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Effects of elevated CO_2 levels on root morphological traits and Cd uptakes of two *Lolium* species under Cd stress*

Yan JIA[1,2,3], Shi-rong TANG[†‡1,2], Xue-hai JU[1,2], Li-na SHU[1,2,3],
Shu-xing TU[3], Ren-wei FENG[1,2], Lorenzino GIUSTI[4]

([1]*Centre for Research in Ecotoxicology and Environmental Remediation, Agro-environmental Protection Institute, Ministry of Agriculture, Tianjin 300191, China*)
([2]*Open Key Laboratory of Agro-environment and Food Safety of Ministry of Agriculture, Tianjin 300191, China*)
([3]*College of Resources and Environment, Huazhong Agricultural University, Wuhan 430070, China*)
([4]*Faculty of Health and Life Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK*)
[†]E-mail: tangshir@hotmail.com

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Abstract: This study was conducted to investigate the combined effects of elevated CO_2 levels and cadmium (Cd) on the root morphological traits and Cd accumulation in *Lolium multiflorum* Lam. and *Lolium perenne* L. exposed to two CO_2 levels (360 and 1000 μl/L) and three Cd levels (0, 4, and 16 mg/L) under hydroponic conditions. The results show that elevated levels of CO_2 increased shoot biomass more, compared to root biomass, but decreased Cd concentrations in all plant tissues. Cd exposure caused toxicity to both *Lolium* species, as shown by the restrictions of the root morphological parameters including root length, surface area, volume, and tip numbers. These parameters were significantly higher under elevated levels of CO_2 than under ambient CO_2, especially for the number of fine roots. The increases in magnitudes of those parameters triggered by elevated levels of CO_2 under Cd stress were more than those under non-Cd stress, suggesting an ameliorated Cd stress under elevated levels of CO_2. The total Cd uptake per pot, calculated on the basis of biomass, was significantly greater under elevated levels of CO_2 than under ambient CO_2. Ameliorated Cd toxicity, decreased Cd concentration, and altered root morphological traits in both *Lolium* species under elevated levels of CO_2 may have implications in food safety and phytoremediation.

Key words: Elevated CO_2 levels, *Lolium multiflorum* Lam., *Lolium perenne* L., Root morphology, Cd uptake, Cd stress
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1 Introduction

The world's industrialization has given rise to increases in the atmospheric carbon dioxide (CO_2) concentrations (from 280 to 380 μl/L) (IPCC, 2007) and environmental pollution. Elevated levels of CO_2 and increased heavy metal concentrations in the agricultural environment potentially affect both plant growth and development, and pose possible hazards to human health through food chain. Consequently, the impacts of elevated levels of CO_2 and metal contamination on plants are receiving more attentions (Tang, 2006). It is now known that under non-contaminated conditions, elevated levels of CO_2 increase photosynthesis, leading to the increased photosynthetic product allocation to roots. This resulted in more highly branched roots and an increase in the capacity of the root system to exploit soil volume through alteration of root morphological traits (Rogers *et al*., 1992; Wechsung *et al*., 1999; Prior

‡ Corresponding author
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et al., 2003; Lee-Ho *et al*., 2007). The changes in root morphology are often associated with a variation in nutrient uptake (Jia and Gray, 2007; Jin *et al*., 2009; Jin and Evans, 2010), though considerable variation between species and systems exists (Bowes, 1993; Kimball *et al*., 2002; Franzaring *et al*., 2008). Previous studies have shown the effect of elevated levels of CO_2 on plant uptakes of essential micronutrients, such as Cu, Fe, Mn, and Zn (Jia *et al*., 2007; Yang *et al*., 2007; Zheng *et al*., 2008; Högy and Fangmeier, 2009; Jin *et al*., 2009; Li *et al*., 2009), but little is known about the effect of elevated levels of CO_2 on the uptakes of non-essential elements such as Cd (Li *et al*., 2010). Few studies have addressed heavy metal uptakes by plants under elevated levels of CO_2 in terms of alterations in root morphological traits.

