Journal of Hazardous Materials 198 (2011) 188–197

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Growth and cesium uptake responses of *Phytolacca americana* Linn. and *Amaranthus cruentus* L. grown on cesium contaminated soil to elevated CO_2 or inoculation with a plant growth promoting rhizobacterium *Burkholderia* sp. D54, or in combination

Shirong Tang[a,b,*], Shangqiang Liao[a,b], Junkang Guo[a,b], Zhengguo Song[a,b], Ruigang Wang[a,b], Xiaomin Zhou[c]

[a] *Centre for Research in Ecotoxicology and Environmental Remediation, Agro-Environmental Protection Institute, Ministry of Agriculture, Tianjin 300191, PR China*
[b] *Key Laboratory of Production Environment and Agro-product Safety of Ministry of Agriculture, Tianjin, PR China*
[c] *Plant Science Department, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9*

ARTICLE INFO

Article history:
Received 18 May 2011
Received in revised form 5 October 2011
Accepted 8 October 2011
Available online 15 October 2011

Keywords:
Elevated CO_2
Bacterial inoculation
Plant growth
Cesium uptake
Contaminated soil

ABSTRACT

Growth and cesium uptake responses of plants to elevated CO_2 and microbial inoculation, alone or in combination, can be explored for clean-up of contaminated soils, and this induced phytoextraction may be better than the natural process. The present study used open-top chambers to investigate combined effects of *Burkholderia* sp. D54 inoculation and elevated CO_2 (860 $\mu L\,L^{-1}$) on growth and Cs uptake by *Phytolacca americana* and *Amaranthus cruentus* grown on soil spiked with various levels of Cs (0–1000 $mg\,kg^{-1}$). Elevated CO_2 and bacterial inoculation, alone or in combination, significantly increased biomass production with increased magnitude, ranging from 22% to 139% for *P. americana*, and 14% to 254% for *A. cruentus*. Total tissue Cs in both plants was significantly greater for bacterial inoculation treatment singly, and combined treatments of bacterial inoculation and elevated CO_2 than for the control treatment in most cases. Regardless of CO_2 concentrations and bacterial inoculation, *A. cruentus* had higher tissue Cs concentration, Cs transfer factors and concentration ratios than *P. americana*, but they had slightly different contents of antioxidant enzymes. It is concluded that combined effects of elevated CO_2 and microbial inoculation with regard to plant ability to grow and remove radionuclides from soil can be explored for CO_2- and microbe-assisted phytoextraction technology.

1. Introduction

Two ways have been attempted thus far to break through the application bottleneck of phytoextraction technology: enhancing the amount of plant biomass in the contaminated soils per unit area or increasing concentrations of metals and radionuclides in plants [1,2]. Inoculation of plants with microorganisms can increase plant biomass and uptake of metals and radionuclides in most cases [3–6]. For this reason, the interactions among metals/radionuclides, inoculated microbes and plants have attracted much attention because of the promise for practical application of microorganisms to metal/radionuclide removal directly from polluted soils or the possible transfer of accumulated metals/radionuclides to higher plants [5–7]. CO_2 enrichment in simplified greenhouses can promote plant growth and enhance uptake of heavy metals and radionuclides as well [2,8]. Elevated CO_2 not only increased above-ground biomass of the *Sorghum vulgare* × *Sorghum vulgare* var. *sudanense* hybrid and *Trifolium pratense* L. species, but also caused more accumulation of Cs [9]. Interestingly, elevated CO_2 induced higher rhizosphere soil microorganism populations of both *Sorghum* and *Trifolium* species, and this process may contribute to enhanced Cs accumulation by plants [9]. Conversely, it can be speculated that inoculation with external microorganisms in combination with elevated CO_2 may promote plant growth, enhance Cs uptake, and affect antioxidant defense systems in plants as well.

Despite recognition that elevated CO_2 or inoculation with plant growth promoting rhizobacteria (PGPR) has individual positive effects on plant growth and pollutant uptake [2,8–11], very little information is available about how plants grown on contaminated soils respond to elevated CO_2 and PGPR inoculation in combination. Also, few studies have been conducted to investigate combined effect of elevated CO_2 and bacterial inoculation on plant growth

* Corresponding author at: Centre for Research in Ecotoxicology and Environmental Remediation, Agro-Environmental Protection Institute, Ministry of Agriculture, Tianjin 300191, PR China. Tel.: +86 22 23003707; fax: +86 22 23003707.
E-mail addresses: tangshir@hotmail.com, tangshirong@cae.org.cn (S. Tang).

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doi:10.1016/j.jhazmat.2011.10.029

and uptake of radionuclides, especially in terms of developing CO_2- and microbe-assisted phytoextraction of contaminated soils. Such research is particularly important, as in reality, simultaneous application of multi-means is commonplace, as shown by microbe-associated phytoextraction in association with chemical soil manipulation. We hypothesized that the combination of elevated CO_2 and bacterial inoculation may positively affect *Phytolacca americana* and *Amaranthus cruentus* grown in Cs contaminated soil, consequently promoting plant growth, enhancing uptake of the pollutants, and altering antioxidant enzymes in plants. This hypothesis was based on two observations: one is that plant species have repeatedly been shown to have better growth and more uptakes of pollutants under elevated CO_2 [2,8–10]; the other is that plant growth is often improved under bacterial inoculation condition [1,3,4,11–13]. If this hypothesis is supported, the association of elevated CO_2 and bacterial inoculation can be developed into CO_2- and microbe-assisted phytoextraction of radiocesium from contaminated soils.

In the present study, we used open-top chambers to investigate the effects of inoculation with PGPR *Burkholderia* sp. D54, isolated from the heavily contaminated paddy field in Shangba village of the Dabaoshan mine in South China, and elevated CO_2 (760 $\mu L\,L^{-1}$) on plant growth, and their associated uptake of stable Cs by *P. americana* and *A. cruentus* grown on soil spiked with various levels of cesium (0, 200, 500 and 1000 mg Cs kg^{-1}). The main objective of the current work was to assess the possibility of using elevated CO_2 as a gas fertilizer in association with bacterial inoculation to promote the biomass production of the two tested plant species, and to increase the accumulation of Cs, making their combination more effective as phytoremediation agents. To our knowledge, this is the first report on the utilization of elevated CO_2 and bacterial inoculation, in combination, to induce phytoextraction of Cs from contaminated soils.

2. Materials and methods

2.1. Tested plant species

The seeds of *A. cruentus* L. were bought from National Crop Germplasm Conservation Centre of China and the seeds of *P. americana* Linn. were provided by Dr. S.G. Xue who first reported the finding of this plant species [14]. Both species were selected for this study due to their potential for phytoextraction of radiocesium contaminated soil [2,15,16]. *P. americana* Linn. was mistook as *Phytolacca acinosa* Roxb. due to their similarities in vegetative growth period without infructescence and inflorescence.

