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Effects of EDTA and plant growth-promoting rhizobacteria on plant growth and heavy metal uptake of hyperaccumulator *Sedum alfredii* Hance

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ABSTRACT

Phytoremediation is a cost-effective and environment-friendly strategy for decontaminating heavy-metal-contaminated soil. However, the practical use of phytoremediation is constrained by the low biomass of plants and low bioavailability of heavy metals in soil. A pot experiment was conducted to investigate the effects of the metal chelator ethylenediaminetetraacetic acid (EDTA) and EDTA in combination with plant growth-promoting rhizobacteria (*Burkholderia* sp. D54 or *Burkholderia* sp. D416) on the growth and metal uptake of the hyperaccumulator *Sedum alfredii* Hance. According to the results, EDTA application decreased shoot and root biomass by 50% and 43%, respectively. The soil respiration and Cd, Pb, Zn uptake were depressed, while the photosynthetic rate, glutathione and phytochelatin (PC) contents were increased by EDTA application. Interestingly, *Burkholderia* sp. D54 and *Burkholderia* sp. D416 inoculation significantly relieved the inhibitory effects of EDTA on plant growth and soil respiration. Compared with the control, EDTA + D416 treatment increased the Cd concentration in shoots and decreased the Pb concentration in shoots and roots, but did not change the Zn concentration in *S. alfredii* plants. Furthermore, EDTA, EDTA + D54 and EDTA + D416 application increased the cysteine and PC contents in *S. alfredii* ($p < 0.05$); among all tested PCs, the most abundant species was PC2, and compared with the control, the PC2 content was increased by 371.0%, 1158.6% and 815.6%, respectively. These results will provide some insights into the practical use of EDTA and PGPR in the phytoremediation of heavy-metal-contaminated soil by *S. alfredii*.

Introduction

The continued elevation of heavy metal concentrations in soil ecosystems has become a major concern all over the world (Pandey et al., 2013). Heavy metal contamination occurs in soil mainly due to anthropogenic activities, such as mining, sewage wastewater irrigation, municipal sewage sludge application, and chemical fertilizer application as well as rapid industrialization (Wuana and Okieimen, 2011; Antonkiewicz et al., 2018). Heavy metal contamination not

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only affects ecosystem functioning, but also poses potential threats to human health since these metals could be absorbed by humans through the food chain. Therefore, remediation of heavy-metal-polluted soil is important for improving the health of both the ecosystem and humans.

Phytoremediation is a strategy of using plants for translocating and accumulating high quantities of heavy metals from contaminated soils to the roots and aboveground parts of the plant (Zhuang et al., 2007; Mahmood, 2010). It is an environment-friendly and cost-effective technique compared with chemical and physical remediation techniques. However, the efficiency of phytoremediation can be limited by the slow growth of heavy-metal-accumulating plants, low root-to-shoot translocation rate as well as low metal bioavailability in soils (Khan et al., 2000). Various strategies have been developed to increase the efficiency of phytoremediation, including applying chelators or organic acids to increase the metal bioavailability in soil, and applying plant growth-promoting rhizobacteria (PGPR) to increase the biomass of metal-accumulating plants (Sobariu et al., 2017; Mahmood et al., 2017; Hassan et al., 2017; Li et al., 2018; Pramanik et al., 2018).

Heavy-metal-resistant PGPR have beneficial effects on plant growth and could stimulate heavy metal uptake by plants. PGPR usually have plant-growth-promoting properties, including the production of indole acetic acids (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and siderophores and solubilization of phosphate (Carlos et al., 2016; Chen et al., 2016; Wang et al., 2017a,b; Rathi and Nandabalan, 2017). PGPR can modulate plant hormone levels, stimulate nutrient uptake and thereby promote plant growth, and they could also improve heavy metal availability and solubility by decreasing the pH of the rhizosphere. Therefore, the presence of PGPR in the rhizosphere could improve the remediation efficiency of heavy-metal-polluted soils.

Ethylenediaminetetraacetic acid (EDTA) is a hexahydric acid that forms strong complexes with metals via its two amine and four carboxylate groups, thus influencing the solubility, mobility and bioavailability of soil-bound heavy metals (Shahid et al., 2014). EDTA is the most efficient and effective chelator, which could increase the solubility of heavy metals in soils (Padmavathiamma et al., 2010; Oh and Yoon, 2014). It forms a soluble metal-EDTA complex with heavy metals, which would be easily taken up by plants (Dipu et al., 2012). Due to these physico-chemical properties, EDTA is widely used to assist the phytoremediation of heavy-metal-contaminated soils (Chen et al., 2004; Awokunmi et al., 2012).

