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RESEARCH ARTICLE

Effects of CO_2 application and endophytic bacterial inoculation on morphological properties, photosynthetic characteristics and cadmium uptake of two ecotypes of *Sedum alfredii* Hance

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Abstract

Plant uptake of cadmium (Cd) is affected by soil and environmental conditions. In this study, hydroponic experiments were conducted to investigate the effects of elevated CO_2 coupled with inoculated endophytic bacteria M002 on morphological properties, gas exchange, photosynthetic pigments, chlorophyll fluorescence, and Cd uptake of *S. alfredii*. The results showed that bio-fortification processes (elevated CO_2 and/or inoculated with endophytic bacteria) significantly ($p < 0.05$) promoted growth patterns, improved photosynthetic characteristics and increased Cd tolerance of both ecotypes of *S. alfredii*, as compared to normal conditions. Net photosynthetic rate (Pn) in intact leaves of hyperaccumulating ecotype (HE) and non-hyperaccumulating ecotype (NHE) were increased by 73.93 and 32.90%, respectively at the low Cd (2 μM), 84.41 and 57.65%, respectively at the high Cd level (10 μM). Superposition treatment increased Cd concentration in shoots and roots of HE, by 50.87 and 82.12%, respectively at the low Cd and 46.75 and 88.92%, respectively at the high Cd level. Besides, superposition treatment declined Cd transfer factor of NHE, by 0.85% at non-Cd rate, 17.22% at the low Cd and 22.26% at the high Cd level. These results indicate that elevated CO_2 coupled with endophytic bacterial inoculation may effectively improve phytoremediation efficiency of Cd-contaminated soils by hyperaccumulator, and alleviate Cd toxicity to non-hyperaccumulator ecotype of *Sedum alfredii*.

Keywords Cadmium uptake · Elevated CO_2 · Endophyte · Photosynthesis · *Sedum alfredii*

Introduction

Cadmium is a toxic element and represents a series of environmental hazards worldwide (Ehsan et al. 2015). Because of dust from atmospheric sedimentation, domestic waste water irrigation, sewage sludge, and long-term fertilizer application, agricultural soils are affected by Cd contamination (Yadav 2010). Cadmium is non-biodegradable, and excessive Cd in agricultural soils can pose a risk to public health through food chains (Liu et al. 2012).

Sedum alfredii is a member of Crassulaceae family and a herbaceous plant with rapid growth, asexual reproduction, and high biomass yield. There are two ecotypes of *S. alfredii* which are native to China, one is a Cd hyperaccumulator plant species identified in mining area (Yang et al. 2004), and the other is the non-hyperaccumulating ecotype occurring in tea gardens.

Plant growth-promoting endophytes have been reported to enhance plant growth, development, nutrient uptake, and resistance to environmental stresses (Afzal et al. 2014; Rehman et al. 2018). Previous studies reported that inoculation with endophytic bacteria can increase biomass yield and heavy metal uptake and accumulation in *Brassica napus* (Jing et al.

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2014; Pan et al. 2017), *B. juncea* (Belimov et al. 2005), *Salix caprea* (Kuffner et al. 2008), *Prosopis juliflora* (Khan et al. 2015), and *Helianthus annuus* (Kolbas et al. 2015). Long et al. (2011) isolated 14 endophytic bacterial strains from the roots of *S. alfredii* and demonstrated their abilities to promote plant growth and heavy metal accumulation. In a previous study, we have identified bacterial strain *Bacillus megaterium sp*. M002, which has shown high Cd stress-tolerant ability. Inoculation with M002 improved plant growth and Cd accumulation in *S. alfredii* growing in moderately Cd-contaminated soil (Tang et al. 2017).

Since last century, the atmospheric CO_2 concentration has increased to 380 mL L^{-1} due to industrial activities and this concentration is expected to raise in the future (IPCC 2007). An increase in CO_2 concentration has profound influences on photosynthesis and nutrient uptake, thus affecting plant growth and development (Long et al. 2004; Ainsworth and Rogers 2007; Jaffrin et al. 2003). This trend has been reported in various plant species, including *Petridium revolutum* (Zheng et al. 2008), *Trifolium pretense* (Wu et al. 2009), and *Sorghum vulgare* (Luo et al. 2011).

Elevated CO_2 concentration or inoculated endophytic bacteria were reported to affect the growth, Cd accumulation and photosynthesis of hyperaccumulator plants (Jia et al. 2010; Chen et al. 2014a; Li et al. 2015; Ashraf et al. 2018; Hussain et al. 2018). However, the combined effects of both elevated CO_2 concentration and inoculation of endophyte isolates on plant growth and Cd uptake, especially for non-hyperaccumulator plants, were rarely studied. The purpose of the present study was to understand effects of combining elevated CO_2 concentration with inoculated endophytic bacteria on morphological properties, photosynthetic characteristics and Cd uptake of two *S. alfredii* ecotypes, and to examine whether this superposed bio-fortification measure is a feasible way to enhance phytoremediation efficiency of Cd contaminated soils by the hyperaccumulator.

Material and methods

Plant materials

Hyperaccumulating ecotype (HE) of *S. alfredii* was collected from an ancient Pb/Zn mining area in Quzhou city (29° 17′ N, 118° 56′ E), Zhejiang Province, China. In order to minimize internal metal accumulation, the hyperaccumulator plants were grown in non-contaminated soil for several generations prior to this experiment. Non-hyperaccumulating ecotype (NHE) of *S. alfredii* was collected from Jiuxi tea garden in Hangzhou city (30° 15′ N, 120° 09′ E), Zhejiang Province, China. Healthy and uniform-sized seedlings with terminal bud were selected; 5 cm top shoots were cultivated in tap water for 2 weeks to initiate roots in growth chamber maintained under 16:8 photoperiod, day/night temperature regime at 28/22 °C, 65% relative humidity, and light intensity of 175 μM m^{-2} s^{-1}. The culture solution was well aerated and replaced every 3 days (Chen et al. 2014b).

Inoculum preparation

Plant growth-promoting endophytic bacterium, *B. megaterium* sp. M002, used in this study was previously isolated from the stem of *S. alfredii*. This endophytic bacterium M002 has proved to be resistant to heavy metals, producing indole-3-acetic acid (IAA), denitrification, and promoting *S. alfredii* growth. The strain was purified by streaking inoculation, and a single clone was cultured in LB broth medium which contained 5.0 g yeast extract, 10.0 g tryptone, and 5.0 g sodium chloride. The pH of growth medium was adjusted to 7.0 and sterilized at 121 °C for 20 min. After incubation overnight at 30 °C, the inoculum was collected by centrifuging at 6000 rpm for 10 min and washing three times with sterilized distilled water and re-suspended to OD 1.0 for subsequent experiments.