2.2. Bacterial isolation, identification, and inoculation

Pure culture of the bacterium strain D54 of the genus *Burkholderia*, isolated from the Dabao Shan mining area, Guangdong Province, Southeast China, was used in this study [11]. Ten grams of soil samples was put into 250 mL conical flasks, and 90 mL of sterile distilled water was added to each. The flasks were shaken at 150 rpm for 30 min at 30 °C in a rotary shaker (SKY-2102C, Sukun, China). One hundred microliters of the suspension were spread over the plates of modified SMN agar medium (mannitol, 1%; $(NH_4)_2SO_4$, 0.2%; peptone, 0.2%; K_2HPO_4, 0.05%; $MgSO_4$, 0.05%; $NaCl_2$, 0.01%; $FeSO_4$, 0.005%; $MnSO_4$, 0.005%; yeast extract, 0.05%; pH 7.0). The plates were incubated for 3 days at 30 °C. The single colonies that grew well on the modified SMN agar were picked up and streaked to other modified SMN agar plates supplemented with 0.5 g L^{-1} of tricalcium phosphate. After incubation for 3 days at 30 °C, the isolates that grew well on the plates were re-streaked three times to fresh modified SMN agar plate and stored on modified SMN agar slants. Genomic DNA extraction and the 16S rRNA gene PCR amplification of the isolate were carried out and identified following the procedures of Chun and Goodfellow [17]. The universal bacterial 16S rRNA gene primers (the forward primer P1: 5′-CGg gat ccA GAG TTT GAT CCT GGC TCA GAA CGA ACG CT-3′ and the reverse primer P6: 5′-CGg gat ccT ACG GCT ACC TTG TTA CGA CTT CAC CCC-3′) were used for the 16S rRNA gene PCR amplification of the isolate. The purified PCR product was directly sequenced by an automated DNA Sequencing System (ABI 3730XL). On the basis of the morphological, physiological and 16S rDNA gene sequence analysis, strain D54 was recognized as a species of the genus *Burkholderia* sp. (GenBank/EMBL/DDBJ accession no. HM467915) [11].

Actively growing cells were centrifuged at 10,000 rpm for 10 min and then washed with 0.85% (w/v) sterilized NaCl twice. The washed cells were re-suspended in sterilized de-ionized water to a final concentration of approximately 10^8 colony-forming units (cfu) mL^{-1} and used for plant inoculation.

Table 1
Physical and chemical characteristics of the soil used in this study.

	Values
Total N (g kg^{-1})	0.96
Total P (g kg^{-1})	0.51
Total K (g kg^{-1})	19.32
Available K (mg kg^{-1})	95.67
Cs concentration in soil (μg kg^{-1})	7.79
Organic matter (g kg^{-1})	16.25
CEC (cmol kg^{-1})	18.32
pH (H_2O)	5.52

2.3. The tested soil and its preparation for this study

The soil used in this study was collected from a long-term experimental rice field at Shenyang Agricultural University, Liaoning Province. The physical and chemical properties of the soil and background Cs concentration are shown in Table 1. The methods for soil property determination and elemental analysis follow Tang et al. [8]; a brief summary follows. The pH of soil was determined by a glass electrode (soil:water ratio, 1:2.5), organic matter by the potassium dichromate oxidation-heat method, total nitrogen by a semi-micro-Kjeldahl method, total phosphorus by the $HClO_4$–H_2SO_4 digestion ammonium molybdate ascorbic acid method, total potassium by $HCl + HNO_3 + HClO_4$ (3:1:1, v/v/v) digestion followed by Atomic Absorption Spectrometer (AAS) and a graphite tube equipped with an automatic sampler (ZEEnit 700, Analytikjena, Germany), and soil texture by the hydrometer method. The soil was digested overnight in 2 mL of concentrated HNO_3 at 120 °C, on a hot plate, and then was dissolved in 5 mL of HNO_3–HCl_4 (1:1, v/v) mixed acid and digested at 220 °C overnight for background Cs content analysis with the AAS. The soil is a sandy, silty loam (FAO classification) with high levels of organic matter (16.25%) and a pH of 5.52.

Fresh soil was drained through a 3-mm sieve and kept in darkness before use. Specified amounts of Cs ($CsCl_2$) in the form of dissolved solution were added and thoroughly mixed into the soil to give three levels: 200, 500 and 1000 mg Cs kg^{-1}. No Cs spiked soil was used as a control. The soil was kept in darkness for 75 days to allow equilibration of the substrate. The balanced soil was then transferred into 192 plastic pots (10 cm diameter and 12 cm height), each containing 1.0 kg dry weight soil. A suitable size plastic saucer was placed under the bottom of the filled pots to avoid leaching of soluble ions. Before planting, pots were fertilized with 200 mg kg^{-1} of N, 100 mg kg^{-1} of P, and 200 mg kg^{-1} of K, watered to maximum water holding capacity (WHC_{max}), and subsequently allowed to equilibrate for two more weeks. The pots

treated with Cs and bacterial inoculation were divided into two sets: one in an open-top chamber with elevated CO_2 and the other with ambient CO_2.

2.4. Conditions of plant growth

P. americana and *A. cruentus* seeds were surface sterilized by soaking them in 1% NaClO for 10 min, rinsed in de-ionized water 5–7 times and germinated in a moist mixture of per liter and vermiculite (1:1), all in a controlled environment growth chamber. Two weeks after germination, healthy and uniformly-sized seedlings were selected for the pot experiment. Two selected seedlings were washed with de-ionized water, and transferred to its respective pot. Five days after growing in pots, the seedlings were thinned to one plant per pot. At the same time, 20 mL of washed bacterial suspension (described above) was added to the plant root for the bacteria inoculated treatments. The pots were placed into six open-top chambers (OTCs), as described in details in Wu et al. [9]. Three were maintained at ambient CO_2 (average of 375 $\mu L\,L^{-1}$) during the daytime, and the other three were ventilated with double CO_2 (average 860 $\mu L\,L^{-1}$) air. CO_2 enrichment was applied to plants from 8:00 to 18:00 h each day of the experiment (9th May to 13th July 2010). Total CO_2 treatment time was 65 days.

During the growth period of plants, a soil moisture analyzer (Field Scout Soil Moisture Meter Model TDR 300, Spectrum Technologies, Inc.) was used to monitor the soil moisture content in pots so that the pots were just watered carefully to 60–70% of the maximum water holding capacity of the soil. Care was taken to ensure that there was no draining water during the growth of plants.

2.5. Harvesting and analysis of Cs in plants and soil

Sixty-five days after growth in the OTC, plants were harvested (2 cm above the soil line) and separated into shoots and roots. Parts of the plant leaves were cut into small pieces (less than 0.5 cm in length) with stainless steel scissors. The cut pieces were wrapped with tin foil, flash frozen in liquid nitrogen, and stored in darkness at −80 °C for superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) determination. The residual shoot material was dried in an oven at 75 °C for 72 h, recorded for a constant dry weight and pulverized to a homogeneous powder with a stainless steel cutter blender (IKA T250D, Germany). Plant roots were removed from the pots, and washed several times with distilled water.