Being in direct contact with the contaminated soil or soil solution, roots may be more easily affected by changes in environmental factors, such as high cadmium (Cd) concentrations (Benavides *et al*., 2005); under elevated levels of CO_2, the root shows a greater size increase than leaves, stems, and reproductive structures, even though leaves are the main site of CO_2 exposure and uptake (Kimball *et al*., 2002; Wang and Taub, 2010). This process usually affects the efficiency of acquisition of resources (Day *et al*., 1996). Cd is a non-essential element that negatively affects plant growth and development processes, such as respiration and photosynthesis (Greger and Ögren, 1991; Baryla *et al*., 2001; Vega *et al*., 2006), water and mineral uptakes (Singh and Tewari, 2003), cell division (Fojtová *et al*., 2002), and cellular redox homoeostasis (Romero-Puertas *et al*., 2004). From the viewpoint of water and nutrient uptakes, roots are of particular physiological importance (Bosac *et al*., 1995; Jia *et al*., 2008). Fine roots are usually more sensitive to exposure to either excessive metal concentrations (Arduini *et al*., 1995) or elevated levels of CO_2 (Day *et al*., 1996; Janssens *et al*., 1998; Phillips *et al*., 2006) when compared to coarse roots. Changes in root morphology may therefore serve as an important indicator of environmental changes (Nishizono *et al*., 1987; Ostonen *et al*., 2007). The changes in root distribution patterns may influence nutrient dynamics, thus influencing crop uptake of metals when plants are grown in contaminated soil. Although plant growth responses to elevated levels of CO_2, when grown in metal-contaminated soil, are known (Tang *et al*., 2003; Zheng *et al*., 2008; Wu *et al*., 2009; Li *et al*., 2010), the relationship between uptakes of metals by plants exposed to elevated levels of CO_2 and metal stress and alteration of root distribution patterns remains poorly understood.

As model plants, *Lolium multiflorum* and *Lolium perenne* species have been frequently studied because of their abilities to survive in metal-contaminated soil and to accumulate metals (Marseille *et al*., 2000; Kiss *et al*., 2002; Palazzo *et al*., 2003; Arienzo *et al*., 2004; Caggiano *et al*., 2005; Guo and Wang, 2009). They contain extensive root systems with high biomass, have high adaptability and low-cost management, and possess the ability to accumulate Cd (Sabreen and Sugiyama, 2008). Understanding the combined effects of elevated levels of CO_2 and metal contamination on their biomass productions, root morphological traits, and metal accumulations will improve both our knowledge of food safety and their survival abilities in metal contaminated environments. It also allows an interspecies comparison of the behaviors of *L. multiflorum* vs. *L. perenne* in metal contaminated environments under elevated levels of CO_2. Elevated levels of CO_2 improve photosynthesis of C_3 plants, reduce stomatal resistance, and as a result, increase water-use efficiency, while aiding in the decrease of photorespiration and oxidative stress (Urban, 2003; Kirschbaum, 2004; Rogers *et al*., 2004). Researches have shown that elevated levels of CO_2 increase the ability of plants to combat abiotic stress, such as O_3, drought, and salt (Sgherri *et al*., 1998; Donnelly *et al*., 2001; Oksanen *et al*., 2001; Geissler *et al*., 2009). We hypothesize that better growth and physiological responses to elevated levels of CO_2 will help plants combat the stress induced by Cd. The objective of this study was to investigate effects of elevated levels of CO_2 on plant growth, root development, and Cd uptakes of *L. multiflorum* and *L. perenne* under Cd stress, and implications for food safety and phytoremediation efficiency.

2 Materials and methods

2.1 Plant materials and growth

Seeds of *L. perenne* L. and *L. multiflorum* Lam. (obtained from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China)

were surface-sterilized by exposure to 0.01 g/ml NaOCl for 10 min, and subsequently washed several times with deionized water, and germinated in a moist mixture of perlite and vermiculite (1:1, v/v) in a controlled growth chamber at a constant temperature (25 °C). After 10 d, healthy and uniformly-sized seedlings were selected for the hydroponic experiment.

Forty-eight pots of 9.2 cm inner diameter and 18 cm height were wrapped with tinfoil, each containing 1 L of 0.50 strength Hoagland nutrient solution and one seedling. Hoagland nutrient solution (pH 6.5) consisted of 4 mmol/L $Ca(NO_3)_2{\cdot}4H_2O$, 6 mmol/L KNO_3, 2 mmol/L $MgSO_4{\cdot}7H_2O$, 1 mmol/L $NH_4H_2PO_4$, 15 μmol/L H_3BO_3, 1 μmol/L $MnSO_4{\cdot}4H_2O$, 0.5 μmol/L $ZnSO_4{\cdot}7H_2O$, 0.2 μmol/L $CuSO_4{\cdot}5H_2O$, 0.01 μmol/L $(NH_4)_6Mo_7O_{24}$, and 100 μmol/L Fe(II)-EDTA. Three days after growth in 0.50 strength nutrient solution, all pots were filled with Hoagland nutrient solution and arranged randomly into two sets. Each set, consisting of 24 pots for both species, was spiked with Cd ($CdCl_2{\cdot}5H_2O$) at 0, 4, and 16 mg/L, with each treatment having four replicates. The nutrient solution was continuously aerated with an aquarium pump and was replaced every two days. The two sets of pots were grown in growth chambers under identical conditions varying only CO_2 level. They were grown under the following conditions: day/night time of 16/8 h, temperature of (25.0±0.5) °C, light intensity of (105±0.8) μmol/(m^2·s), and a humidity of (60±1)%. Elevated levels of CO_2 were supplied by a compressed CO_2 cylinder (CO_2 purity of 99.9%) from Tianjin Saint-Nan Gases Supply Co., Ltd., China. The CO_2 concentration was monitored with an infrared gas analyzer equipped with an automatic switching solenoid used to maintain the constant CO_2 concentration of (1000±80) μl/L. The other control growth chamber was ventilated with an ambient CO_2 concentration of (360±12) μl/L.