Experimental treatments

After pre-culturing of *S. alfredii* plants for 2 weeks in tap water, roots were immersed in the bacterium suspension for 2 h, and for control plants sterilized distilled water was used (Pan et al. 2017). Healthy and uniform plants were transferred to Hoagland nutrient solution, which contain $Ca(NO_3)_2$ 2.0 mM, KH_2PO_4 0.1 mM, $MgSO_4$ 0.5 mM, KCl 0.1 mM, K_2SO_4 0.7 mM, H_3BO_3 10.0 μM, $MnSO_4$ 0.5 μM, $ZnSO_4$ 1.0 μM, $CuSO_4$ 0.2 μM, $(NH_4)_6MO_7O_{24}$ 0.01 μM, and Fe-EDTA 100 μM, with pH 5.8 (Tao et al. 2016). The nutrient solution was renewed every 3 days.

Each hydroponic pot contained 2.5 L nutrient solution, and seven plants were transplanted in each pot. All hydroponic pots were transferred to growth chambers (Conviron® E7/2, Canada) with day/night temperature of 26/20 °C, humidity of 70/85%, and light intensity of 200 μmol m^{-2} s^{-1} during a 14-h light cycle. Plants were subjected to different CO_2 concentration (ambient CO_2: 350 μL L^{-1}; elevated CO_2: 800 μL L^{-1}) conditions in growth chambers for 14 days. Then different Cd (in the form of $CdCl_2$) concentration (CK: 0 μM Cd; low Cd: 2 μM; high Cd: 10 μM) was applied to corresponding treatments. *S. alfredii* plants were subjected to Cd stress for 3 days in growth chambers. Each treatment consisted of four independent replicates. The detailed experimental design and treatments are listed in Table 1.

Table 1 Detail experimental treatments of hydroponics experiment

Treatments	Ecotype	Cd concentration (μM)	CO_2 concentration (μL L^{-1})	Endophyte inoculated	Abbreviation
1	HE	0	350	No	HE-CK
2	HE	0	800	No	HE-CK + CO_2
3	HE	0	350	Yes	HE-CK + M
4	HE	0	800	Yes	HE-CK + CO_2 + M
5	HE	2	350	No	HE-L
6	HE	2	800	No	HE-L + CO_2
7	HE	2	350	Yes	HE-L + M
8	HE	2	800	Yes	HE-L + CO_2 + M
9	HE	10	350	No	HE-H
10	HE	10	800	No	HE-H + CO_2
11	HE	10	350	Yes	HE-H + M
12	HE	10	800	Yes	HE-H + CO_2 + M
13	NHE	0	350	No	NHE-CK
14	NHE	0	800	No	NHE-CK + CO_2
15	NHE	0	350	Yes	NHE-CK + M
16	NHE	0	800	Yes	NHE-CK + CO_2 + M
17	NHE	2	350	No	NHE-L
18	NHE	2	800	No	NHE-L + CO_2
19	NHE	2	350	Yes	NHE-L + M
20	NHE	2	800	Yes	NHE-L + CO_2 + M
21	NHE	10	350	No	NHE-H
22	NHE	10	800	No	NHE-H + CO_2
23	NHE	10	350	Yes	NHE-H + M
24	NHE	10	800	Yes	NHE-H + CO_2 + M

Sample analysis

Morphological property analysis

After harvest, ten representative plants for all treatments were separately recorded for plant heights, stem diameters, root lengths, as well as shoots and root fresh weights. Shoot samples were rinsed with tap water and carefully washed with deionized water for three times. Root samples were immersed in 20 mM Na_2-EDTA for 15 min to remove metal ions and debris particles on the external root surface and thoroughly rinsed with deionized water for three times. Both shoots and roots were dried in an oven at 105 °C for 30 min, followed by 65 °C until constant weight was attained. Biomass weight of shoots and roots was recorded. Dried plant samples were ground to < 100-mesh powder using stainless mill (Retsch MM301, Germany) for Cd analysis as previously described (Tang et al. 2018).

Gas exchange analysis

Top 4 to 6 leaves were sampled for measuring gas exchange at the end of the experiment. Leaf gas exchange, net photosynthetic rate (Pn), transpiration rate (Tr), intercellular CO_2 concentration (Ci), and stomatal conductance (Gs) were measured using the Li-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA). Water use efficiency (WUE) of leaves was calculated based on Pn/Tr. All measurements were carried out in growth chambers under the same environmental conditions (Li et al. 2015).

Photosynthetic pigments analysis

Leaves were harvested from the petiole region by sharp stainless steel scissor and gas exchange was measured. Fresh leaf samples (0.2 g) were placed into 20 mL acetone and ethyl alcohol mixed solution (2:1) in dark for 24 h, then extracts were tested for the absorbance at the 663 nm and 645 nm to measure chlorophyll a and chlorophyll b concentrations, respectively, (Tang et al. 2016), and at 470 nm to measure carotenoid concentration using ultraviolet spectrophotometer (Lambda 350 V-vis, PerkinElmer, Singapore) (Jia et al. 2010).

Chlorophyll fluorescence analysis

Four representative plants from each treatment were taken and sucked dry to remove residual solution. After 0.5 h dark

adaptation, PSII electron transports, including maximum photochemistry efficiency (Fv/Fm), dark-adapted minimum fluorescence (Fo), effective quantum yield (Φ (II)), photochemical quenching coefficient (qP), quantum yield of non-regulated energy (Y(NO), non-photochemical quenching coefficient (NPQ) and quantum yield of regulated energy (Y(NPQ)) were measured using the IMAGING-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). Color-coded images of fluorescence parameters were scaled on demand using a rainbow lookup table. Leaves of all treatments were measured at the same ambient CO_2 concentration.

Plant Cd analysis

Dry plant samples (0.1 g) were digested in a polytetrafluoroethylene (PTFE) tube with a mixture of HNO_3 (5 mL) and $HClO_4$ (1 mL). The mixture was heated at 170 °C for 4 h, cooled to room temperature. The digested solution was filtered and transferred to a 25-mL volumetric flask and made to the volume with deionized water. The concentrations of Cd in the filtrates were determined using inductively coupled plasma mass spectrometer (ICP-MS, 7500a, Agilent, USA) (Hamid et al. 2019). Standard reference rice flour (SRM 1568a) from National Institute of Standards and Technology (Gaithersburg, MD, USA) was used to check the accuracy of Cd analysis. The standard reference rice flour was digested in the same way as plant samples. The recovery of standard was 90 to 110%.