Plant subsamples were digested in a mixture of HNO_3 and H_2O_2 (8:1, v/v) using a microwave digestion method (CEM, Mars: 240/50, USA), and the extractants were analyzed for Cs content by Atomic Absorption Spectrometer (see Wu et al. [9])

2.6. Determination of chlorophyll and carotenoid content

Chlorophyll and carotenoids were extracted by homogenizing ca 0.1000 g fresh leaves in chilled ethanol solution (95%), with the extractant vessels filled to 25 mL. Chlorophyll and carotenoid contents in supernatants were determined spectrophotometrically (Specord S600, Analytik Jena, Germany) at 665, 649 and 470 nm. The calculation method followed Lichtenthaller and Wellburn [18].

2.7. Determination of SOD, CAT and MDA

Leaf material (0.2000 g) was taken from the −80 °C refrigerator, immediately ground in liquid nitrogen in a mortar and pestle, and the crude enzymatic extracts of each part were homogenized in 3 mL of 50 mM potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 8000 × *g* for 20 min at 4 °C [19]. The supernatant (i.e., the enzyme extract) was used for determinations of enzyme activities (SOD, CAT) and the content of MDA.

A SOD assay was performed according to the method of Giannopolitis and Ries [20], with some modifications. Briefly, 3 mL reaction mixtures contained 0.3 mL of each: 750 $\mu mol\,L^{-1}$ nitroblue tetrazolium (NBT), 20 $\mu mol\,L^{-1}$ riboflavin, 130 $mmol\,L^{-1}$ methionine, and 100 $\mu mol\,L^{-1}$ EDTA–Na_2, 1.5 mL of 50 $mmol\,L^{-1}$ phosphate buffer (pH 7.8), 0.25 mL of de-ionized water, and 0.05 mL of enzyme extract. The test tubes were placed under light with an average photon flux density of 78 $\mu mol\ photons\,s^{-1}\,m^{-2}$ for 20 min, and the absorbance of the reaction mixture was recorded at 560 nm. Reaction solution placed in the dark was used as control. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 50% inhibition of the rate of nitroblue tetrazolium reduction.

A CAT assay was modified from the method of Brennan and Frenkel [21]. 0.1 mL enzyme extract was added to mixture solution of 1 mL of 0.3% H_2O_2 and 1.9 mL of 50 $mmol\,L^{-1}$ phosphate buffer (pH 7.0) to initiate the reaction. The activity of CAT was measured by determining the rate of change of H_2O_2 absorbance in 60 s at 240 nm. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 1% absorbance reduction in 60 s.

Lipid peroxides were measured by thiobarbituric acid test for MDA according to Heath and Packer [22]. Supernatant (1.5 mL) was added to 2.5 mL of 20% trifluoroacetic acid containing 0.5% thiobarbituric acid. The mixture was held at 100 °C in a water bath for 20 min and cooled quickly in an ice bath followed by centrifugation at 10,000 × *g* for 10 min. The absorbances of the resulting solutions were determined at 450, 532 and 600 nm.

2.8. Calculation of transfer factor (TF), Cs concentration ratios (CR), and total Cs uptake

The TF, used to express plant uptake of Cs, was calculated for each plant sample as:

$$\text{Cs TF} = \frac{\text{shoot Cs concentration}}{\text{soil Cs concentration}} \quad (1)$$

Cs concentration ratio (CR) was calculated as:

$$\text{CR} = \frac{\text{shoot Cs concentration}}{\text{root Cs concentration}} \quad (2)$$

Total Cs uptake, expressed on a per-seedling basis, was calculated separately for shoots and roots as:

$$\text{total Cs uptake} = \text{plant tissue Cs concentration} \times \text{plant tissue biomass} \quad (3)$$

2.9. Statistical analysis

Statistical analysis was performed using the SPSS software program (SPSS Inc., Chicago, IL, Version 16.0). The data were analyzed with a three-way analysis of variance (ANOVA) approach, i.e. microbe (with or without bacterial inoculation), CO_2 treatment (2 levels), and Cs treatment (4 levels). To examine the statistical significant differences ($p < 0.05$) between means, the Tukey test was performed.

3. Results

3.1. Plant biomass

There are large differences in both shoot- and root biomass production and shoot: root ratio between *P. americana* and *A. cruentus*. *P. americana* had higher biomass production than *A. cruentus* for the soils spiked with Cs in general (Fig. 1). Plant biomass decreased with increasing Cs concentration in the soil, regardless of CO_2 concentration. For the same spiked Cs level, elevated CO_2 and bacterial

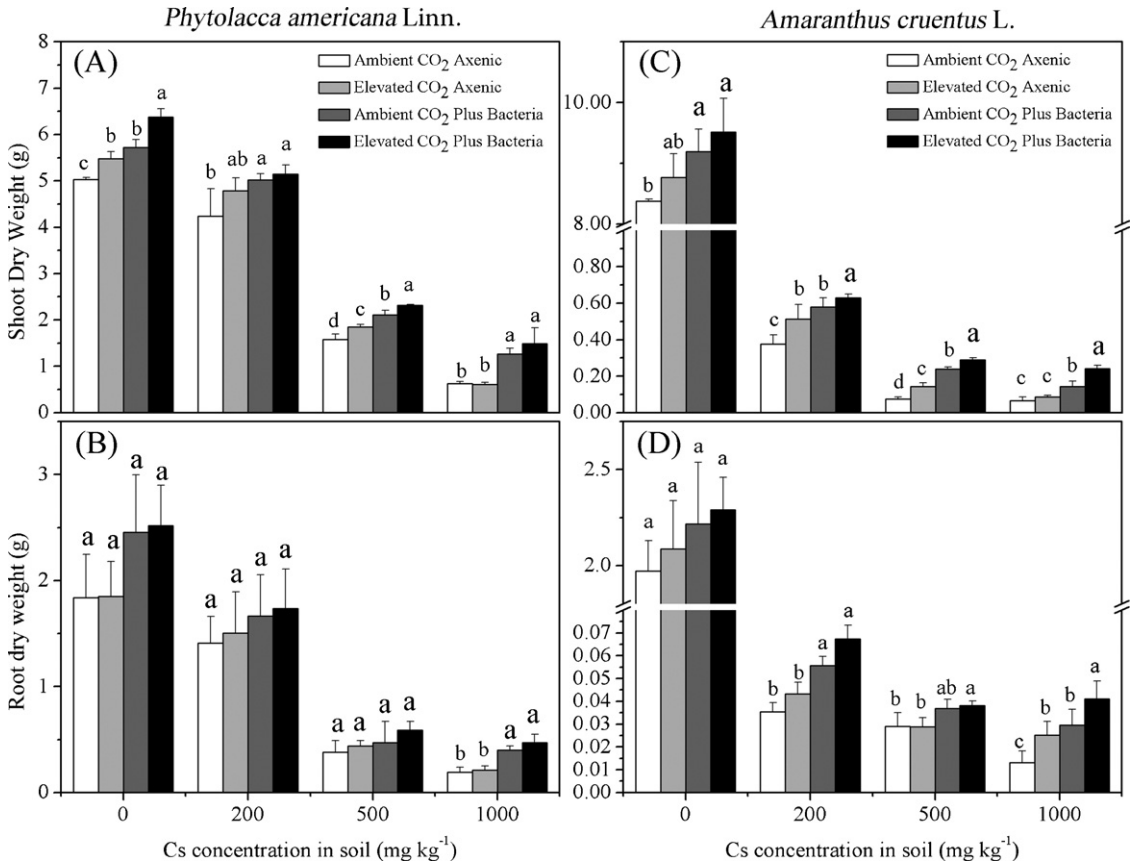

Fig. 1. Effect of inoculation with *Burkholderia* sp. D54 on biomass of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

inoculation, alone and in combination, increased the biomass production, with much greater magnitude in shoots than in roots. Plant growth was stimulated more under conditions of elevated CO_2 and bacterial inoculation in combination than alone. The increase in magnitude of biomass at the Cs level of 0, 200, 500 and 1000 mg kg^{-1} was 29, 22, 48, and 139% for *P. americana*; 14, 70, 215, and 254% for *A. cruentus*, respectively. Maximum increase of biomass production of both plant species due to combined effect of elevated CO_2 and bacterial inoculation occurred at a Cs exposure level of 1000 mg kg^{-1}.

