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# Applying carbon dioxide, plant growth-promoting rhizobacterium and EDTA can enhance the phytoremediation efficiency of ryegrass in a soil polluted with zinc, arsenic, cadmium and lead

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ABSTRACT

This study was conducted to investigate the use of elevated carbon dioxide ($CO_2$), plant growth-promoting rhizobacterium *Burkholderia* sp. D54 (PGPR) and ethylenediaminetetraacetic acid (EDTA) to enhance the phytoextraction efficiency of ryegrass in response to multiple heavy metal (or metalloid)-polluted soil containing zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb). All of the single or combined $CO_2$, PGPR and EDTA treatments promoted ryegrass growth. The stimulation of ryegrass growth by $CO_2$ and PGPR could primarily be attributed to the regulation of photosynthesis rather than decreased levels of Zn, As and Cd in the shoots. Most treatments seemed to reduce the Zn, As and Cd contents in the shoots, which might be associated with enhanced shoot biomass, thus causing a “dilution effect” regarding their levels. The combined treatments seemed to perform better than single treatments in removing Zn, As, Cd and Pb from soil, judging from the larger biomass and relatively higher total amounts (TAs) of Zn, As, Cd and Pb in both the shoots and roots. Therefore, we suggest that the $CO_2$ plus PGPR treatment will be suitable for removing Zn, As, Cd and Pb from heavy metal (or metalloid)-polluted soils using ryegrass as a phytoremediation material.

## 1. Introduction

The contamination of heavy metals (metalloids, HMs) in soils and waters occurs frequently in many countries as a result of increasing anthropogenic activities. For example, the contamination of arsenic (As) has led to a gravest natural disaster in Bangladesh, India (West Bengal), China, Vietnam, United States of America, Argentina, Chile, Mexico (Azizur Rahman et al., 2008). The northeast part of the Tiexi Industrial District of Shenyang in China was reported to be heavily polluted by lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd) and As due to the discharge of waste from smelting plant (Li et al., 2013). A large number of studies have reported the excess accumulation of heavy metals in plants, especially in crops growing on heavy metal polluted soils, which brought serious health risks to people via food chain. It is important to control the influxes of heavy metals into the food chain from the polluted environment, and to reach this goal, many technologies have been developed to clean up or reduce the contamination of heavy metals.

Compared to other remediation technologies, phytoremediation (which is mainly referred to phytoextraction) is ascendant because of its environmental friendliness, low cost and relative ease of implementation (Feng et al., 2011, 2013; Tangahu et al., 2011). However, this technology is often limited by practical factors. The first limiting factor is the screening of suitable plants to remediate HM-contaminated environments because most phytoextraction plants yield a low biomass and grow relatively slowly (Kärenlampi et al., 2000; Tian et al., 2013). For example, the Cd and Zn hyperaccumulator *Thlaspi caerulescens* showed a low biomass and required 391 days to remove 43% of the Cd and only 7% of the Zn from an industrially contaminated soil in a study by Lombi et al. (2001). The second factor is the high selectivity of a hyperaccumulating plant for a certain HM, suggesting limited applications of this plant in sites contaminated with multiple HMs (McIntyre, 2003). The third factor is the lower bioavailability of HMs in contaminated soils. The bioavailability of HMs is thought to be partially dependent on their intensity of adsorption to soil particles and on their interactions with soil microorganisms (Vamerali et al., 2010).

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Phytoremediation prerequisites include the selection of suitable plants with fast growth rates accompanied with a relatively high capacity to accumulate multiple HMs in their above-ground parts. Grass species are preferred for phytoremediation as compared to most hyperaccumulators (Sarma, 2011) because they produce high biomasses and are easier to manage; thus, using them for phytoremediation is economical (Ghosh and Singh, 2005; Sabreen and Sugiyama, 2008; Tian et al., 2013; Vamerali et al., 2010). However, the concentrations of HMs taken up by grass species are often lower than those of hyperaccumulators. Many strategies can be employed to enhance the uptake of HMs in plants, such as enhancing the bioavailability of soil elements through chelator-assisted strategies (Jabeen et al., 2009) and/or microorganism-assisted strategies (Burd et al., 2000; Guo et al., 2011; Kumar et al., 2008; Tang et al., 2011; Zhuang et al., 2007). Many studies in the previously mentioned fields have been performed using hyperaccumulating plants (Lombi et al., 2001) or crops, but few have employed grass species. Nevertheless, the phytoremediation efficiency in many studies was often not high. To enhance the phytoremediation efficiency of *Sedum alfredii* Hance, we attempted to inoculate the plant with the plant growth-promoting rhizobacterium *Burkholderia* sp. D54 (PGPR), and the results showed that after inoculation, the biomass, Cd concentration, and total uptake of Cd, Pb and Zn by *S. alfredii* Hance were all significantly enhanced compared to the non-inoculation treatment (Guo et al., 2011). However, in the above study, the biomass of *S. alfredii* Hance was very small, and the growth rate was slow, which restricted phytoremediation efficiency. Aside from the genetic modification of plants (Kärenlampi et al., 2000; Krämer, 2005; Jabeen et al., 2009; LeDuc and Terry, 2005), the use of carbon dioxide ($CO_2$) fertilizer is also known to improve phytoremediation efficiency. Elevated $CO_2$ can significantly enhance the biomass of *Brassica juncea* L. Czern., *Helianthus annuus* L., *Pteridium revolutum* and *Pteridium aquilinum*, and it induces more Cu uptake in these plants (Tang et al., 2003; Zheng et al., 2008). Similar growth stimulation by elevated $CO_2$ was also reported in cesium-stressed (Wu et al., 2009; Song et al., 2012; Tang et al., 2011) and Cd-stressed plants (Li et al., 2010, 2012; Jia et al., 2010; Wang et al., 2012).