Statistical analyses

The statistical analyses were performed using the SPSS (SPSS Inc., USA, version 20.0). For all the data, a multiple-way ANOVA was conducted with four factors (ecotype, CO_2 application, endophyte inoculation and Cd stress) as the explanatory variables. Morphological property data were statistically analyzed and mean values were presented as ±standard error (SE) of 10 replicates. Photosynthetic characteristics and Cd uptake data were presented as ±standard error (SE) of 4 replicates. Significant differences in mean values were determined using Duncan's multiple range test ($p < 0.05$). Graphical representation was generated by Origin Pro 8.0.

Results

Effect of elevated CO_2 and inoculated endophyte on morphological properties

Tables 2 and 3 showed that increasing Cd concentration decreased all the morphological properties of NHE, but plant height and stem diameter of HE were not affected regardless of bio-fortification, and root length of HE was even increased. Elevated CO_2 improved morphological properties of HE regardless of Cd concentrations except for the inoculated endophyte treatments, as compared to NHE. Superposed bio-fortification treatments greatly increased the morphological properties, as compared to single bio-fortification, and the increase was greater for high Cd than low Cd level. Six morphological properties were all significantly affected by ecotype, CO_2 application, endophyte inoculation and Cd stress ($p < 0.001$ or $p < 0.01$). However, there was no interaction among the four factors ($p > 0.05$) (Table S1).

Effect of elevated CO_2 and endophyte on photosynthetic characteristics

Photosynthetic pigments in leaves

Fig. 1a, c showed that the photosynthetic pigments in leaves of NHE were significantly higher, as compared to HE regardless of bio-fortification treatments and Cd level. Bio-fortification decreased chlorophyll a and b contents in NHE leaves (Fig. 1a and b), but increased carotenoids contents regardless of Cd level (Fig. 1c), whereas chlorophyll a/b ratio was not affected by bio-fortification (Fig. 1d). Increasing Cd concentration proportionally increased carotenoids contents in HE leaves and chlorophyll a and b contents in NHE leaves. A decreased in carotenoids contents occurred in NHE, irrespective of bio-fortification treatment. Multiple-way ANOVA showed that chlorophyll a, b and carotenoids were significantly affected by the four factors ($p < 0.001$ or $p < 0.01$). Chlorophyll a/b was only significantly affected by ecotype and Cd stress ($p < 0.001$) (Table S2).

Gas exchange

Intact leaf Pn was significantly different and had highly significant interaction among the ecotype, CO_2 application, endophyte inoculation, and Cd stress ($P < 0.001$) (Table S3). Bio-fortification significantly enhanced Pn of intact leaves in two ecotypes of *S. alfredii* ($p < 0.05$), irrespective of Cd concentrations. Superposition treatments resulted in the highest Pn in both HE and NHE, as compared to non-bio-fortification treatment except for CO_2 treatment in HE under the low Cd level. Intact leaf Pn of HE was increased by 154.3, 17.92, and 208.5%, respectively for elevated CO_2, inoculating strain M002 (M), and superposition (CO_2 + M) treatment, respectively without Cd stress, by 113.20, 61.39, and 73.93%, respectively under low Cd level, and by 43.05, 22.71, and 84.41%, respectively under high Cd level. For NHE, the corresponding values obtained by the above treatments were 78.47 (CO_2), 30.56 (M), and 97.92% (CO_2 + M) without Cd stress; 5.16 (CO_2), 25.16 (M), and 32.90% (CO_2 + M) under low Cd level; and 8.24 (CO_2), 43.53 (M), and 57.65% (CO_2 + M) under high Cd level (Fig. 2a).

Table 2 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on plant height, stem diameter, and root length of two *S. alfredii* ecotypes under different Cd concentrations in hydroponics. (SA: *Sedum alfredii*, M: endophytic bacterium, HE: hyperaccumulating ecotype, NHE: non-hyperaccumulating ecotype, L: Cd^{2+} = 2 μM, H: Cd^{2+} = 10 μM. Data are means ± SE (n = 10). Different letters within the same group of bars indicate significant difference at $p < 0.05$ based on Duncan's multiple range test

Treatment		Plant height (cm)		Stem diameter (cm)		Root length (cm)	
		HE	NHE	HE	NHE	HE	NHE
CK	SA	13.69 ± 1.44ab	10.46 ± 0.36bcd	0.28 ± 0.05a	0.18 ± 0.01abc	9.59 ± 1.35e	9.33 ± 0.72bcde
	SA + CO_2	14.90 ± 0.54ab	12.31 ± 0.40ab	0.34 ± 0.02a	0.20 ± 0.02ab	13.38 ± 2.38 cd	10.29 ± 1.25abcd
	SA + M	13.74 ± 0.54ab	12.35 ± 0.10ab	0.32 ± 0.02a	0.21 ± 0.02a	12.89 ± 2.27 cd	11.87 ± 1.20ab
	SA + CO_2 + M	16.84 ± 1.57a	13.66 ± 0.95a	0.35 ± 0.01a	0.22 ± 0.03a	15.28 ± 2.45bc	14.67 ± 1.88a
L	SA	13.80 ± 1.48ab	10.26 ± 0.54bcd	0.31 ± 0.03a	0.17 ± 0.02abc	11.04 ± 0.99de	9.19 ± 1.33bcde
	SA + CO_2	13.96 ± 1.02ab	10.87 ± 0.92bc	0.33 ± 0.01a	0.19 ± 0.02ab	14.09 ± 1.54 cd	7.89 ± 1.22bcde
	SA + M	13.95 ± 0.21ab	10.87 ± 0.74bc	0.33 ± 0.03a	0.19 ± 0.01ab	14.57 ± 2.34c	11.78 ± 1.88ab
	SA + CO_2 + M	16.76 ± 0.44a	11.19 ± 1.10abc	0.35 ± 0.02a	0.21 ± 0.03a	17.88 ± 1.56ab	14.67 ± 1.88abc
H	SA	12.31 ± 0.36b	8.00 ± 0.94d	0.30 ± 0.03a	0.12 ± 0.02c	12.18 ± 2.45cde	5.21 ± 0.98e
	SA + CO_2	12.72 ± 1.17b	8.50 ± 1.36 cd	0.33 ± 0.03a	0.14 ± 0.01bc	13.61 ± 1.33 cd	6.16 ± 0.97de
	SA + M	12.72 ± 0.41b	9.23 ± 0.36 cd	0.32 ± 0.01a	0.16 ± 0.01abc	14.72 ± 1.54c	6.99 ± 1.54 cde
	SA + CO_2 + M	14.20 ± 0.63ab	9.56 ± 0.84bcd	0.34 ± 0.01a	0.17 ± 0.02abc	18.79 ± 1.54a	8.44 ± 1.55bcde

Intact leaf Tr of NHE significantly decreased by biofortification for the majority of treatments regardless Cd levels (except M treatment under CK). Inoculating strain M002 increased Tr of intact leaves in HE, but these values decreased under CO_2 and CO_2 + M treatments. All Tr in NHE under Cd stressed were significantly greater, as compared to HE under the same Cd level (Fig. 2b); this phenomenon may be due to reduced photosynthesis by Cd stress and promoted transpiration rate of NHE. All of ecotype, CO_2 application, endophyte inoculation and Cd stress had a significant effect on Tr ($p < 0.001$ or $p < 0.01$). However, no significant interaction was observed when the four factors were applied together (Table S3).