3.2. Cs concentration in plants

P. americana and *A. cruentus* had different ability to accumulate Cs. In general, *A. cruentus* had higher Cs concentration than *P. americana* for the same spiked Cs level either in shoots or in roots (Fig. 2). Regardless of CO_2 concentration and bacterial inoculation treatments, shoot and root Cs concentration (Fig. 2) were significantly increased with increasing Cs concentration in soil. Significant differences in Cs concentration were observed in plants grown in soil spiked with various Cs concentrations. For soil treated with 1000 mg Cs kg^{-1}, shoot Cs concentrations ranged from 11,000 to 17,000 mg Cs kg^{-1} in *A. cruentus*, and 7000 to 12,000 mg Cs kg^{-1} in *P. americana*. By contrast, root Cs concentration in the highest Cs treatments ranged from 9500 to 13,000 mg Cs kg^{-1} for *A. cruentus*, and 7500 to 11,000 mg Cs kg^{-1} for *P. americana*. The highest mean shoot Cs concentration for the two species was present in the 1000 mg Cs kg^{-1} treatment under conditions of elevated CO_2 and bacterial inoculation together.

Total shoot and root Cs in both plants were significantly greater for bacterial inoculation treatment singly and combined treatments of bacterial inoculation and elevated CO_2 than for the control treatments, with the exception being the response of the *P. americana* roots exposed to 200 mg Cs kg^{-1} (Fig. 3). Elevated CO_2 treatment alone affected the total uptake of Cs in roots and shoots of both *P. americana* and *A. cruentus* positively, but not significant enough in most cases.

Cesium (TFs) showed sharply contrasting values, depending upon plant species and treatments (including spiked Cs levels, elevated CO_2 levels, and bacterial inoculation) (Table 2). *P. americana* had lower Cs TFs and CR values than *A. cruentus*. For *P. americana*, average cesium TFs ranged from 7.65 to 20.34 whereas for *A. cruentus*, average Cs TFs from 11.34 to 42.31. Concentration ratios for Cs ranged from 0.63 to 1.10, and 1.17 to 1.41 for *P. americana* and *A. cruentus*, respectively, showing no significant differences in most cases between bacterial inoculation and elevated CO_2 treatments.

3.3. Chlorophyll and carotenoid contents in plants

Increasing spiked Cs concentrations in soil caused a continual decrease in the contents of chlorophylls a and b, and carotenoids, irrespective of CO_2 concentrations and bacterial inoculation treatments (Fig. 4). For the same spiked Cs level treatment, elevated CO_2 and bacterial inoculation alone, and their combination, significantly increased the contents of chlorophylls a and b in *P. americana*, but no significant difference in the chlorophylls a and b contents was observed for the Cs control treatment (Fig. 4(A) and (B)). Elevated CO_2 and bacterial inoculation alone resulted in a significant increase in carotenoid content of the plants exposed to 200 and 500 mg Cs kg^{-1}, but no significant difference was noted under 1000 mg Cs kg^{-1} exposure and the Cs control condition (Fig. 4(C)).

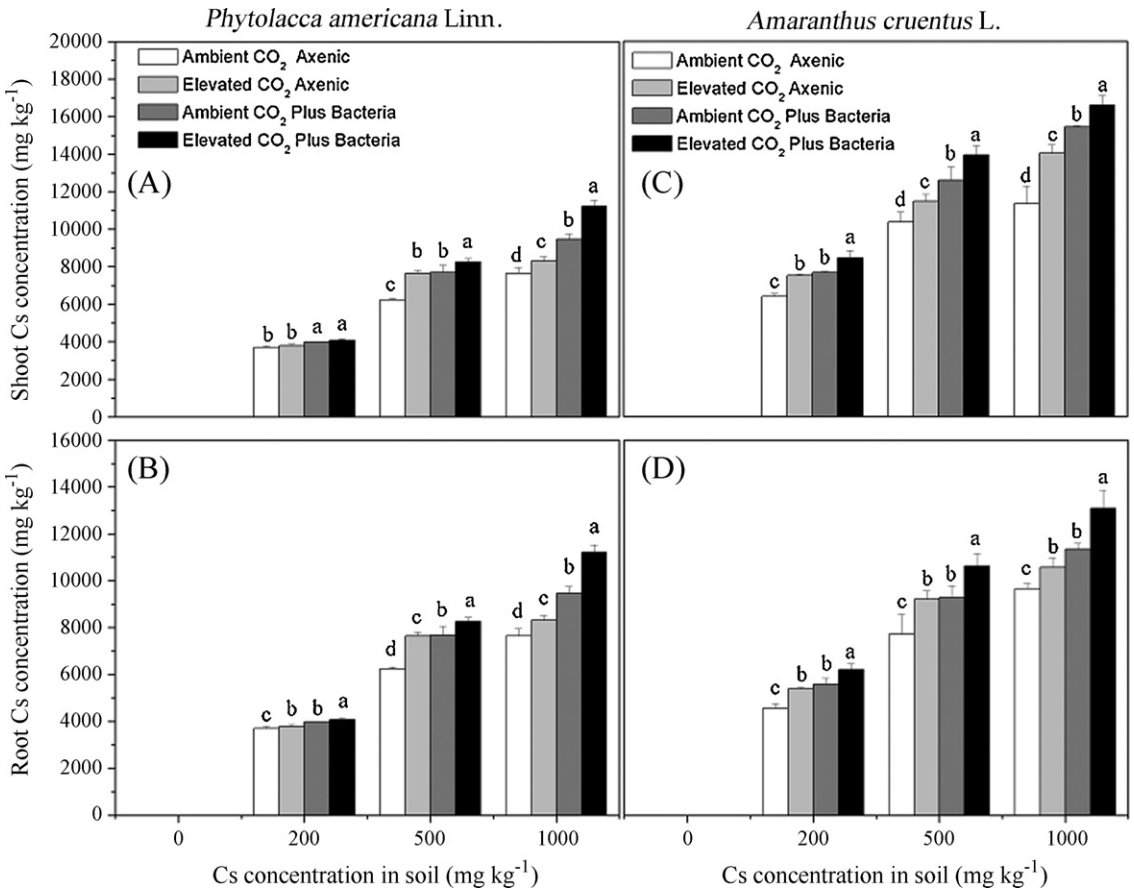

Fig. 2. Effect of inoculation with *Burkholderia* sp. D54 on Cs concentrations in shoots and roots of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

By comparison, elevated CO_2 and bacterial inoculation, singly and in combination, significantly increased the contents of chlorophylls a and b and carotenoids in the leaves of *A. cruentus* exposed to 1000 mg Cs kg^{-1}. For the 0, 200, and 500 mg kg^{-1} treatments, the contents of chlorophylls a and b and carotenoids were increased but not significant (Fig. 4(E) and (F)). Compared to the control treatment, elevated CO_2 and bacterial inoculation alone, and their combination, significantly increased the carotenoid contents in the

Table 2
Effect of inoculation with *Burkholderia* sp. D54 on TFs and CRs of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2. Values represent mean ± SD, with 6 replicates per treatment. Different letters indicate significant differences ($p \leq 0.05$) between CO_2 treatments and bacteria treatments (within one Cs level).