2.2 Harvest, root scanning, and chemical analyses

The plants were subjected to three weeks of the CO_2 treatment, and then harvested and separated into shoots and roots. Root scanning was carried out immediately using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada). Root length, surface area, volume, average diameter, number of tips, and root length distribution in different size-categories were measured and recorded through a root image analysis system using image analysis software WinRHIZO. The root average diameter was expressed as the total root width divided by the length of roots.

After scanning, the fresh plant samples were washed with deionized water, dried in an oven at 65 °C for 72 h, and weighted and pulverized with a stainless steel cutter blender (T250D, IKA, Germany). Dry plant samples (0.200 g) were digested in 10 ml of HNO_3-$HClO_4$ (4:1, v/v) at 220 °C on a hot plate, filtered with Whatman filter paper, and the filtrate was diluted to 25 ml with 5% (v/v) HNO_3 solution. The Cd concentration was determined by using an atomic absorption spectrometer (AAS) and a graphite tube equipped with an automatic sampler (ZEEnit 700, Analytik Jena, Germany). The quality control included blanks and certified samples (GBW10016) with each batch of samples.

2.3 Bioconcentration factor (BCF), transport index (TI), and total Cd uptake in mass per plant

The Cd BCF was calculated on the basis of dry weight using Eq. (1). It represents an index of the ability of plants to accumulate Cd with respect to its concentration in the hydroponic substrate (Ghosh and Singh, 2005):

$$\mathrm{BCF}=c_{\mathrm{Cd,sh/r}}\times 100\%/c_{\mathrm{Cd,s}}, \quad (1)$$

where $c_{\mathrm{Cd,sh/r}}$ (mg/kg) and $c_{\mathrm{Cd,s}}$ (mg/L) represent Cd concentrations in shoots or roots and the hydroponic solution, respectively.

The TI was computed as the Cd concentration in plant shoots (mg/kg dry weight) divided by the Cd concentration in roots (mg/kg dry weight) using Eq. (2) (Ghosh and Singh, 2005). The TI represents the ability of plants to transport Cd from root to shoot under elevated levels of CO_2 or ambient CO_2:

$$\mathrm{TI}=c_{\mathrm{Cd,sh}}\times 100\%/c_{\mathrm{Cd,r}}. \quad (2)$$

Total shoot or root Cd uptake in mass-per-plant was calculated by shoot or root dry weight biomass multiplied by Cd concentration in the corresponding plant part. It represents the ability of plants to remove Cd from contaminated environments under elevated levels of CO_2 or ambient CO_2.

2.4 Statistical analysis

The experiment was performed using a three-factor completely randomized design (CRD) with four replications. Statistical analysis was performed by using SPSS statistical software (Version 16.0, SPSS Inc., Chicago, Illinois, USA). The data were analyzed using analysis of variance (ANOVA). To examine the statistical significance of differences (P<0.05) between means, the Tukey test was performed.

3 Results

3.1 Growth of plants

Elevated levels of CO_2 increased the tiller number, root dry weight, shoot dry weight, and total plant dry weight significantly as compared to the ambient CO_2 controls in all the three Cd concentrations, regardless of the negative effect of Cd stress (Table 1). Toxic symptoms, as shown by brown roots and withered leaf tips, were first observed in fine root tips and leaf tips of both *Lolium* species at 16 mg/L Cd exposure. The plants grown under elevated levels of CO_2 showed less Cd toxicity symptoms (data not shown), implying that increased root growth due to elevated levels of CO_2 may alleviate Cd toxicity. Cd exposure increased dry weight ratios of root/shoot in both plant species, but the elevated levels of CO_2 had the opposite effect (Table 1). Apparently, there were interacting effects between Cd and CO_2 on the shoot dry weight, root dry weight, and total plant dry weight for both plant species (P<0.05; Table 2). Significant differences in tiller numbers and root/shoot ratios were noted between the two plant species in terms of the response to elevated levels of CO_2 (P<0.05).