Upon exposure to heavy metals, plants experience serious oxidative stress and cellular ionic homeostasis disturbance, which ultimately lead to cellular damage or cell death (Wei et al., 2018). To ameliorate the toxic effects of heavy metals, plants have developed various detoxification mechanisms, including the production of thiol metabolites, viz. cysteine (Cys), glutathione (GSH) and phytochelatin (PCs). PC is cysteine-rich heavy-metal-binding peptides known to play a key role in sequestration and detoxification of heavy metals in plants, which are enzymatically synthesized from gluthatione (GSH) by the enzyme PC synthase (Jia et al., 2011). These substrates show high affinity for toxic metals and play important roles in heavy metal chelation and subcellular compartmentalization (Yadav et al., 2010; Awasthi et al., 2018). Garg and Kaur (2013) found that *G. mosseae* colonization significantly increased the level of total non-protein thiols in *Cajanus cajan* under Cd and/or Zn stress. A consortium application of an alga (*Chlorella vulgaris*) and bacterium (*Pseudomonas putida*) increased the Cys and non-protein thiol content and ameliorated the arsenic toxicity in rice (Awasthi et al., 2018). These findings indicate that microbial inoculation can affect the thiol levels in plants and thereby influence their resistance against heavy metal stress.

Sedum alfredii Hance (*S. alfredii*) is a well-recognized hyperaccumulator. It has the ability to hyperaccumulate cadmium (Cd) as well as zinc (Zn) and lead (Pb) (Yang et al., 2005; Guo et al., 2011). As mentioned above, phytoremediation efficiency could be limited by the slow growth of plants as well as low metal bioavailability in soil (Khan et al., 2000). PGPR play importance roles in terms of plant growth, and EDTA could promote metal bioavailability. Therefore, the object of this study was to investigate the effects of (1) EDTA and PGPR on the plant growth, leaf photosynthetic rate (Pn), glutathione (GSH) and phytochelatin (PC) production of *S. alfredii*; (2) EDTA and PGPR on the heavy metal uptake of the hyperaccumulator *S. alfredii* and soil respiration, which is associated with plant roots and rhizobacteria activity. To our knowledge, this is the first study to investigate the combined effect of EDTA and PGPR on the growth and metal uptake of the hyperaccumulator *Sedum alfredii* Hance.

1. Materials and methods

1.1. Soil collection and characterization

The heavy-metal-contaminated soil used in this study was originally from a paddy field in Daxing County, Guangxi Zhuang Autonomous Region, southwest of China. It consists of hydromorphic anthrosols according to the World Reference Base (WRB) for soil resources 2014 (IUSS Working Group WRB, 2015). The surface (0–20 cm) soil was collected and brought back to the lab, air-dried, sieved through a 2-mm aperture and homogenized prior to the determination of physico-chemical properties. The pH of the soil was measured in a 1:10 (W/V) solid/deionized water suspension using a digital pH meter. Organic matter (OM) and cation exchangeable capacity (CEC) were measured according to standard procedures (Sparks et al., 1996; Grossman and Reinsch, 2002). Total N, P, K and total Cd, Pb, Zn and Cu were determined as previously described (Jiang et al., 2008; Bloem et al., 2017).

1.2. Bacterial strains

Burkholderia sp. D54 (GenBank accession No.HM467915) is a multi-heavy-metal resistant bacterium that belongs to the genus *Burkholderia*. It was originally isolated from soil samples collected from the heavy-metal-contaminated paddy fields near the Dabaoshan mine, Guangdong Province, China. Its minimum inhibitory concentrations for Pb, Cd, Cu and Zn were 800, 1500, 150, and 2500 mg/L, respectively (Guo et al., 2011). Besides, *Burkholderia* sp. D54 has several plant-growth promoting properties, such as the production of IAA,

siderophores, ACC deaminase, and the solubilization of inorganic phosphate (Guo et al., 2011). *Burkholderia* sp. D416 (GenBank accession No.KJ672505) is also a member of the genus *Burkholderia;* it has high 16S rRNA gene sequence similarity with strain D54 (>98%), and also has similar plant-growth-promoting traits (unpublished data).