Species	Cs (mg kg^{-1})	Treatments	TF	CR
P. americana	200	Ambient CO_2 axenic	18.45 ± 0.44^{c}	0.72 ± 0.01^{a}
		Elevated CO_2 axenic	18.96 ± 0.42^{bc}	0.66 ± 0.03^{ab}
		Ambient CO_2 plus bacteria	20.34 ± 0.42^{a}	0.63 ± 0.05^{b}
		Elevated CO_2 plus bacteria	19.92 ± 0.05^{ab}	0.67 ± 0.01^{ab}
	500	Ambient CO_2 axenic	12.48 ± 0.14^{c}	0.96 ± 0.03^{a}
		Elevated CO_2 axenic	15.30 ± 0.32^{b}	0.99 ± 0.05^{a}
		Ambient CO_2 plus bacteria	15.36 ± 0.77^{ab}	0.80 ± 0.02^{b}
		Elevated CO_2 plus bacteria	16.53 ± 0.34^{a}	0.81 ± 0.02^{b}
	1000	Ambient CO_2 axenic	7.65 ± 0.30^{c}	1.04 ± 0.02^{b}
		Elevated CO_2 axenic	8.31 ± 0.20^{c}	0.90 ± 0.04^{c}
		Ambient CO_2 plus bacteria	9.45 ± 0.29^{b}	1.10 ± 0.01^{a}
		Elevated CO_2 plus bacteria	11.22 ± 0.30^{a}	0.99 ± 0.01^{b}
A. cruentus	200	Ambient CO_2 axenic	31.98 ± 0.76^{c}	1.41 ± 0.10^{a}
		Elevated CO_2 axenic	37.56 ± 0.37^{b}	1.40 ± 0.01^{a}
		Ambient CO_2 plus bacteria	38.33 ± 0.36^{b}	1.38 ± 0.08^{a}
		Elevated CO_2 plus bacteria	42.31 ± 1.87^{a}	1.36 ± 0.02^{a}
	500	Ambient CO_2 axenic	20.73 ± 1.07^{c}	1.35 ± 0.09^{a}
		Elevated CO_2 axenic	22.93 ± 0.78^{bc}	1.25 ± 0.03^{a}
		Ambient CO_2 plus bacteria	25.16 ± 1.47^{ab}	1.36 ± 0.02^{a}
		Elevated CO_2 plus bacteria	27.88 ± 0.95^{a}	1.31 ± 0.02^{a}
	1000	Ambient CO_2 axenic	11.34 ± 0.94^{c}	1.17 ± 0.07^{b}
		Elevated CO_2 axenic	14.03 ± 0.45^{b}	1.33 ± 0.01^{a}
		Ambient CO_2 plus bacteria	15.44 ± 0.06^{ab}	1.36 ± 0.03^{a}
		Elevated CO_2 plus bacteria	16.59 ± 0.51^{a}	1.27 ± 0.04^{ab}

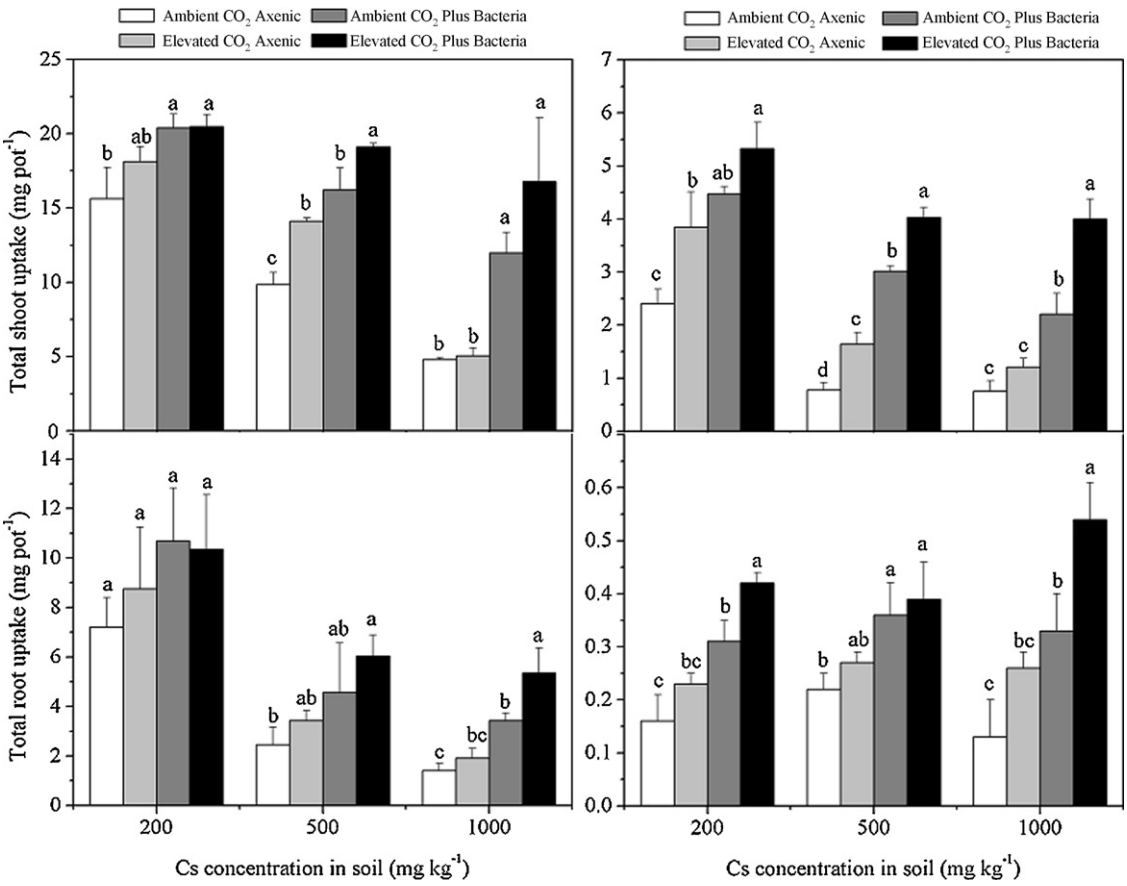

Fig. 3. Effect of inoculation with *Burkholderia* sp. D54 on Cs uptake by *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

leaves of *A. cruentus* exposed to 200 and 500 mg Cs kg^{-1}, but had no significant effect of the chlorophyll a/b ratio.