Table 1 Effects of Cd and CO_2 levels on number of tillers, shoot dry weight, root dry weight, total plant dry weight, and root/shoot ratio in *L. multiflorum* and *L. perenne*

Species	CO_2 level	c_{Cd} (mg/L)	n	Shoot DW (g)	Root DW (g)	TPDW (g)	Root/shoot ratio
L. multiflorum	Ambient CO_2	0	18.0^{a}	1.04^{a}	0.249^{a}	1.29^{a}	0.244^{b}
		4	15.3^{b}	0.53^{b}	0.223^{a}	0.75^{b}	0.409^{a}
		16	10.5^{c}	0.40^{c}	0.165^{b}	0.57^{c}	0.418^{a}
	Elevated CO_2	0	20.5^{A}	1.75^{A*}	0.411^{A*}	2.16^{A*}	0.234^{B}
		4	15.8^{B}	1.02^{B*}	0.344^{B*}	1.36^{B*}	0.338^{A*}
		16	11.8^{C}	0.76^{C*}	0.249^{C*}	1.01^{C*}	0.346^{A*}
L. perenne	Ambient CO_2	0	14.8^{a}	1.11^{a}	0.249^{a}	1.36^{a}	0.225^{b}
		4	11.3^{b}	0.54^{b}	0.186^{b}	0.73^{b}	0.327^{a}
		16	7.5^{c}	0.42^{b}	0.148^{c}	0.57^{c}	0.348^{a}
	Elevated CO_2	0	23.5^{A*}	1.83^{A*}	0.366^{A*}	2.20^{A*}	0.200^{B}
		4	17.8^{B*}	0.87^{B*}	0.310^{B*}	1.23^{B*}	0.302^{A}
		16	12.3^{C*}	0.70^{B*}	0.236^{C*}	0.94^{C*}	0.321^{A}

c_{Cd}: concentration of Cd; n: number of tillers; DW: dry weight; TPDW: total plant dry weight. Values are means of four replicates. For each species, different small superscript letters or capital superscript letters following values in the same column refer to significant differences (P<0.05) between Cd treatments at ambient or elevated CO_2 levels, respectively. Within a Cd concentration, '*' indicates significant differences (P<0.05) between the two CO_2 treatments in each species

Table 2 ANOVA test for number of tillers, shoot dry weight, root dry weight, total plant dry weight, and root/shoot ratio in *L. multiflorum* and *L. perenne*

Factor	F value				
	n	Shoot DW	Root DW	TPDW	Root/shoot ratio
Species	5.2^{*}	n.s	5.7^{*}	n.s	5.0^{*}
CO_2	81.0^{***}	257.2^{***}	193.0^{***}	323.7^{***}	6.6^{*}
Cd	116.3^{***}	4.5^{***}	90.8^{***}	294.7^{***}	39.3^{***}
Species×CO_2	37.2^{***}	n.s.	n.s.	n.s.	11.6^{**}
Species×Cd	n.s.	n.s.	n.s.	n.s.	n.s.
CO_2×Cd	n.s.	37.6^{***}	11.4^{***}	37.7^{***}	n.s.
Species×CO_2×Cd	n.s.	n.s.	n.s.	n.s.	n.s.

n: number of tillers; DW: dry weight; TPDW: total plant dry weight. n.s.: no significant differences; * P<0.05, ** P<0.01, *** P<0.001

Elevated levels of CO_2 triggered an increase in total plant dry weight (Table 1). The increased stimulation due to elevated levels of CO_2 was 67%, 77%, and 81%, for *L. multiflorum*, and 62%, 65%, and 68% for *L. perenne* under 0, 4, and 16 mg/L Cd treatments, respectively. The increased shoot biomass was likely dependent on the increase in tiller number for *L. perenne*, and more relied on leaf expansion for *L. multiflorum* (data not shown). The elevated CO_2 treatment showed more dry weight increase in shoots than in roots, resulting in reduced root/shoot ratio compared to the ambient CO_2 control.