All of Ci, Gs, and WUE were significantly affected by ecotype, CO_2 application, endophyte inoculation, and Cd stress. Moreover, there were significant interactions between the four factors and the three gas exchange traits ($p < 0.001$) (Table S3). Elevated CO_2 and superposition treatment increased Ci of intact leaves of both HE and NHE, but they were decreased when strain M002 was inoculated. Increasing Cd concentrations in nutrient solution caused a steady decrease in Ci of NHE; however, Ci of HE with the same treatments became greater when Cd was spiked (Fig. 3a).

For HE, Gs of intact leaves was significantly decreased when plants were grown under elevated CO_2 (CO_2 and

Table 3 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on shoot fresh weight, shoot dry weight, and root fresh weight of two *S. alfredii* ecotypes under different Cd concentrations in hydroponics

Treatment		Shoot FW (g $plant^{-1}$)		Shoot DW (g $plant^{-1}$)		Root FW (g $plant^{-1}$)	
		HE	NHE	HE	NHE	HE	NHE
CK	SA	6.43 ± 0.22abcd	4.22 ± 0.11bcd	0.51 ± 0.02bc	0.36 ± 0.01bcd	1.89 ± 0.11f	1.07 ± 0.10bcd
	SA + CO_2	6.79 ± 0.15abc	4.40 ± 0.24bc	0.55 ± 0.02abc	0.37 ± 0.03bcd	2.44 ± 0.20cdef	1.30 ± 0.12ab
	SA + M	6.35 ± 0.17bcd	4.97 ± 0.24ab	0.52 ± 0.01bc	0.41 ± 0.02ab	2.34 ± 0.22def	1.40 ± 0.20abc
	SA + CO_2 + M	6.86 ± 0.09ab	5.55 ± 0.30a	0.57 ± 0.01ab	0.46 ± 0.04a	2.78 ± 0.22bcde	1.88 ± 0.13a
L	SA	6.57 ± 0.24abcd	3.24 ± 0.22ef	0.54 ± 0.02abc	0.28 ± 0.02de	2.21 ± 0.14ef	1.13 ± 0.10bcd
	SA + CO_2	6.98 ± 0.17ab	3.46 ± 0.23def	0.57 ± 0.02ab	0.30 ± 0.03 cd	2.88 ± 0.19bcde	1.22 ± 0.11bc
	SA + M	6.63 ± 0.11abcd	4.39 ± 0.35bc	0.55 ± 0.01abc	0.35 ± 0.03bcd	2.33 ± 0.31def	1.72 ± 0.26ab
	SA + CO_2 + M	7.10 ± 0.26a	4.46 ± 0.22bc	0.60 ± 0.03a	0.38 ± 0.03abc	3.33 ± 0.20ab	1.79 ± 0.14ab
H	SA	5.97 ± 0.19d	2.23 ± 0.39 g	0.48 ± 0.02c	0.21 ± 0.03e	2.65 ± 0.20bcdef	0.69 ± 0.11e
	SA + CO_2	6.16 ± 0.22 cd	2.66 ± 0.35 fg	0.50 ± 0.03bc	0.22 ± 0.05e	3.15 ± 0.13bc	0.79 ± 0.11de
	SA + M	6.13 ± 0.30 cd	3.73 ± 0.16cde	0.50 ± 0.02bc	0.31 ± 0.01 cd	3.05 ± 0.42bcd	1.11 ± 0.12bcd
	SA + CO_2 + M	6.33 ± 0.18bcd	3.94 ± 0.14cde	0.52 ± 0.02bc	0.33 ± 0.02bcd	3.89 ± 0.17a	1.14 ± 0.17ab