3.4. SOD and CAT activities and MDA contents in plants

Leaf SOD and CAT activities in both *P. americana* and *A. cruentus* showed a trend proportional with increasing spiked Cs concentration in the soil, regardless of CO_2 concentrations and bacterial inoculation (Fig. 5). In the same level of Cs treatment, elevated CO_2 and bacterial inoculation alone significantly increased leaf SOD and CAT activities in *P. americana*, with maximum increase occurring under the combined treatment condition (Fig. 4(A) and (C)). When exposed to 500 mg Cs kg^{-1}, elevated CO_2 and bacterial inoculation, singly and in combination, significantly increased SOD activity in the leaves of *A. cruentus*. For the Cs control, elevated CO_2 alone had no significant effect of SOD activity. Bacterial inoculation alone had no significant effect on leaf SOD activity in the leaves of *A. cruentus* exposed to 200 mg Cs kg^{-1} (Fig. 5(B)). CAT activity in the leaves of *A. cruentus* was, however, significantly increased under conditions of elevated CO_2 and bacterial inoculation, singly and in combination (Fig. 5(D)).

MDA concentration in both *P. americana* and *A. cruentus* showed an increasing trend with increasing spiked Cs concentration in the soil (Fig. 6). Overall, elevated CO_2 and bacterial inoculation alone and their combination significantly decreased the MDA concentration in leaves of *P. americana* under Cs stress. For *A. cruentus*, the variation trend is slightly different. Only when exposed to 200 and 500 mg Cs kg^{-1} did MDA in the leaves of *A. cruentus* decrease significantly under conditions of elevated CO_2 and bacterial inoculation alone and their combinations. For the Cs control, elevated CO_2 and bacterial inoculation had no individual significant effect on leaf MDA.

4. Discussion

4.1. Combined effect of Cs on plant growth and development

Chlorosis and necrosis are typical toxic symptoms for plants grown in soils contaminated with high level of Cs [23]; these symptoms are considered to be associated with Cs interference with K metabolism [24]. In our previous study we observed severe Cs phytotoxicity with *S. vulgare* × *S. vulgare* var. *sudanense* hybrid and *T. pratense* grown on soil spiked with Cs concentration of more than 300 mg kg^{-1} [9]. Cook et al. [25] found a reduction in root growth of both *Agropyron cristatum* and *Bromus tectorum* grown in the 50 mg Cs kg^{-1} treatments, but they observed no significant difference in root growth in *Agropyron spicatum* and *Leymus cinereus*. Our present study revealed a similar toxic symptom as shown by the rolled leaves of *A. cruentus* grown at spiked Cs level of more than 200 mg kg^{-1}.

A lot of research has been conducted on growth stimulation either at elevated CO_2 [8–10,26–29] or under condition of bacterial inoculation [7,11,30–32], but very little information is available as to the combined effect of both elevated CO_2 and bacterial inoculation on plant growth and development under Cs stress. In this experiment, we observed that elevated CO_2 alone enhanced the growth and biomass of plants. We showed that bacterial inoculation alone had significant positive effects on the biomass accumulation and its yield as well. We also observed similar response patterns in the combined treatments with CO_2 and

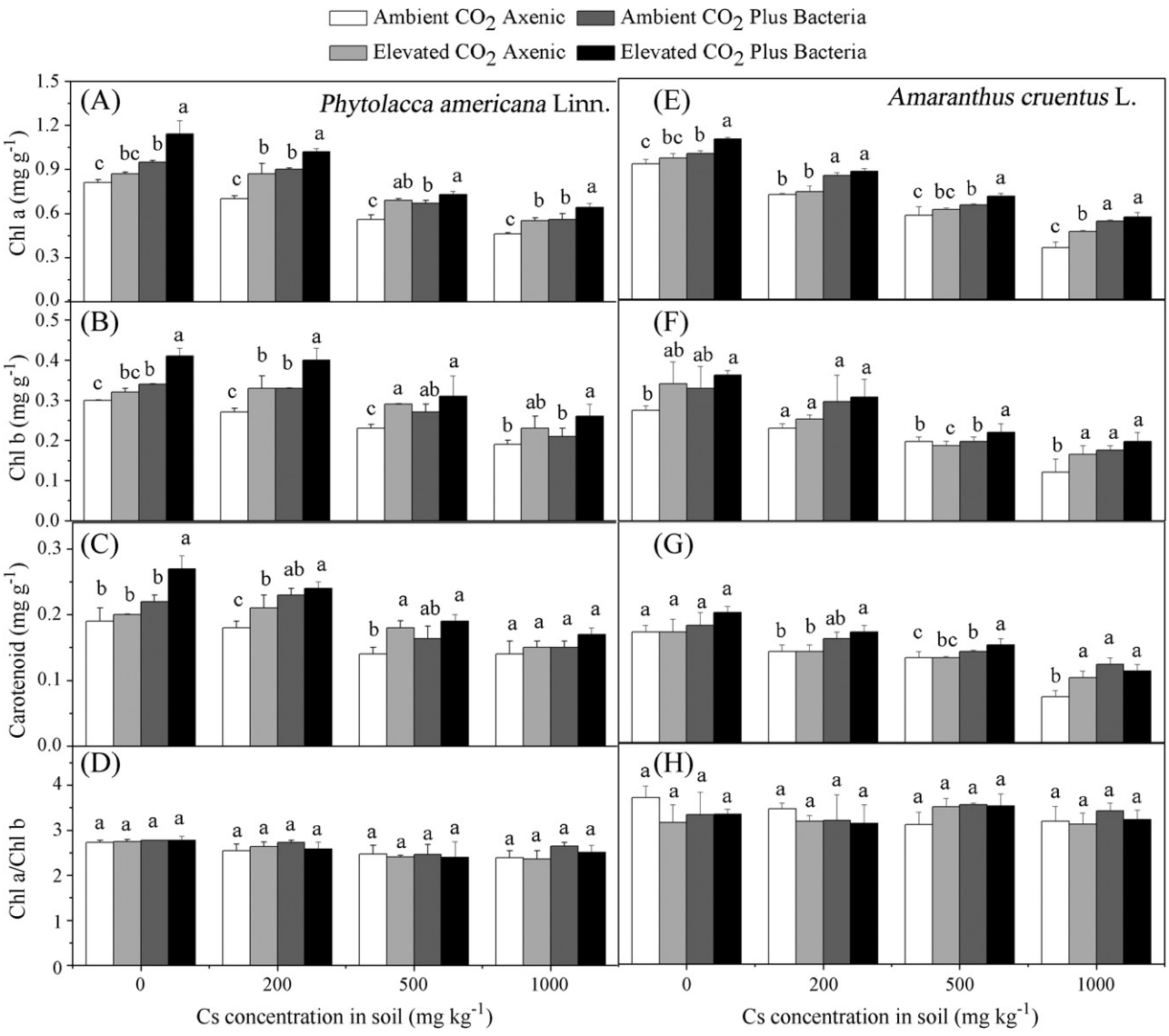

Fig. 4. Effect of inoculation with *Burkholderia* sp. D54 on photosynthetic pigments in leaves of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

PGPR *Burkholderia* sp. D54 inoculation. Since the efficiency of the phytoextraction of radionuclide contaminated soil is often connected with high biomass production by plant species and their accumulation of the contaminants; the increase in the biomass of both *P. americana* and *A. cruentus* grown in the combination treatments with elevated CO_2 and PGPR inoculation may imply improvement in phytoremediation efficiency if they are applied to field practice. By comparison, elevated CO_2 had more effect on shoot than on root development. *P. americana* grew better on soils spiked with 1000 mg Cs kg^{-1} without visible injury symptoms or significant growth retardation than *A. cruentus*, suggesting that the former was more tolerant to Cs stress than the latter. This phenomenon was more likely related to higher tissue Cs concentration in *A. cruentus* than *P. americana* (Fig. 2).