3.2 Root morphological traits

It was clear that for the same Cd level, elevated levels of CO_2 improved the root growth of the two tested species (Tables 3 and 4). Elevated levels of CO_2 increased the root length by 8.6%, 17.0%, and 14.0% for *L. multiflorum*, and by 35.8%, 42.9%, and 46.9% for *L. perenne* under 0, 4, and 16 mg/L Cd treatments, respectively; it also increased the root tip numbers by 0.7%, 8.8%, and 7.9% for *L. multiflorum*, and by 4.8%, 39.4%, and 47.7% for *L. perenne* under 0, 4, and 16 mg/L Cd treatments, respectively. When both plant species were exposed to Cd under elevated levels of CO_2, the negative effect of Cd on the roots of both plant species was mitigated and consequently roots with a larger volume were observed under the elevated CO_2 condition compared to the ambient CO_2 control (Tables 3 and 4). The elevated CO_2 treatment increased the average root diameter of *L. multiflorum*, but decreased average root diameter of *L. perenne*.

Table 3 Effects of Cd and CO_2 levels on length, surface area, volume, average diameter, number of tips of the roots in *L. multiflorum* and *L. perenne*

Species	CO_2 level	c_{Cd} (mg/L)	l (cm)	S (cm^2)	V (cm^3)	d (mm)	n_{tips}
L. multiflorum	Ambient CO_2	0	185^{a}	299^{a}	3.86^{a}	0.516^{b}	2519^{a}
		4	133^{b}	216^{b}	2.83^{b}	0.511^{b}	2044^{b}
		16	93^{c}	161^{c}	1.73^{c}	0.549^{a}	1805^{b}
	Elevated CO_2	0	201^{A}	359^{A*}	5.91^{A*}	0.637^{AB*}	2539^{A}
		4	156^{B}	296^{B*}	4.55^{B*}	0.610^{B*}	2225^{AB}
		16	106^{C}	179^{C}	2.77^{C*}	0.647^{A*}	1947^{B}
L. perenne	Ambient CO_2	0	201^{a}	304^{a}	3.67^{a}	0.582^{b}	4467^{a}
		4	84^{b}	145^{b}	2.03^{b}	0.565^{b}	1466^{b}
		16	49^{c}	87^{c}	1.21^{c}	0.677^{a}	1016^{c}
	Elevated CO_2	0	273^{A*}	549^{A*}	5.82^{A*}	0.544^{AB*}	4680^{A}
		4	120^{B*}	178^{B*}	3.23^{B*}	0.507^{B*}	2044^{B*}
		16	72^{C*}	13^{C*}	2.06^{C*}	0.586^{A*}	1501^{C*}

c_{Cd}: concentration of Cd; l: length; S: surface area; V: volume; d: average diameter; n_{tips}: number of tips. Values are means of four replicates. For each species, different small superscript letters or capital superscript letters following values in the same column refer to significant differences ($P<0.05$) between Cd treatments at ambient or elevated CO_2 levles, respectively. Within a Cd concentration, '*' indicates significant differences ($P<0.05$) between the two CO_2 treatments in each species

Table 4 ANOVA test for length, surface area, volume, average diameter, number of tips of the roots in *L. multiflorum* and *L. perenne*

Factor	F value				
	l	S	V	d	n_{tips}
Species	11.8^{**}	n.s.	n.s.	n.s.	n.s.
CO_2	37.7^{***}	29.0^{***}	141.6^{***}	4.3^{*}	n.s.
Cd	227.3^{***}	79.0^{***}	155.4^{***}	n.s.	71.8^{***}
Species×CO_2	9.2^{**}	4.9^{*}	12.2^{**}	n.s.	5.6^{*}
Species×Cd	33.1^{***}	14.7^{***}	23.5^{***}	n.s.	35.4^{***}
CO_2×Cd	n.s.	3.7^{*}	22.0^{***}	4.2^{*}	n.s.
Species×CO_2×Cd	n.s.	4.3^{*}	9.5^{**}	n.s.	n.s.

l: length; S: surface area; V: volume; d: average diameter; n_{tips}: number of tips. n.s.: no significant differences; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

Increasing Cd concentrations resulted in a significant decrease in number and proportion of fine roots in both species (Fig. 1). Elevated levels of CO_2 alleviated the decreases of fine roots in both Cd-stressed species, but the percentage of thick roots showed a positive response to elevated levels of CO_2 for *L. multiflorum* and a negative response for *L. perenne*.