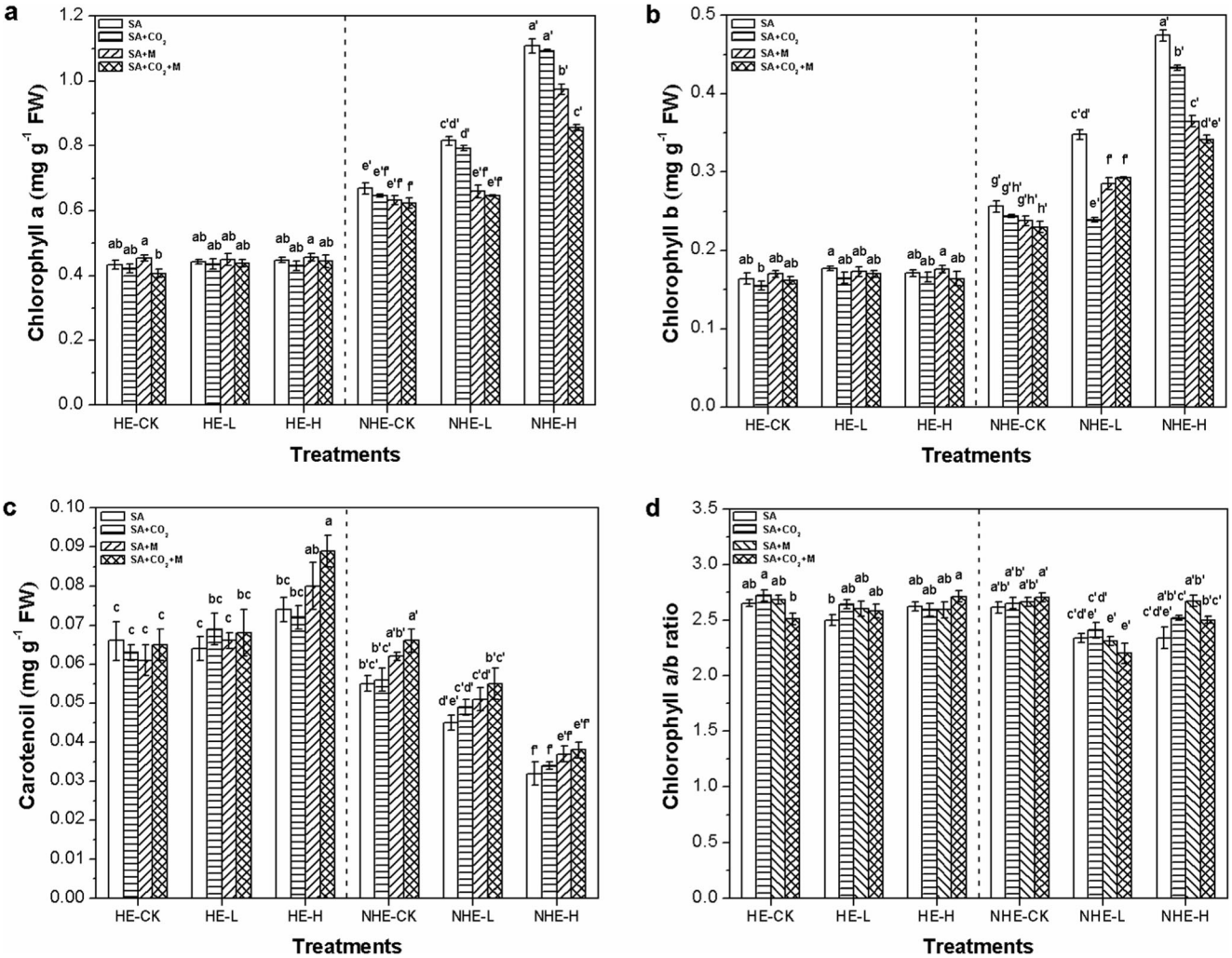

Fig. 1 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on photosynthetic pigment concentrations in two *S. alfredii* ecotypes under different Cd concentration treatments in hydroponics. SA: *Sedum alfredii*, M: endophytic bacterium, HE: hyperaccumulating ecotype, NHE: non-hyperaccumulating ecotype, L: $Cd^{2+} = 2$ μM, H: $Cd^{2+} = 10$ μM. Data are means ± SE ($n = 4$). Different letters within the same group of bars indicate significant difference at $p < 0.05$ based on Duncan's multiple range test. The same below

CO_2 + M) ($p < 0.05$), but was significantly increased by inoculation with stain M002 ($p < 0.05$), as compared to CK, which is contrast with Ci. For NHE without Cd stress, effects of biofortification on Gs were the same with HE; however, these

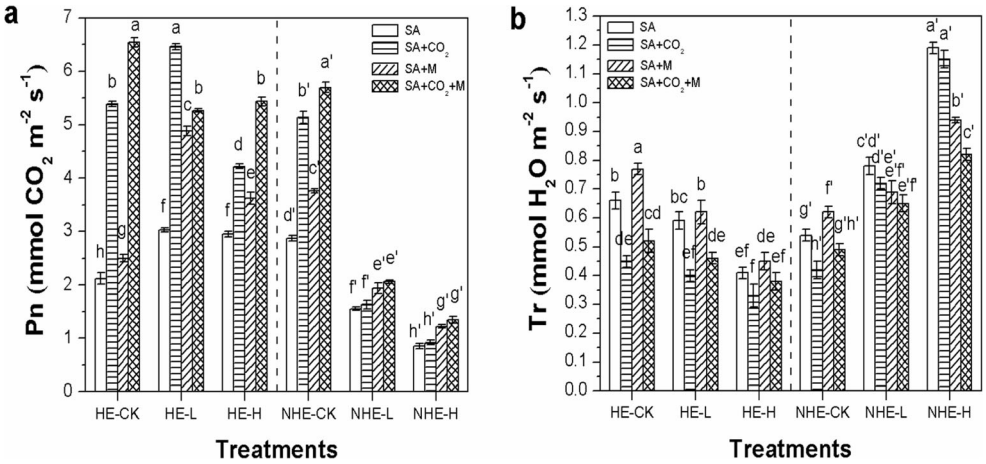

Fig. 2 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on net photosynthetic rate (Pn) and transpiration rate (Tr) in intact leaves of two *S. alfredii* ecotypes under different Cd concentration treatments in hydroponics

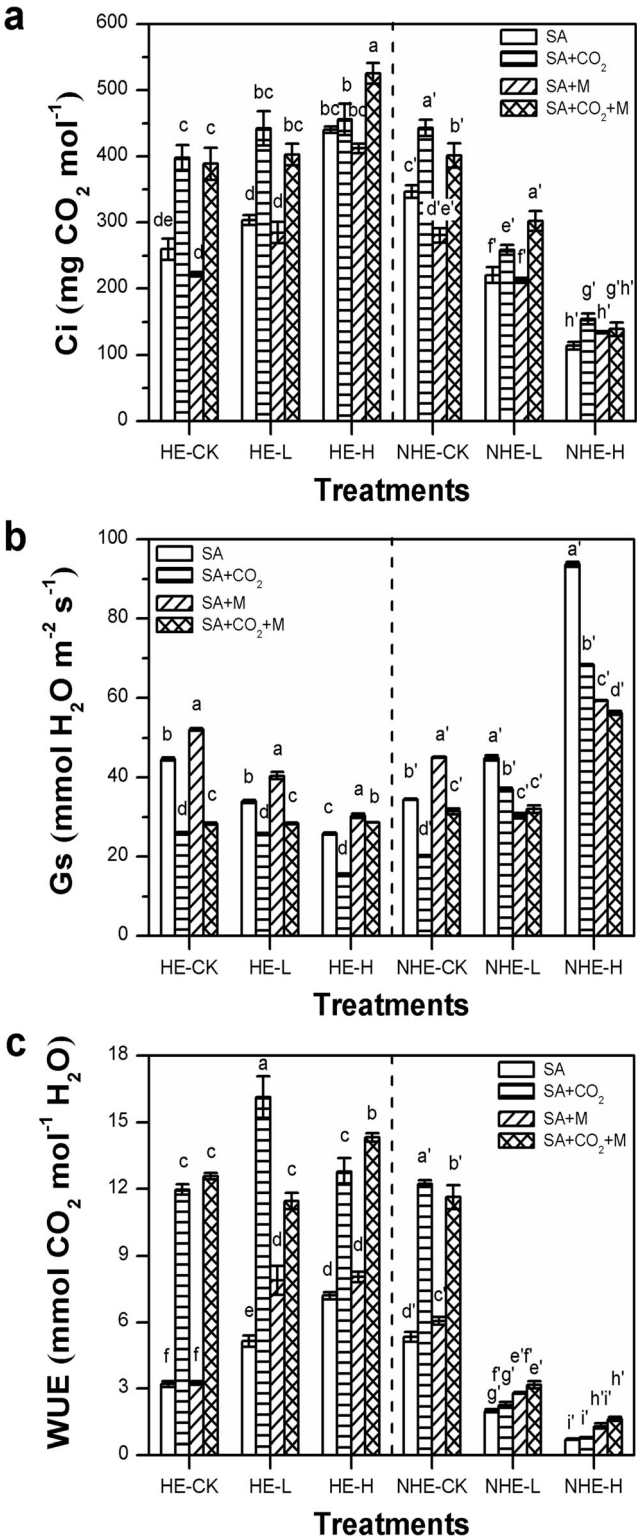

Fig. 3 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on intercellular CO_2 concentration (Ci), stomatal conductance (Gs), and water use efficiency (WUE) in intact leaves of two *S. alfredii* ecotypes under different Cd concentration treatments in hydroponics

values rapidly declined when NHE plants were grown under light or severe Cd stress (Fig. 3b), which may be attributed to inefficiency to regain normal physiological functions in the leaves of NHE under Cd stress.