4.2. Combined effect on total Cs uptake by plants, and its implication for phytoremediation

Most efforts in the past have attempted to understand and predict how plants respond to either elevated CO_2 concentration or inoculation with microorganisms on total Cs uptake by plants, although little attention has been given to their combined effect. There are several studies [8–10] that reported positive effect of elevated CO_2 on plant uptake of metals, but very few studies focus on such effect on radionuclide uptake by plants. The only paper, published by Wu et al. [9] on the effect of CO_2 on plant uptake of cesium, documented the increased biomass production of *S. vulgare* × *S. vulgare* var. *sudanense* and *T. pratense* and enhanced plant uptake of Cs at elevated CO_2 [9]. This positive effect of elevated CO_2 on plant uptake of Cs singly might be attributed to the lowered soil pH values, and changes in number and kind of microorganisms in the rhizospheres of the *Sorghum* and *Trifolium* species growing on Cs spiked soils at elevated CO_2.

By contrast, there are much more investigations that have been conducted to address the effect of microorganism inoculation on plant growth and uptake of radionuclides [5–7,32]. Roger and Williams [7] reported that *Melilotus offinialis* and *Sorgham sudanense*, when infected with vesicular-arbuscular mycorrhizae, could take up only 0.93–2.5 and 0.6–1.42 parts per million cesium-137 compared with the concentration in the pots in which they were grown, respectively. Entry et al. [6] showed that *Pinus ponderosa* and *Pinus radiata* seedlings inoculated with

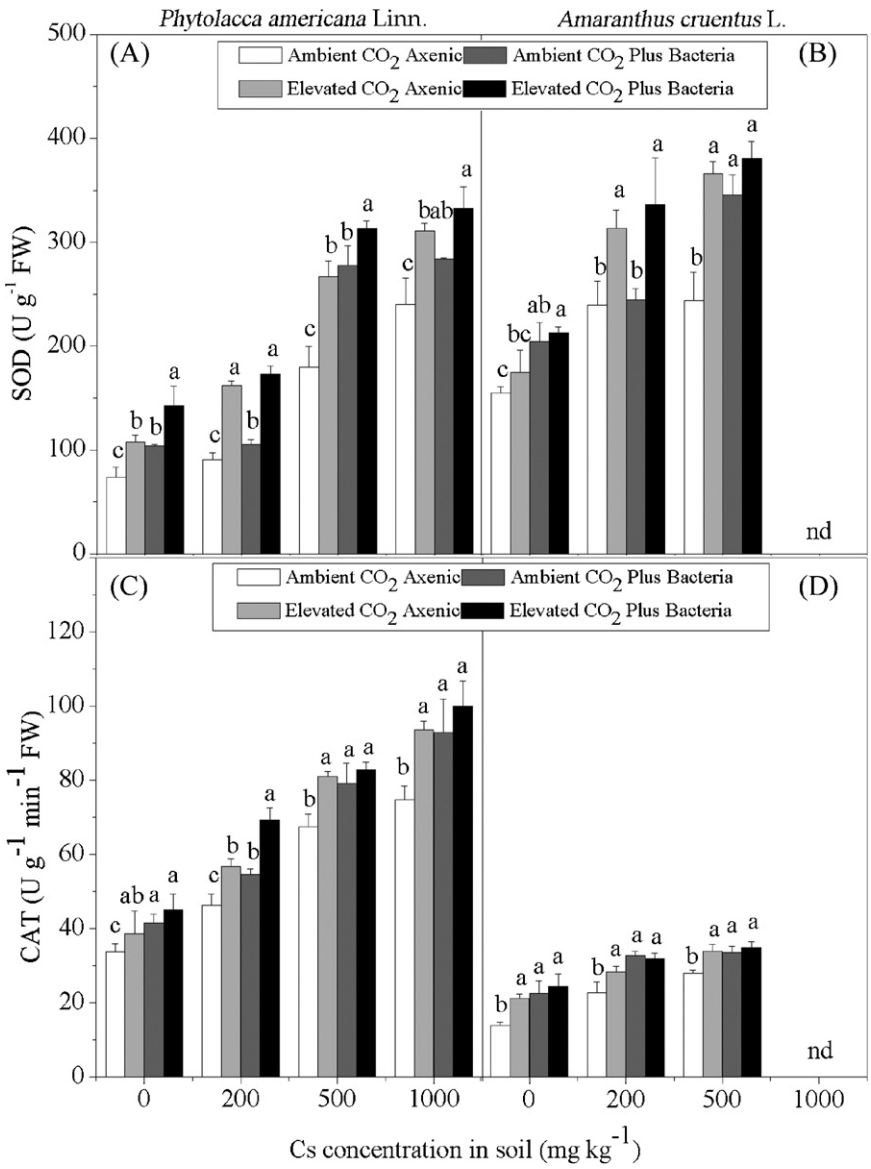

Fig. 5. Effect of inoculation with *Burkholderia* sp. D54 on SOD and CAT activities in leaves of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

ectomycorrhizal fungi were able to remove 3–5 times more ^{90}Sr from contaminated soil than seedlings without ectomycorrhizae. Similar results were presented in their further study [32] in which they found that inoculation with mycorrhizal fungi, *Glomus mosseae* and *Glomus intraradices* increased the above-ground biomass of *Eragrostis bahiensis*, *Sorghum halepense* and *Panicum virgatum* while also increasing the accumulation of ^{137}Cs in a relatively short period of time.

Little is known, however, about the combined effect of elevated CO_2 and microorganism inoculation on plant growth and uptake of radiocesium. Our present experiment addressed individual and combined effects of CO_2 and external bacterial inoculation on total Cs uptake by plants, along with their potential use in phytoextraction. Treatments of CO_2 and bacterial inoculation, alone and in combination, produced significant positive effects on Cs uptake by both *P. americana* and *A. cruentus*. Elevated CO_2 not only increased plant growth and yield of both *P. americana* and *A. cruentus*, but also enhanced the positive effects of bacterial inoculation on Cs uptake. This finding is of great importance since the increase in Cs uptake together with increased biomass under conditions of elevated CO_2 and bacterial inoculation implies that the use of elevated CO_2 and bacterial inoculation, alone and in combination, might be an alternative way to improve phytoextraction efficiency.