3.3 Cd concentration and total uptake

Plants grown under elevated levels of CO_2 had lower Cd in both shoots and roots, compared to the ambient CO_2 control. The effect of interaction between Cd and CO_2 on Cd concentration in shoots and roots was significant (P<0.05). The decrease of Cd concentration in shoots and roots triggered by elevated levels of CO_2 was more substantial in 16 mg/kg Cd treatment than in 4 mg/kg Cd treatment (Fig. 2). However, the total Cd uptake calculated on the basis of per-pot dry weight biomass was significantly higher (42% to 73% increase in shoots) at elevated levels of CO_2 than at ambient CO_2 levels (Fig. 3).

At ambient levels of CO_2 and high Cd levels (16 mg/L), the measured levels of Cd were 417 and 466 mg/L in the shoots, and 3469 and 4460 mg/L in the roots of *L. multiflorum* and *L. perenne*, respectively (Fig. 2). The BCF of root was much higher than that of shoot (Tables 5 and 6). Both BCFs decreased with increasing spiked Cd concentration in the growth media regardless of CO_2 treatment. At a lower Cd concentration (4 mg/L) and ambient CO_2 levels, the BCFs of roots and shoots were as high as 388 and 39 L/kg for *L. multiflorum*, and 436 and 39 L/kg for *L. perenne*, respectively. However, the BCFs of roots and shoots decreased with elevated levels of CO_2 regardless of Cd levels. TI values throughout the treatments were less than 1, and lower for the 16 mg/L Cd treatment than for the 4 mg/L Cd treatment. Elevated levels of CO_2 induced a higher TI in both species under the same Cd treatment level.

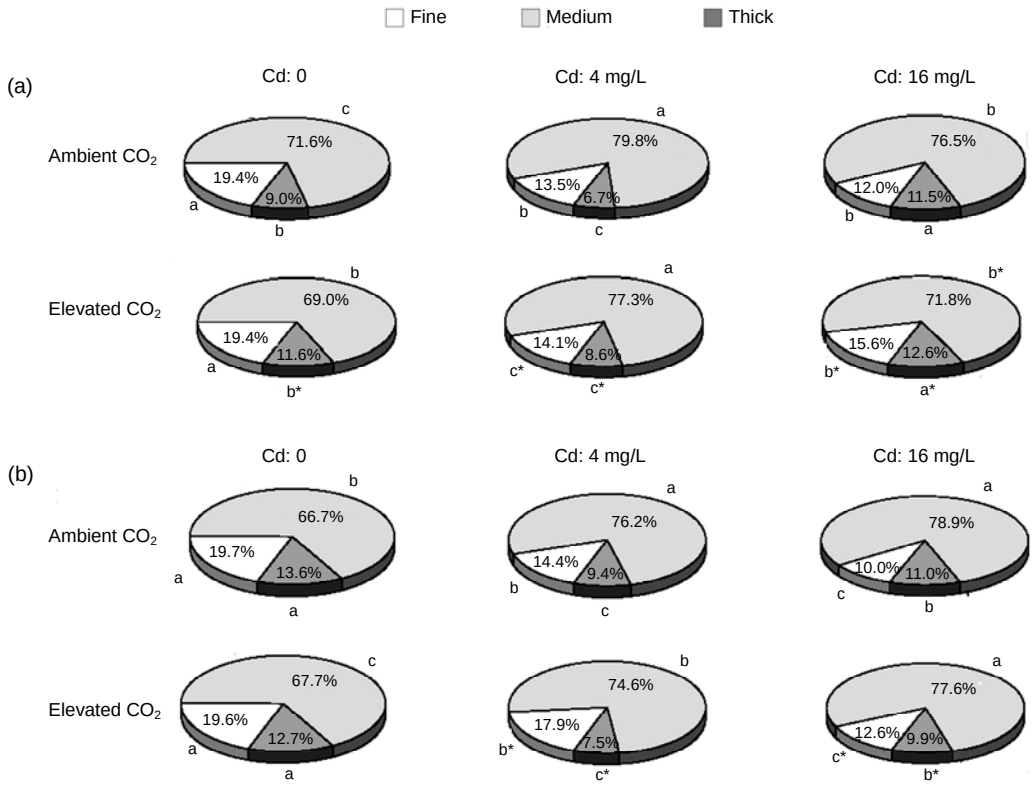

Fig. 1 Root distributions of *L. multiflorum* (a) and *L. perenne* (b) grown hydroponically at various Cd concentrations and CO_2 levels in different size-categories

Dameter: fine, <0.1 mm; medium, 0.1–1.0 mm; thick, >1.0 mm. For each species, different letters refer to significant differences (P<0.05) between Cd treatments at ambient or elevated CO_2 levels for each size-category, respectively. Within a Cd concentration, '*' indicate significant differences (P<0.05) between the two CO_2 treatments in each size-category for each species