1.3. Pot experiment

The pot experiment was conducted in a growth chamber at the Shaanxi University of Science and Technology (34°22′44″N, 108°58′20″E). The soil was air-dried and passed through a 4-mm sieve. 1 kg of soil was transferred to plastic pots (12 cm in diameter and 9 cm in depth), then 200 mg N (as ammonium-N), 100 mg P (as phosphate) and 140 mg K (as KCl) were added to each pot, which was left to stand for two weeks to reach equilibrium (Guo et al., 2011). *S. alfredii* seedlings of uniform size were selected, surface-sterilized in 3% NaOCl for 15 min and rinsed 3 times with sterile distilled water before use in the pot experiment. The following treatments were established: (1) control, plants grown in heavy-metal-contaminated soil; (2) EDTA, plants grown in contaminated soil that was amended with 100 mL EDTA solution (5 mmol/L); (3) D54 + EDTA, plants were grown in contaminated soil inoculated with *Burkholderia* sp. D54 and amended with 100 mL EDTA solution (5 mmol/L); (4) D416 + EDTA: plants were grown in contaminated soil inoculated with *Burkholderia* sp. D416 and amended with 100 mL EDTA solution (5 mmol/L); EDTA was added one week before plant harvest. For each treatment, 10 pots of plants were established, and the experiment was repeated 4 times; in total, 160 pots of plants were used in this study.

Bacterial inoculation was performed as previously reported (Zhang et al., 2015). Briefly, pure cultures of *Burkholderia* sp. D54 and *Burkholderia* sp. D416 were grown in Luria-Bertani's (LB) broth for 24 hr in a shaking incubator at 28°C. Cells in the exponential phase were harvested by centrifugation at 12,000 r/min for 10 min. The bacterial concentration was then adjusted to 10^8 cfu/mL by resuspension in sterile saline solution. Bacterial inoculation was performed by soaking the roots of *S. alfredii* seedlings in a bacterial suspension for 2 hr and followed by transplanting into pots. Three days later, seedlings were inoculated again by spraying 10 mL of bacterial cell suspension on the root area of seedlings. For EDTA treatment, 100 mL EDTA (as Na_2-EDTA salt) solution (5 mmol/L) was added to the soil and mixed well one week before plant harvest. Plants were placed in a growth chamber with temperatures of (25 ± 2)°C (day, 16 hr) and (18 ± 2)°C (night, 8 hr), and relative humidity of 60%–80%. Plants were watered with deionized water and soil moisture was maintained at about 60% water holding capacity. 60 days after growth in the chamber, plants were carefully removed from the pots and washed with tap water and deionized water to remove the attached soils.

1.4. Determination of metal concentration in plants

Roots and shoots of *S. alfredii* were separated and dried in the oven at 65°C to a constant weight. Heavy metal content was determined as previously described (Wei et al., 2018). Briefly, dry samples (0.25 g) were ground and acid-digested using HNO_3. The digests were then filtered through a 0.45 μm membrane, and the heavy metal (Cd, Pb and Zn) content was analyzed by ICP-MS (Agilent 7500a, Agilent, USA).

1.5. Determination of photosynthetic parameters

Photosynthesis parameters, including photosynthetic rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs), were determined by an infrared gas analyzer (LI-COR 6400, LI-COR Inc, Lincoln, NE, USA). The water-use efficiency (WUE) was determined by dividing the Pn by Tr. Measurements were conducted at 9:30 a.m. and 4:30 p.m. Beijing time.

1.6. Determination of soil respiration rate

The soil respiration rate was determined using a soil CO_2 flux chamber connected to a portable photosynthesis infrared gas analyzer (LI-COR 6400, LI-COR Inc., Lincoln, NE, USA). The depth of 1 cm was chosen for testing and 8 soil respiration measurements were performed for each treatment, and the obtained data were then averaged to obtain a mean soil respiration rate for each treatment.

1.7. Determination of Cys, GSH and PC content

The Cys and GSH contents were determined as previously described (Mishra et al., 2006). The homogenate preparation of PCs for analysis was performed according to the method of Grill et al. (1991), and the separation and analysis of PCs were performed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Germany) and Agilent Zorbax Eclipse XDB-C18 column (4.6 mm ID×30 mm, 1.8 μm; Agilent Technologies Inc., USA) as described by Jia et al. (2011).

1.8. Statistical analysis

Statistical analysis was performed by one-way ANOVA in SPSS 21.0 for Windows (SPSS Inc, Chicago, IL, USA). Significant differences between treatments were calculated at 5% probability levels ($p < 0.05$).