WUE of two ecotypes of *S. alfredii* for all Cd concentrations were increased by bio-fortification processes. Elevated CO_2 (CO_2 and CO_2 + M) triggered a greater WUE increase for HE regardless of Cd level and NHE grown without Cd stress. We observed a steady decline in WUE in NHE with increasing Cd concentration in nutrient solution, and the WUE under the same bio-fortification processes was significantly lower without Cd treatment (Fig. 3c). This result indicates that water use efficiency of NHE was affected by Cd stress.

Chlorophyll fluorescence

After grown in medium supplemented with Cd, Fo, Y(NO), NPQ, and Y(NPQ) of both ecotypes *S. alfredii* increased, but Φ(II) and qP decreased. However, elevated Cd concentration of nutrient solutions increased Fv/Fm of HE, but not NHE. Bio-fortification significantly affected the chlorophyll fluorescence parameters of both ecotypes of *S. alfredii*, but there was no consistent pattern (Fig. 4). Ecotype or Cd stress had significant effects on all chlorophyll fluorescence traits ($p < 0.001$), and CO_2 application had significant effects on all chlorophyll fluorescence traits except for Fo. Meanwhile, endophyte inoculation had significant effects on all chlorophyll fluorescence traits ($p < 0.001$) except for Fo and Y(NPQ) (Table S4).

Effect of elevated CO_2 and inoculated endophyte on Cd uptake

Regardless of plant ecotypes and bio-fortification treatments, Cd concentration in shoots and roots of both *S. alfredii* ecotypes increased with increasing Cd concentrations in nutrient medium. After growth in hydroponic solution with varying Cd concentration, Cd accumulation in shoots of HE was much higher, as compared to roots, irrespective of bio-fortification treatments (Fig. 5a), whereas in NHE Cd concentration was higher in roots than shoots (Fig. 5b). Ecotype, CO_2 application, endophyte inoculation and Cd stress had significant effects on Cd concentration of both shoot and root ($p < 0.001$), but there was no significant interaction when the four factors were applied together (Table S5).

Compared to the controlled conditions, HE with bio-fortification treatment had increased Cd concentrations in both shoots and roots. The increases in Cd concentrations were 5.79, 3.97, and 8.93%, respectively for CO_2, M, and CO_2 + M treatments in shoots; and 4.15, 9.36, and 9.61% in roots of HE without Cd; and the corresponding values were 26.26, 22.38, and 50.87% in shoots; and 31.21, 42.70, and 82.12% in roots at the low Cd; and 26.79, 16.79, and 46.75% in shoots; and 38.14, 36.85, and 88.92% in roots at the high Cd level (Fig. 5a).

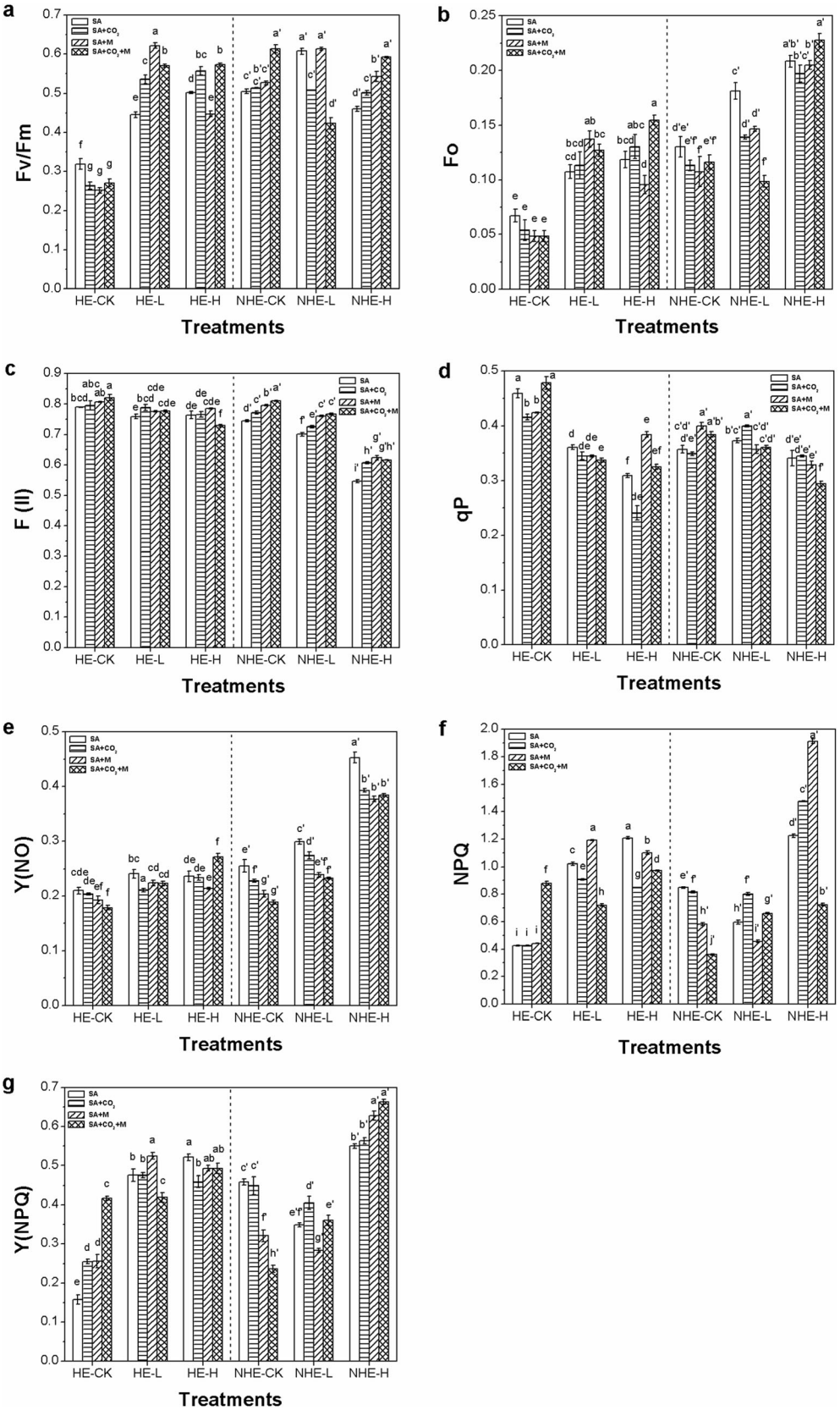

Fig. 4 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on chlorophyll fluorescence parameters in intact leaves of two *S. alfredii* ecotypes under different Cd concentration treatments in hydroponics