At present, one phytoremediation technology is often used separately or in combination with another, usually with hyperaccumulators or accumulators of a certain HM. Few phytoremediation investigations with grass species in combination with $CO_2$, chelators and microorganisms have been conducted, and little is known about whether these supplemental methods can help grass species to remediate soils contaminated with multiple HMs. Our previous studies have shown that a ryegrass species of *Lolium multiflorum* Lam. can tolerate high levels of Cd and accumulate a large amount of Cd in its shoots (Jia et al., 2010, 2011). We did not know whether this plant species can simultaneously accumulate a large amount of Zn, Pb and As. Therefore, the main purpose of this study was to investigate the phytoremediation potential of ryegrass (*L. multiflorum* Lam.) assisted by $CO_2$, a chelator (EDTA) and PGPR. To our knowledge, this is the first report on the use of ryegrass in concert with $CO_2$, EDTA and microorganisms to enhance phytoextraction efficiency in a multiple HM-polluted soil.

## 2. Materials and methods

### 2.1. Soil characteristics and plant materials

The soil employed in this study was sampled from the arable layers (0–25 cm) of a multiple HM-contaminated paddy field in Hechi, Guangxi province, China where soil contamination resulted from a sudden wastewater discharge from a tailings dam after flash-flooding. Selected physical and chemical properties of the soil are listed in Table 1, and the methods for the determination of soil properties were described in the study of Tang et al. (1999). The Zn, As, Cd and Pb concentrations were much higher than the individual National Standards of China (15618-2008, pH within a range of 5.5–6.5 for a paddy field, 200 mg $kg^{-1}$ for Zn, 30 mg $kg^{-1}$ for As, 0.3 mg $kg^{-1}$ for Cd and 80 mg $kg^{-1}$ for Pb). Soil was air-dried, sieved (2 mm medium sieve), homogenized and stored in the dark before use. One kilogram of dry soil was fertilized with 200 mg of ammonium-N, 100 mg of P ($P_2O_5$) and 140 mg of K (KCl) and then placed in a plastic pot. The soil was fully watered with de-ionized water and maintained at equilibrium for 2 weeks.

Ryegrass seeds were purchased from the Chinese Academy of Agricultural Sciences. To inhibit microbe growth, the seeds were surface-sterilized with 3% NaOCl for 15 min, and rinsed several times with sterile distilled water, and then separated into two parts. One portion of the seeds was inoculated with a pellet suspension of PGPR at logarithmic growth phase ($OD_{600nm} = 1.0$, approximately $10^8$ CFU $ml^{-1}$) for 2 h. The other part was soaked in sterile distilled water. Five to six seeds with (or without) the PGPR treatment were sown in one pot.

### 2.2. Experimental design and treatments

Pot experiments were performed in six open-top chambers (OTC), and the conditions, such as $CO_2$ purity and structure of the chambers, were described by Wu et al. (2009). In this study, there were two levels of $CO_2$, i.e., ambient air (360 μl $L^{-1}$) and elevated $CO_2$ (860 μl $L^{-1}$). The six chambers were separated into two groups, each group contained three chambers, and each chamber was regarded as a replication. One group was ventilated with ambient air, and the other group was treated with elevated $CO_2$. $CO_2$ application was performed from 8:30 to 18:00 each day during the experiment, which lasted for 60 d. The $CO_2$ level was monitored with an infrared gas analyzer (GMP343, Vaisala, Finland).

There were a total of eight treatments, and each treatment was replicated three times in this study. The treatments included: (1) a control treatment (CK); (2) the B treatment (treated with PGPR); (3) the C treatment (added EDTA); (4) the $CO_2$ treatment (added elevated $CO_2$); (5) the B + C treatment (added PGPR and EDTA); (6) the $CO_2$ + B treatment (added PGPR and elevated $CO_2$); (7) the $CO_2$ + C treatment (added EDTA and elevated $CO_2$); (8) the $CO_2$ + B + C treatment (added PGPR, EDTA and elevated $CO_2$). The eight treatments were arranged in the corresponding chambers. Per chamber ventilated with ambient air contained one replication of CK, B, C and B + C treatments, and per chamber ventilated with $CO_2$ contained one replication of $CO_2$, $CO_2$ + B, $CO_2$ + C and

**Table 1**
Selected physical and chemical properties of the soil.

| Properties | Values |
|---|---|
| Organic matter content (g $kg^{-1}$) | 23.53 |
| pH | 5.52 |
| Total P (mg $kg^{-1}$) | 0.74 |
| Available P (mg $kg^{-1}$) | 29.84 |
| Total K (g $kg^{-1}$) | 5.42 |
| Total N (mg $kg^{-1}$) | 0.74 |
| Available N (mg $kg^{-1}$) | 80.85 |
| Available K (mg $kg^{-1}$) | 47.4 |
| CEC (cmol $L^{-1}$) | 4.9 |
| Clay | 0.08 |
| Silt | 0.1 |
| Sand | 0.82 |
| Zn (mg $kg^{-1}$) | 410.2 |
| As (mg $kg^{-1}$) | 38.6 |
| Cd (mg $kg^{-1}$) | 2.4 |
| Pb (mg $kg^{-1}$) | 340.1 |