2. Results

2.1. Soil properties

The physico-chemical properties of the experimental soil were determined. As shown in Table 1, the soil was slightly acidic as evidenced by the pH of 6.3. OM content was 35.6 g/kg, and CEC was 11.2 cmol/kg. Total N, P, K content was 2.5, 0.9 and 22.2 g/kg, respectively. Total Pb, Cd, Zn, Cu content was 790, 46, 4131 and 29 mg/kg, respectively. The above-mentioned heavy metal contents far exceeded their background concentrations in natural soils of China as well as the national standards (250, 0.3, 200, 50 mg/kg for Pb, Cd, Zn, and Cu, respectively, GB 15618-1995), indicating that the soil is severely polluted and needs to be remediated.

2.2. Effects of EDTA, EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on pH of soil

Soil pH is considered as one of the most important parameters that affect heavy metal availability and mobility in the soils. Therefore, the effect of PGPR and EDTA on soil pH was determined. The control, EDTA, EDTA + D54, and EDTA + D416 treated soils were all acidic after the plant harvest, and the pH was 4.75, 4.61, 4.70 and 4.765, respectively (data not shown), indicating that there was no significant difference in pH among these treatments; however, an obvious decrease in soil pH was observed before and after the pot experiment.

2.3. Effects of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on plant growth

The influence of EDTA, EDTA + D54 and EDTA + D416 on the growth of *S. alfredii* was evaluated and the dry weight of underground and aboveground parts is shown in Fig. 1. Compared with the control, EDTA application depressed the root and shoot growth by 50% and 43%, respectively. Both EDTA + D54 and EDTA + D416 treatments significantly increased the shoot and root dry mass compared with the EDTA treated plants ($p < 0.05$), and the dry mass of EDTA + D54 and EDTA + D416 treated plants was similar to that of the control plants, indicating that *Burkholderia* sp. D54 and *Burkholderia* sp. D416 exerted a positive effect in terms of plant biomass accumulation, and could compensate for the growth inhibition caused by EDTA.

2.4. Effects of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on heavy metal concentration of plants

The effects of EDTA, EDTA + D54, and EDTA + D416 on the metal concentrations in *S. alfredii* plants were determined (Fig. 2). Compared with the control, EDTA and EDTA + D54 treatment did not influence the Cd level in either the roots or aerial parts of *S. alfredii* (Fig. 2a). Interestingly, EDTA + D416 application significantly increased the Cd concentration in shoots of the plants compared with the control ($p < 0.05$), with an increase of 33%. Pb concentrations in both roots and shoots were highest in the control plants (Fig. 2b). Exogenous application of EDTA or EDTA + D416 markedly decreased the Pb concentration in both roots and shoots of *S. alfredii* ($p < 0.05$). In EDTA + D54 treated plants, the Pb concentration in shoots was similar to that of the control, and the Pb concentration in roots decreased by 70% compared with that of the control plants. When the soil was amended with EDTA, the Zn concentration in shoots was significantly decreased ($p < 0.05$). In EDTA + D54 and EDTA + D416 treated plants, the Zn concentration in roots and shoots was similar to that of the control plants.

Table 1 – Physico-chemical characteristics and heavy metal content of the studied soils.

Property	Soil for pot experiment
pH	6.3
OM (g/kg)	35.6
CEC (cmol/kg)	11.2
Total N (g/kg)	2.5
Total P (g/kg)	0.9
Total K (g/kg)	22.2
Total Cd (mg/kg)	46
Total Pb (mg/kg)	790
Total Zn (mg/kg)	4131
Total Cu (mg/kg)	29

OM: organic matter; CEC: cation exchangeable capacity.

2.5. Effects of EDTA, EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on photosynthetic performance of *S. alfredii*

The impacts of EDTA, EDTA + D54 and EDTA + D416 on photosynthetic parameters, including photosynthetic rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs), were evaluated (Table 2). According to the results, EDTA treatment significantly increased Pn compared with the control ($p < 0.05$). The Pn level in EDTA + D54 treated plants was similar to that of the control plants, and no significant difference was observed. In EDTA + D416 treated plants, the Pn was the lowest compared with other treatments. Similarly, the WUE was higher in EDTA treated plants, followed by control, EDTA + D54 and EDTA + D416 treated plants.