4.3. Combined effect of elevated CO_2 and bacterial inoculation on oxidative stress

Plants respond to changing environment by changing antioxidative systems [33]. Variation of environmental factors alone, such as salinity [34], drought [35,36], inoculation with microorganisms [37], elevated levels of heavy metals [29], and O_3 [38,39], may result in oxidative stress and decrease the concentrations of chlorophylls and carotenoids in plants to a various degree [40]. Elevated CO_2 can ameliorate oxidative stress and decrease in chlorophyll and carotenoid contents induced by environmental stress [29,41–43]. In spite of much research on the effect of elevated levels of Cs on plant growth and development, little information is available about how plants grown on Cs contaminated soils respond to both elevated CO_2 and bacterial inoculation in combination in terms of

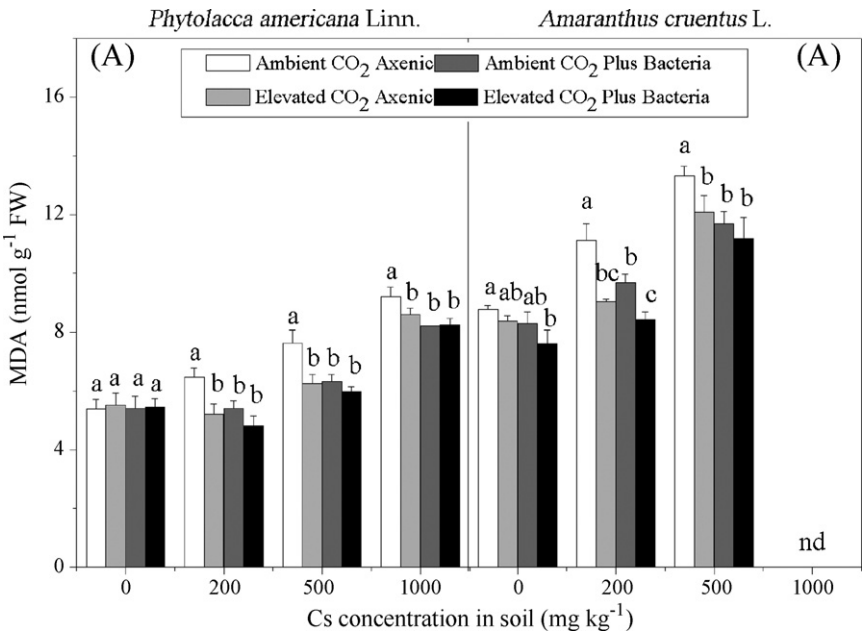

Fig. 6. Effect of inoculation with *Burkholderia* sp. D54 on MDA contents in leaves of *P. americana* and *A. cruentus* grown on soils spiked with different Cs level under ambient and elevated CO_2.

plant antioxidant apparatus, chlorophyll and carotenoid contents [9].

MDA reflects the extent to which free radical is produced and consequent tissue is damaged [44], and is routinely used as an index of lipid peroxidation under heavy metal stress conditions. Previous studies have shown significant effect of individual salt and heavy metal stress on the MDA level of plants [45–47], but little research has been conducted to investigate combined effects of elevated Cs levels and other environmental factors such as elevated CO_2 and inoculation with microorganisms. Our present results showed a significant decrease in MDA concentration in the leaves of *P. americana* and *A. cruentus*, suggesting some extent of alleviation of Cs induced oxidative damage under conditions of elevated CO_2 and bacterial inoculation, singly and in combination.

Plants under stressed conditions of individual environments factor may produce antioxidative enzymes SOD, CAT, and so on, that directly or indirectly detoxify reactive oxygen species (ROS) [48]. Elevated SOD activities are beneficial for improved protection of plants against oxidative stresses [48]. Variation in SOD and CAT activities of wheat seedlings under low temperature stress was related to the degree of cold tolerance of plants [48]. Individual elevated CO_2 significantly affects the activity of antioxidative enzymes in plants [36], but the results are contradictory [38–40]. Our previous studies showed that the alleviation of Cd toxicity with elevated CO_2 for the *Lolium* species may be more dependent on enhanced antioxidant capacity [29]. In the present study, we showed that elevated CO_2 and bacterial inoculation, singly and in combination, significantly increased leaf SOD and CAT activities in the leaves of *P. americana* and *A. cruentus* (Fig. 4(A)–(D)), and that the two Cs stressed species exhibited slightly different responses to elevated CO_2 and inoculation with PGPR *Burkholderia* sp. D54, singly and in combination. This new information suggests that under conditions of elevated CO_2 and inoculation with inoculated PGPR *Burkholderia* sp. D54, singly and their combinations, ROS can be scavenged more effectively by SOD and CAT, and the plants survive better on Cs contaminated soil.

Chlorophylls a and b are the pigments that make plants look green; carotenoids are part of a larger collection of plant derived compounds called terpenes. Their contents in the leaves of plants can be used to evaluate the photosynthetic apparatus [41]. The concentrations of chlorophylls and carotenoids are usually decreased to a different degree when plants are under heavy metal and salinity stress, and this variation often provides information concerning the effect of the stress on plant growth, and physiological biochemical processes [49,40]. Although Jia et al. [29] reported that elevated CO_2 increased chlorophylls a and b contents in *Lolium* species grown on soils spiked with a high level of Cd, little information is available in the literature regarding the relationships between variations in chlorophylls and carotenoids, and elevated Cs concentration in soil. In our present study, the difference in chlorophyll and carotenoid content under Cs stress condition between *P. americana* and *A. cruentus* might indicate their difference in ability to plant responsiveness to light intensity. Two implications may be drawn from this conclusion: the first implication is that the two plant species use different strategies with respect to the mechanism of accumulation and uses of photosynthetic pigments under Cs stressed condition; and the second one is that individual and combined treatments with elevated CO_2 and bacterial inoculation had positive effect on these processes. The increases in photosynthetic pigments under the same Cs stressed condition may be related to the alleviation of oxidative stress with elevated CO_2 and bacterial inoculation [50].

5. Conclusions

We concluded that elevated atmospheric CO_2 concentration increased the beneficial effects of inoculation with a PGPR on growth and Cs uptake by *P. americana* and *A. cruentus*, contributing to a greater plant biomass production as well as total uptake of Cs by plants. In a context of enriching CO_2 as an assisting tool in phytoremediation, we suggest that the inoculations with PGPR be used as an effective way to promote plant growth and improve radionuclide uptake by plants, thus contributing to an improvement in phytoextraction practice. As the conclusion was based on the data obtained from plants grown in Cs spiked contaminated soil under OTC and pot growth conditions, the growth and Cs uptake response might be exaggerated or overestimated. Therefore, more research is needed on plants grown under realistic field conditions

with elevated CO_2 and microbial inoculation combination treatments in order to make reasonable predictions on combined effects of elevated CO_2 and microbial inoculation combination on plant growth and radionuclide uptake in terms of developing CO_2- and microbe-assisted phytoextraction technology.

Acknowledgements

The work was financially supported by the projects of Grant Nos. 40773078 and 2011-S2. The authors acknowledge the partial funding from Central Public Research Institutes Basic Funds for Research and Development and the Fund for First-class Distinguished Talent Program of Chinese Academy of Agricultural Sciences.

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