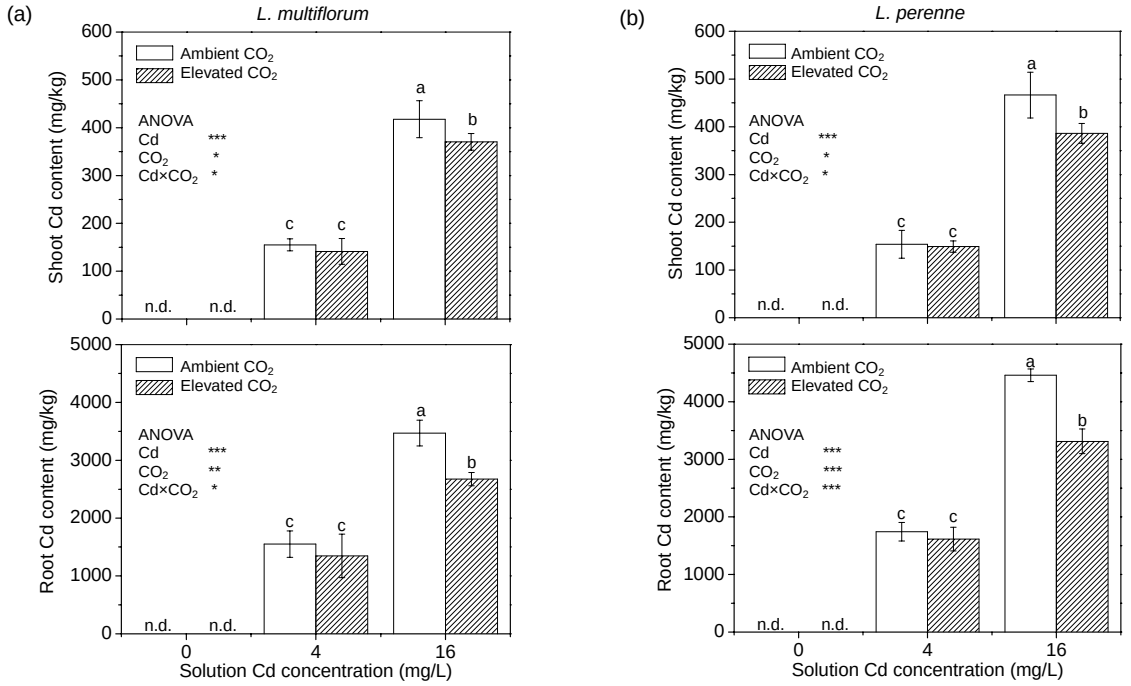

Fig. 2 Effects of Cd and CO_2 levels on Cd distributions in shoots and roots of *L. multiflorum* (a) and *L. perenne* (b)
For shoot or root of each species: different letters refer to significant differences (P<0.05) between treatments; n.d.: not determined. For ANOVA: n.s.: no significant differences; * P<0.05, ** P<0.01, *** P<0.001