2.6. Effects of EDTA, and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on Cys, GSH and PC content of *S. alfredii*

The contents of Cys, GSH and PCs in the tested plants were determined (Table 3). EDTA, EDTA + D54, and EDTA + D416 treatments significantly increased Cys content compared with the control ($p < 0.05$). The GSH contents in EDTA and EDTA + D54 treated plants were increased by 29.7% and 30%, respectively. The content of PC2, PC3, PC4 and PC5 was also significantly increased by EDTA, EDTA + D54 and EDTA + D416

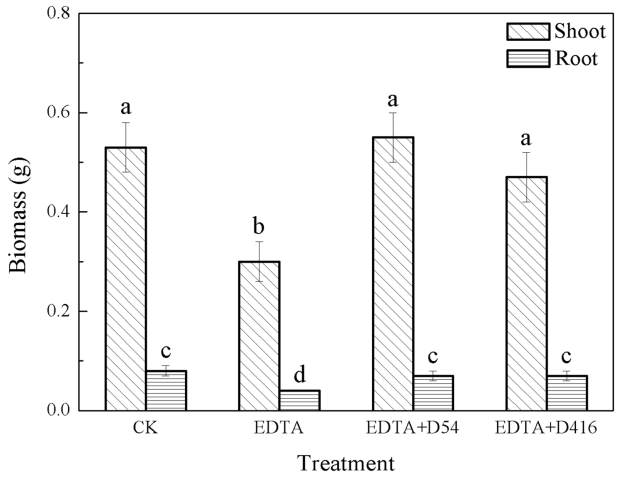

Fig. 1 – Effects of different treatments on dry mass of *Sedum alfredii* Hance. The bar on the columns is the SD (standard deviation). Different lowercase letters above a bar graph indicate significant difference from the corresponding control and other treatments ($p < 0.05$).

($p < 0.05$). Among all the tested PCs, PC2 was more abundant than the others, indicating that it was the dominant PC peptide under the experimental conditions, and compared with the control, the PC2 contents were increased by 371.0%, 1158.6% and 815.6% in EDTA, EDTA + D54, and EDTA + D416 treated plants, respectively.

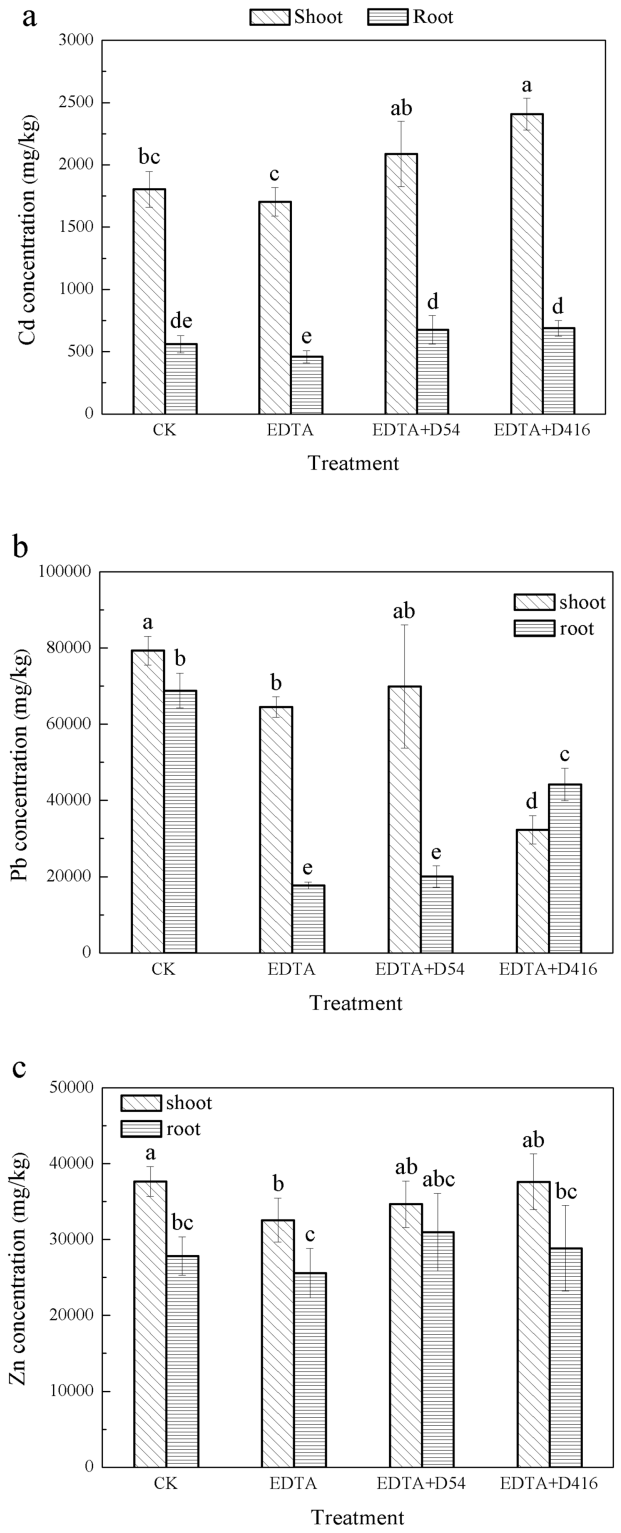

Fig. 2 – Effects of different treatments on heavy metal concentrations in *Sedum alfredii* Hance. (a) Cd; (b) Pb; (c) Zn. The bar on the columns is the SD (standard deviation). Different lowercase letters above a bar graph indicate significant difference from the corresponding control and other treatments ($p < 0.05$).