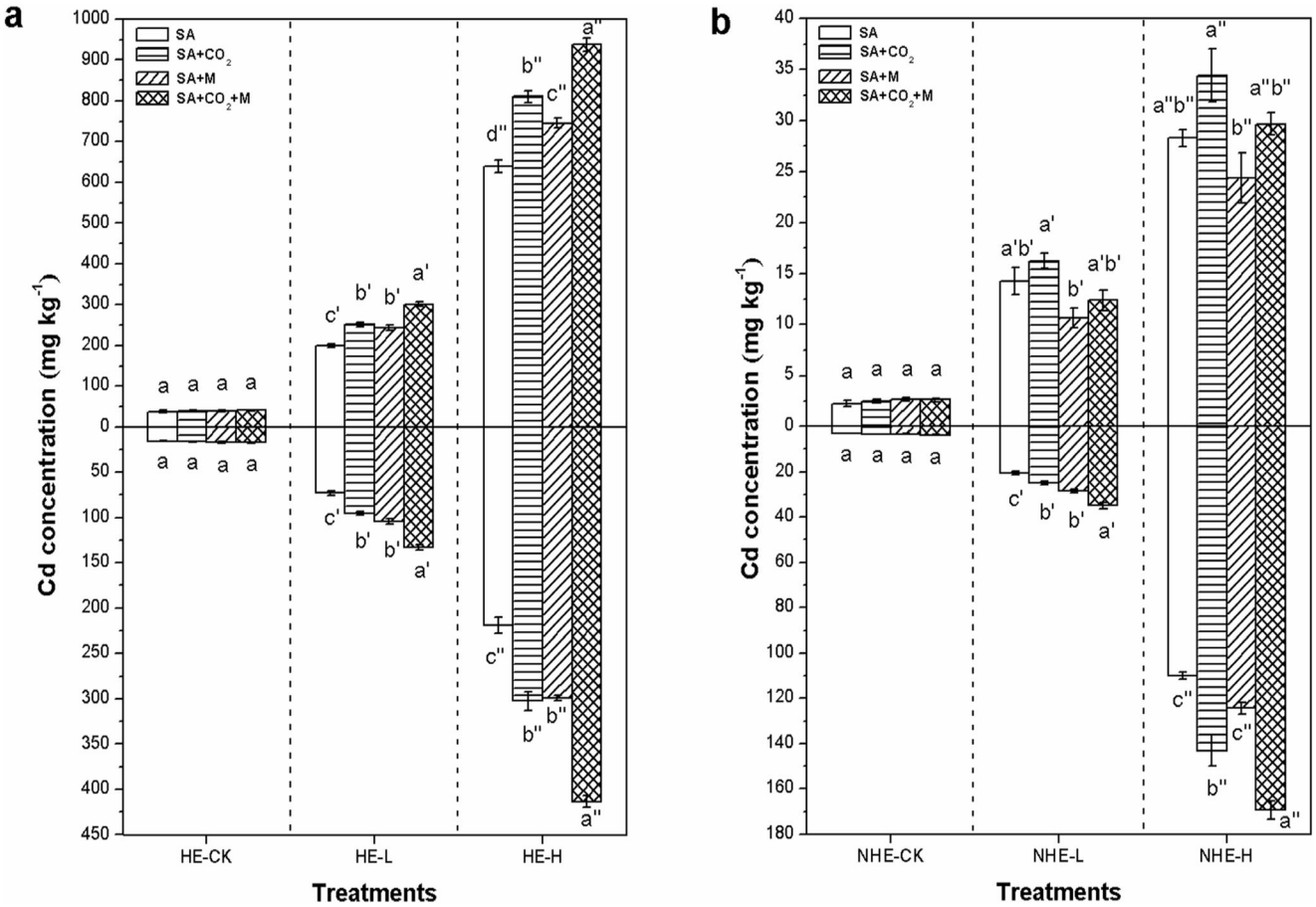

Fig. 5 Effect of elevated CO_2 and inoculated endophytic bacterium treatments on Cd concentration and distribution in roots and shoots of two *S. alfredii* ecotypes under different Cd concentration treatments in hydroponics

When NHE grown in no-Cd medium, the increase in shoots Cd concentrations was 10.23, 18.02 and 16.36% for CO_2, M, and CO_2 + M treatments, respectively, and the corresponding values were 2.99, 5.52, and 15.95% for roots, respectively. However, this phenomenon did not occur to NHE grown in medium with Cd stress. As shown in Fig. 5b, inoculated stain M002 (M and CO_2 + M) reduced Cd concentrations in shoots of NHE, and the decrease in Cd concentration might result from the dilution effect, as plant growth was much improved by inoculating stain M002. This is in agreement with previously reported results that inoculating stain M002 increased biomass of NHE regardless of Cd concentrations. All the bio-fortification processes promoted Cd uptake by roots of NHE, as compared to control.

Discussion

Application of CO_2 and/or inoculation with endophyte showed a significant and positive effect on morphological properties of both ecotypes of *S. alfredii* under Cd stress (Tables 2 and 3). These results were consistent with previous reports of other HE or NHE grown in heavy metal-contaminated soils (Jing et al. 2014; Guo et al. 2015; Song et al. 2015). In this study, increasing Cd concentration in nutrient solutions decreased all morphological properties of NHE. However, Cd stress did not reduce and even increased some agronomic traits of HE, which is a recognized characteristic of hyperaccumulator plants.

Hyperaccumulating ecotype grown under different bio-fortification treatments and Cd concentrations showed less statistically detectable variation in photosynthetic pigments and their ratio; it may be attributed to the hyperaccumulation characteristic of HE. The Cd concentration in nutrient solution of this experiment is not toxic to the hyperaccumulator, so the advantages of bio-fortification were not effectively conveyed in photosynthetic characteristics of HE.

