 2016b); however, factors such as plant root exudates can modify rhizosphere communities (Dzantor 2007; Shukla *et al.* 2011; Badri *et al.* 2013). It is noteworthy that levels of OSI upon inoculation were even lower than those of plants grown on non-polluted sediments, indicating that oxidative stress induced by metal (loid)s can be fully mitigated by PGPR inoculation (Yang *et al.* 2009). Rhizosphere microorganisms enhance plant induced systemic tolerance (IST) towards abiotic factors such as cold, heat, salinity, drought and heavy metals through modulation of plant gene expression (Pieterse *et al.* 2014); however, the mechanisms underlying this protective effect remain elusive.

In a second approach, expression levels of 12 genes considered as markers of metal(loid)s stress in plants were investigated. Only three of these genes could be amplified with our primers and conditions, besides the housekeeping genes. The genes amplified were: *GR* encoding glutathione reductase that catalyses reduction of glutathione disulphide (GSSG) to reduced glutathione (GSH), involved in thiol redox signalling and a major redox buffer against ROS,

thus helping to maintain a reducing environment *in vivo* (Yadav 2010); *ETR* encoding a receptor for ethylene, a phytohormone controlling numerous aspects of plant growth and development, including biotic and abiotic stresses (Dey & Vlot 2015); and *PAL* encoding phenylalanine ammonia lyase that catalyses conversion of L-phenylalanine to ammonia and trans-cinnamic acid (Olsen *et al.* 2008). Our case results suggest a dominant role of *PAL* in adaptation of this plant to metal stress in the presence of suitable PGPR, pointing to a relevant role of secondary metabolism in alleviating metal stress, as previously shown (Pawlak-Sprada *et al.* 2011; Xia *et al.* 2011). It is known that plants can modify the quantity and quality of root exudates, particularly under stress situations, as a way to modify rhizosphere communities and recruit beneficial bacteria that better suit them to tolerate stressful conditions (Huang *et al.* 2014). Plants are able to secrete an array of flavonoids and other metabolites to attract beneficial bacteria to their rhizosphere, particularly under stress situations (Broughton *et al.* 2000; Lafuente *et al.* 2015). Recent reports indicated the relevance of phenolic compounds in determining the rhizosphere microbiome (Badri *et al.* 2013). *S. densiflora* might be sensing the presence of PGPR and increasing expression of *PAL* in order to synthesise and excrete secondary metabolites such as flavonoids to attract beneficial rhizosphere bacteria that tolerate metals present in the sediments. These beneficial bacteria could, in turn, activate or enhance *PAL* expression to improve adaptation to metal stress. In fact, secondary metabolites such as (iso) flavonoids and brassinosteroids have ROS-scavenging activity on the aromatic rings in their molecules (Xia *et al.* 2011). *PAL* also participates in lignin synthesis (Olsen *et al.* 2008; Labeeuw *et al.* 2015) and hence root strengthening, a mechanism widely used to protect roots against metal toxicity (Kováčik & Klejdus 2008; Pawlak-Sprada *et al.* 2011).

In contrast, *GR* and *ETR* did not differ significantly in polluted and non-polluted sediments, despite these genes being widely involved in Cd and As detoxification (Hossain *et al.* 2012). It is possible that many copies of these genes are present, so that in this plant, as in *Arabidopsis thaliana* and rice (Trivedi *et al.* 2013), specific regulation of a particular gene copy could be masked through amplification of several gene copies. Also *S. densiflora* is a heptaploid species (Fortune *et al.* 2008). Genome-wide analysis using NGS (new generation sequencing) approaches could help to identify genes/pathways involved in metal stress alleviation by PGPR, since almost no sequence is available for this species, in contrast to other *Poaceae* for which these techniques have allowed identification of genes involved in biotic and abiotic stresses (Muthusamy *et al.* 2017; Verma *et al.* 2017).

CONCLUSIONS

Our results indicate that inoculation with appropriate PGPR ameliorates metal oxidative stress of *Spartina densiflora*, as deduced from lower levels of ROS-scavenging enzymes and of lipid peroxidation. On the other hand, inoculation strongly induces expression of *PAL*, pointing to an important role for secondary metabolites in attracting suitable rhizobacteria for stress attenuation in the presence of metals, besides their role as ROS scavengers. In addition, enhanced lignin synthesis could be another adaptation to metal stress upon inoculation. Finally, we revealed the difficulty in doing molecular studies in this plant due to the scarcity of genetic information, which seems to be a bottleneck but also a challenge for future research. In particular, NGS approaches could shed light on the abovementioned mechanisms and help to identify other genes/pathways involved in stress attenuation upon PGPR inoculation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Investigating the mechanisms of phytoprotection by PGPR in the presence of heavy metals.

Table S1. Pairs of primers and conditions for qRT-PCR amplification of stress-related genes and housekeeping genes based on conserved sequences available in database. Positive: gene amplified (confirmed by sequencing); Negative: no amplification under conditions used or identity of the product was not confirmed by sequencing.

File S1. Highlights.

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