2.7. Effects of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 on soil respiration rate

The effects of EDTA, EDTA + D54 and EDTA + D416 on the soil respiration rate were determined (Fig. 3). The soil respiration rate was strongest in the control. EDTA application significantly decreased the soil respiration rate ($p < 0.05$), indicating that the amount of EDTA used in this experiment exerted a negative effect on soil respiration. EDTA + D54 treatment markedly increased the soil respiration compared with EDTA-treated plants, which is likely attributed to the existence of *Burkholderia* sp. D54 in the rhizosphere. Similarly, an increase in soil respiration was also observed in EDTA + D416-treated plants compared with the EDTA-treated plants; however, the soil respiration rates in EDTA + D54 and EDTA + D416 treated plants were still lower than that of the control plants.

3. Discussion

S. alfredii has been identified as a hyperaccumulator with the potential to uptake and accumulate heavy metals (Guo et al., 2011). However, the practical use of metal-accumulating plants for phytoremediation is constrained by their relatively low biomass and the low bioavailability of heavy metals in the soil. Previous studies indicated that heavy-metal-resistant PGPR could assist heavy metal phytoremediation via promoting plant growth and improving heavy metal accumulation (Sessitsch et al., 2013). A number of studies have shown that soil amendments, such as EDTA, could increase heavy metal bioavailability in soil, and thus enhance heavy metal uptake from soil to the roots and shoots of the plants (Wang et al., 2017a,b; Gul et al., 2019). Therefore, the effects of EDTA and EDTA with PGPR on phytoremediation by *S. alfredii* were evaluated.

Table 2 – Effects of different treatments on photosynthetic parameters of *Sedum alfredii* Hance.

Treatment	Pn (μmol/(m²·sec))	Tr (g/(m²·hr))	WUE (g/kg)
CK	38.40 ± 0.10 b	0.07 ± 0.04 b	619.84 ± 332.44 ab
EDTA	39.23 ± 0.12 a	0.04 ± 0.02 bc	1246.44 ± 622.61 a
EDTA + D54	38.80 ± 0.17 b	0.15 ± 0.01 a	255.18 ± 10.48 c
EDTA + D416	37.83 ± 0.15 c	0.07 ± 0.01 c	582.17 ± 88.93 ab

Mean values are presented as mean ± SD (standard deviation). Different letters indicate significant differences between treatments ($p < 0.05$).
Pn: net photosynthetic rate; Tr: transpiration rate; WUE: water use efficiency, WUE= Pn/Tr.

Table 3 – Effects of different treatments on cysteine, gluthatione and phytochelatin content of *Sedum alfredii* Hance.

Treament	Cys	GSH	PC2	PC3	PC4	PC5
	(nmol/g)	(nmol/g)	(nmol/g)	(nmol/g)	(nmol/g)	(nmol/g)
CK	0.000 ± 0.000 c	11.662 ± 1.401 b	0.626 ± 0.071 c	0.363 ± 0.097 c	0.050 ± 0.015 b	0.009 ± 0.001 b
EDTA	0.142 ± 0.043 b	15.128 ± 1.967 a	4.712 ± 1.050 b	2.085 ± 0.482 b	0.111 ± 0.034 a	0.015 ± 0.003 a
EDTA + D54	0.380 ± 0.366 ab	15.063 ± 1.851 a	7.879 ± 1.567 a	3.221 ± 0.509 a	0.117 ± 0.054 ab	0.012 ± 0.002 a
EDTA + D416	0.549 ± 0.273 a	10.935 ± 0.722 b	5.732 ± 2.135 ab	2.894 ± 0.082 a	0.154 ± 0.106 ab	0.013 ± 0.004 ab

Mean values are presented as mean ± SD (standard deviation). Different letters denote significant difference from the corresponding control and other treatments ($p < 0.05$).
Cys: cysteine; GSH: glutathione; PC: phytochelatins.