Chlorophyll a and b concentrations in NHE leaves were lower when applied with CO_2 and/or inoculated with endophyte treatments with or without Cd treatment. The reduction may be attributed to a dilution effect of increased fresh weight as stimulated by bio-fortification; these findings were consistent with those reported by Croonenborghs et al. (2009) and Jia et al. (2010). Singh et al. (2017) reported that elevated CO_2 reduced chlorophyll a and b but increased carotenoids concentration in soybean, which agrees with the present study. Zare-

Maivan et al. (2017) obtained similar results on maize with fungal inoculation.

Zhou and Qiu (2005) found an increase in chlorophyll a and b concentration in leaves of *S. alfredii* under Cd treatments. This phenomenon was also reported by Li et al. (2015), which is similar to our present study on NHE. Some researchers suggested that this result may be due to Cd acting as a promoter to produce more chlorophyll in leaves. However, in our present study, this phenomenon was more likely attributed to the wilting of NHE plants caused by Cd stress, because there was no or little increment in chlorophyll a and b concentration in HE leaves with Cd treatments.

Bio-fortification significantly increased Pn of both ecotypes *S. alfredii* under all the Cd concentrations. Elevated CO_2 processes (CO_2 and CO_2 + M) had greater ability to increase Pn than M treatment. Dilution effect resulted from fresh weight increment was caused by bio-fortification, which decreased photosynthetic pigment concentration but increased Pn of intact leaves. This suggests that lower chlorophyll concentrations can inhibit photosynthesis of both ecotypes of *S. alfredii* since photosynthetic and growth promotion of *S. alfredii* by elevated CO_2 under Cd stress had little influence on chlorophyll concentrations, which is similar to most CAM plants (Drennan and Nobel 2000). The increment of Pn by inoculated endophyte may be attributed to the improvement of plant growth and development. The positive effect of bio-fortification on Pn of both ecotypes of *S. alfredii* was comparatively more crucial than effect of Cd stress, as the former facilitates plants to resist Cd stress.

In addition, elevated CO_2 (CO_2 and CO_2 + M) significantly increased Ci and WUE of HE regardless of Cd concentration and NHE without Cd spiking. Elevated CO_2 generally increases leaf Ci level and WUE (Long et al. 2004; Donohue et al. 2017). Higher Ci under elevated CO_2 was sufficient to offset a decrease in leaf photosynthesis resulting from the lower Gs (Bernacchi et al. 2005), which provides a partial explanation for the Pn stimulation under elevated CO_2 condition.

In the present study, inoculating stain M002 reduced Ci and WUE of HE at all the Cd concentrations compared to NHE without Cd stress only. Monne et al. (2005) inoculated *Neotyphodium lolii* to *L. perenne* grown in Zn excess soil and nutrient solution, and the results showed that Ci and WUE of *L. perenne* decreased after inoculation. Xia et al. (2016) inoculated *Blumeria graminis* to *Achnatherum inebrians* and obtained similar results. However, little information is available concerning the mechanisms of improved Ci and WUE involved in endophyte inoculation.

When plants exposed to elevated CO_2 (CO_2 and CO_2 + M), contrary to the increase in Pn, Ci and WUE, Tr and Gs of both ecotypes of *S. alfredii* decreased regardless of Cd stress. Intact leaf Tr is a major driving force of nutrient mass flow in the xylem, with the effective transport, the nutrients were translocated from roots to the leaves and fruits via stem (Houshmandfar et al. 2015), which is further influenced by Gs. Gs of plants decreased when elevated CO_2 is an effective way to suppress canopy transpiration (Cao et al. 2009). Barton et al. (2012) reported elevating CO_2-reduced Tr and Gs of *Eucalyptus saligna*, which was in agreement with the present results. The decrease of Tr and Gs may be attributed to stomatal closure, as a result, the sensitivity of guard cells to environmental factors does not appear to acclimate with growth at elevated CO_2 (Ainsworth and Rogers 2007).

On the contrary, inoculation with strain M002 increased Tr and Gs of HE under all the Cd conditions and NHE without Cd stress. However, bio-fortification increased Pn, Ci, and WUE but reduced Gs and Tr of NHE grown under low or high Cd stress. In NHE plants, photosynthesis is the most sensitive process negatively affected by heavy metals (Pietrini et al. 2003; Solti et al. 2008). The change of these indexes might attribute to destruction of normal physiological functions of NHE under Cd stress. The negative effects can be controlled by bio-fortification to keep normal photosynthetic activity.

PSII has frequently been identified as the main target of heavy metal stresses (Küpper et al. 2007). Chlorophyll fluorescence analysis has become one of the most powerful techniques used to estimate the photochemical activities of PSII in leaves and the operating quantum efficiency of electron transport through PSII (Baker and Rosenqvist 2004), which can provide insight into intra-cellular photosynthetic responses to abiotic stress (Redondo-Gómez et al. 2010).

Changes in fluorescence occur as a result of variation in the redox state of the reaction center complex of PS II (Haldimann and Strasser 1999), which provides a partial explanation for the irregular change of chlorophyll fluorescence parameters under different bio-fortification and Cd stress. A survey of published literature revealed that little information is available concerning the effect of heavy metals and superposition treatment on chlorophyll fluorescence parameters; therefore, this is a meaningful topic deserving further study.

Our results from the present study showed that bio-fortification treatments caused an increment in Cd concentration in shoots and roots of both ecotypes of *S. alfredii* especially under severe Cd stress, which is in agreement with the report of Li et al. (2013), showing the possibility of using bio-fortification to promote Cd uptake by *S. alfredii* in phytoremediation practices. Previous studies have reported that elevated CO_2 and/or inoculated endophyte under Cd stress conditions enhanced root growth and development; this may be another reason of *S. alfredii* to absorb more Cd under bio-fortification (Li et al. 2012). Concentration of Cd in shoots and roots of both ecotypes under CO_2 treatments was greater than M treatments, but the reason for this phenomenon was entirely different. For HE, elevating CO_2 has stronger ability to increase biomass than inoculated stain M002. However, for

NHE, plants gained greater biomass with M than CO_2 treatments, the lower Cd concentration in plants of NHE might result from dilution effect, due to rapid plant growth.

Conclusions

The results from the present study showed that biofortification (elevated CO_2 and/or inoculated endophytic bacterial) promoted morphological properties, improved photosynthetic characteristics and increased Cd tolerance ability of both ecotypes of *S. alfredii*, as compared to control. The knowledge gained in this investigation constitutes an important advancement in our understanding of the interactions between CO_2 concentration, endophyte inoculation, and heavy metal contamination, in terms of growth and development of hyperaccumulators and non-hyperaccumulators. However, more elaborated investigation is mandatory to understand the involved mechanisms, and the potential applications of the findings to phytoremediation practices.

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