3.1. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 reduced the soil pH

Soil pH directly influences heavy metal mobility since it affects metal/metalloid solubility and capacity of forming chelates in soil. In the present study, the pH of the original experimental soil was determined, and it is slightly acidic (pH 6.3) (Table 1). Interestingly, the soil pH decreased to about 4.6 for all treatments after the pot experiment (data not shown), but no significant difference in pH was detected among these treatments. These results indicated that *Burkholderia* sp. D54, *Burkholderia* sp. D416 and EDTA played a limited role in soil pH change. It is likely that heavy metals in soil stimulated the defense response of plants and promoted the exudation of low-molecular-weight organic acid in the root area (Lu et al., 2007), thus resulting in decreased soil pH. Besides, bacteria inoculation could decrease soil pH via secretion of amino acids, organic acids and protons through bacterial metabolic activities (Van der et al., 1999; Huang et al., 2002). The change of soil pH would ultimately influence the solubility and mobility of heavy metals. A previous study showed that the content of cations in soil solution increased under a low pH environment (Huang et al., 2002).

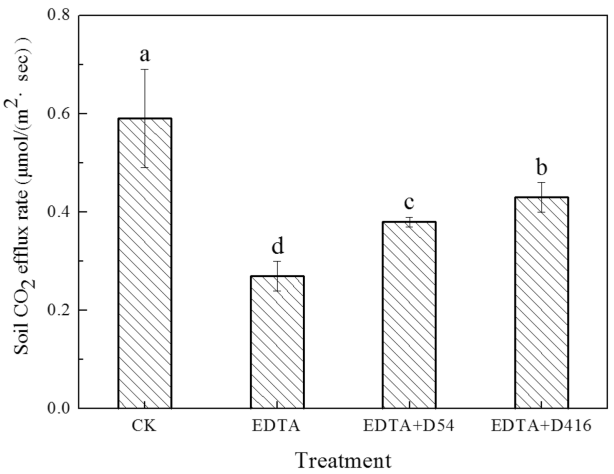

Fig. 3 – Effects of different treatments on soil respiration rate. The bars on columns are the SD (standard deviation). Different letters above a bar graph indicate significant difference from the corresponding control and other treatments ($p < 0.05$).

3.2. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 influenced biomass of *S. alfredii*

Heavy metal stress usually leads to growth inhibition in plants (Wei et al., 2018). Compared with the control plants, the exogenous application of EDTA had a strong inhibitive effect on the biomass accumulation of *S. alfredii*, as evidenced by the significant reduction of the plant dry weight (Fig. 1). The adverse effects of adding this synthetic chelator were also observed by others (Rengel, 2002; Lai and Chen, 2005). It is possible that the applied concentration of this chelator was not appropriate and caused phytotoxicity in the tested plants. Application of EDTA + D54 and EDTA + D416 significantly increased plant dry weight compared with EDTA-treated plants, indicating that bacterial inoculation was able to compensate for the negative growth effect caused by EDTA; the increase of plant growth was likely due to the plant growth properties of the bacteria (Guo et al., 2014).

3.3. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 influenced the heavy metal concentration in *S. alfredii*

EDTA treatment did not influence the Cd level in either the roots or shoots of the *S. alfredii* compared with the control. In EDTA + D54 treated plants, the Cd concentration was also similar to that of the control. EDTA + D416 noticeably increased the Cd concentration in the shoots of the tested plants (Fig. 2a). Interestingly, compared with the EDTA-treated plants, EDTA + D54 and EDTA + D416 significantly increased the Cd concentration in both the shoots and roots of plants, indicating that the microbial inoculation stimulated Cd uptake; a previous study by Guo et al. (2011) also demonstrated that *Burkholderia* sp. D54 could enhance metal uptake by the hyperaccumulator *S. alfredii*. It is likely that the mobility and availability of metal contaminants in rhizosphere soil were enhanced by these microbes via the exudation of organic compounds, soil acidification, and redox changes. In addition, bacterial inoculation may improve the physiological status of the plants via its plant growth properties, thus increasing the Cd uptake. EDTA, EDTA + D54 and EDTA + D416 treatments decreased the Pb concentration in *S. alfredii* plants (Fig. 2b). EDTA + D54 and EDTA + D416 did not influence the Zn concentration in *S. alfredii*, while EDTA alone decreased the Zn concentration in plant shoots (Fig. 2c). These results indicated that EDTA alone or in combination

with PGPR could modulate different heavy metal concentrations differentially. A previous study indicated that EDTA application increased phytoremediation efficiency (Shahid et al., 2014), but in this study, EDTA did not facilitate Cd, Pb, or Zn accumulation in roots and shoots of *S. alfredii* compared with the control. This is possibly due to the concentration used exerting an inhibitive effect on the root and shoot development (Fig. 2). A previous study indicated that utilization of a metal chelator (e.g., EDTA) in soil remediation may cause extra damage to plant growth and increase eco-risks (Baldock, 2007).

3.4. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 influenced the photosynthesis of *S. alfredii*

Photosynthesis is usually negatively affected by heavy metal stress, in this study, EDTA treatment significantly increased the Pn compared with the control ($p < 0.05$) (Table 2), and this phenomenon was also observed by others (Markovska et al., 2013). EDTA + D54 treatment increased the Pn compared with the control, although this increase was not significant. It is possible that application of bacteria helped the plants to maintain a better physiological status and thereby increased Pn. A previous study demonstrated that *Burkholderia* sp. D54 alone could stimulate the Pn of ryegrass compared with that grown on multi-heavy-metal-contaminated soil (Guo et al., 2014); however, when combined with EDTA, this stimulative effect was decreased. This phenomenon was even more obvious in EDTA + D416 treated plants, since Pn was significantly decreased when compared with the control. The underlying mechanism still needs further investigation.

3.5. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 increased the phytochelatin content of *S. alfredii*

Heavy metal exposure induces production of reactive oxygen species in plants, which could attack biomolecules, such as proteins and nucleic acids, leading to irreparable metabolic dysfunction or cell death. To counteract heavy metal stress, plants have developed other strategies. GSH and PCs also play important roles in chelating heavy metals (Yadav, 2010). PCs, the cysteine-rich oligopeptides synthesized from GSH, play an important role in intracellular binding of Cd and other metal ions through thiolate coordination (Gupta et al., 2004), thus PC and GSH synthesis is considered to be one of the heavy metal detoxification mechanisms in higher plants. EDTA, EDTA + D54, and EDTA + D416 treatments enhanced the levels of PC2, PC3, PC4 and PC5 compared with the control plants (Table 3), indicating that EDTA and the bacteria promoted the synthesis of PCs and increased the detoxification ability of plants. The GSH level in EDTA- and EDTA + D54-treated plants was also significantly enhanced, while the Cys content was enhanced by all treatments when compared with the control, indicating that the synthesis of these biomolecules plays an important role in detoxification of heavy metal stress in *S. alfredii*.

3.6. Application of EDTA and EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 reduced the soil respiration rate

Soil respiration is the combined CO_2 flux from roots and microorganisms (Qiao et al., 2009). In the present study, the strongest soil respiration rate was detected in the control plants. EDTA, EDTA + D54, and EDTA + D416 application significantly decreased soil respiration; among them, the lowest soil respiration was detected in EDTA-treated soil (Fig. 3). It is likely that the EDTA concentration used in this study was not optimal, and adversely affected the root development of *S. alfredii* as well as microbe diversity, and thus led to the observed decrease in soil respiration. This assumption is also supported by the fact that the lowest dry weight of plant roots was observed in EDTA-treated plants (Fig. 1). The application of EDTA with *Burkholderia* sp. D54 or *Burkholderia* sp. D416 significantly enhanced soil respiration compared with that of the EDTA-treated soil, not only due to the introduction of an increased amount of bacteria in the soil, but also due to the positive effect of PGPR on the root biomass (Fig. 1). Taken together, these findings in our study indicate that inoculation with the above-mentioned bacteria may relieve the inhibitory effect of EDTA on root growth and increase the rhizosphere microbe diversity (Whitaker et al., 2014).

4. Conclusions

In the present study, we attempted to enhance the phytoremediation efficiency of the hyperaccumulator *S. alfredii* by application of the metal chelator EDTA, or EDTA in combination with plant growth-promoting rhizobacteria. Unfortunately, EDTA application negatively affected plant biomass and soil respiration as well as the Cd, Pb and Zn concentration compared with the control, although it improved Pn and the content of Cys, GSH and PCs. Interestingly, PGPR inoculation compensated for the negative effects of EDTA on plant biomass accumulation, and the Cd concentration in shoots and PC content of *S. alfredii* were increased compared with the control. The influences of EDTA and PGPR on plant growth, metal uptake and phytochelatin content may provide some new knowledge on metal chelator and PGPR application in soil remediation.

Conflicts of interest

The authors declare that they have no conflicts of interest with this work.

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