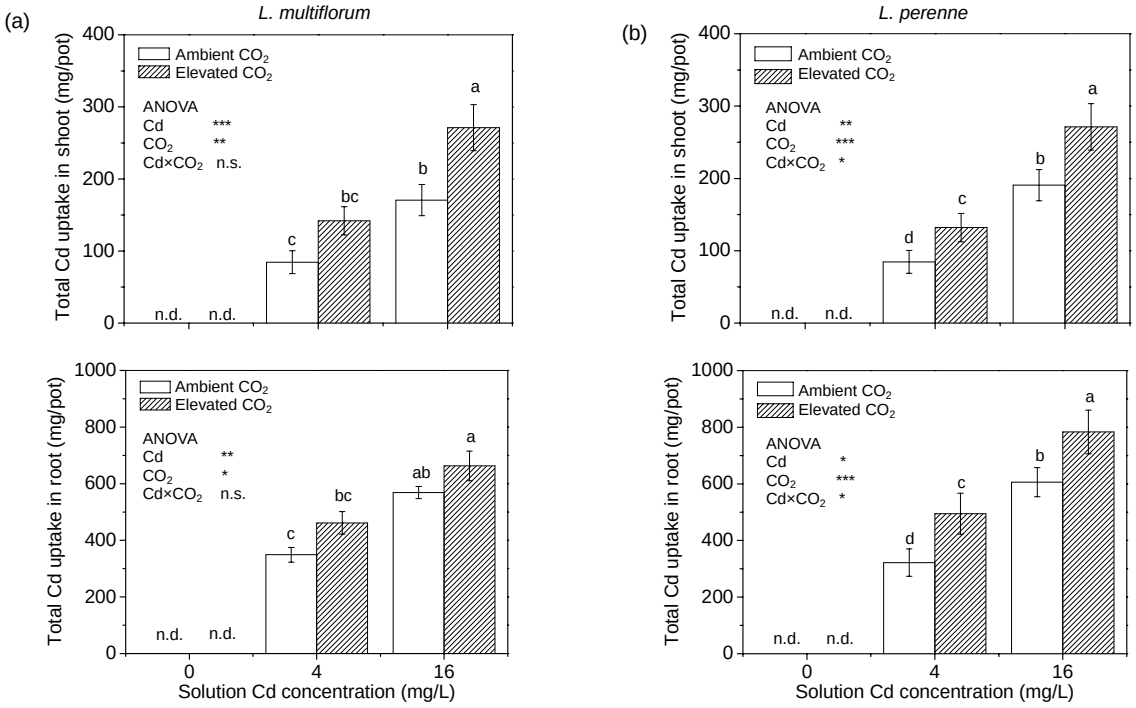

Fig. 3 Effects of Cd and CO_2 levels on total shoot or root Cd uptake per pot in *L. multiflorum* (a) and *L. perenne* (b)
For shoot or root of each species: different letters refer to significant differences (P<0.05) between treatments; n.d.: not determined. For ANOVA: n.s.: no significant differences; * P<0.05, ** P<0.01, *** P<0.001

Table 5 Effects of Cd and CO_2 levels on Cd bioconcentration factor and Cd transport index in *L. multiflorum* and *L. perenne*

Species	CO_2 level	c_{Cd} (mg/L)	BCF (L/kg)		TI
			Shoot	Root	
LM	Ambient CO_2	4	39^{a}	388^{a}	0.100^{a}
		16	26^{b}	217^{b}	0.088^{b}
	Elevated CO_2	4	35^{A}	337^{A}	0.105^{A*}
		16	23^{B*}	167^{B*}	0.092^{B}
LP	Ambient CO_2	4	39^{a}	436^{a}	0.120^{a}
		16	29^{b}	279^{b}	0.105^{b}
	Elevated CO_2	4	37^{A}	404^{A}	0.139^{A*}
		16	24^{B*}	207^{B*}	0.117^{B*}

LM: *L. multiflorum*; LP: *L. perenne*; c_{Cd}: concentration of Cd; BCF: bioconcentration factor; TI: transport index. Values are means of four replicates. For each species, different small superscript letters or capital superscript letters following values in the same column refer to significant differences (P<0.05) between Cd treatments at ambient or elevated CO_2, respectively. Within a Cd concentration, '*' indicates significant differences (P<0.05) between the two CO_2 treatments in each species

Table 6 ANOVA test for Cd bioconcentration factor and transport index in *L. multiflorum* and *L. perenne*

Factor	*F* value		
	BCF (L/kg)		TI
	Shoot	Root	
Species	n.s.	18.02^{***}	9.93^{**}
CO_2	5.02^{*}	15.91^{**}	4.62^{*}
Cd	57.96^{***}	183.43^{***}	21.75^{***}
Species×CO_2	n.s.	n.s.	n.s.
Species×Cd	n.s.	n.s.	n.s.
CO_2×Cd	n.s.	n.s.	n.s.
Species×CO_2×Cd	n.s.	n.s.	n.s.

BCF: bioconcentration factor; TI: transport index; n.s.: no significant differences; * P<0.05, ** P<0.01, *** P<0.001

4 Discussion

4.1 Combined effects of Cd concentrations and elevated CO_2 levels on plant growth

We observed a significant effect of elevated levels of CO_2 on the dry weights of both *Lolium* plants (P<0.001) (Tables 1 and 2); however interestingly, the elevated levels of CO_2 had an increasing effect on the total plant dry weight but it was different for different plant species under 0, 4, and 16 mg/L Cd treatments. Enhanced plant growth under elevated levels of CO_2 and uncontaminated soil conditions has been widely documented, and the magnitude of increase was dependent on plant species and genotype (Ziska *et al*., 1996; Moya *et al*., 1998; Horie *et al*., 2000; Kimball *et al*., 2002; Long *et al*., 2004; Lobell and Field, 2008; Cheng *et al*., 2009). The increased stimulation, due to elevated levels of CO_2, was higher under Cd stress than under non-Cd stress for both species, showing the effectiveness of elevated levels of CO_2 in ameliorating Cd toxicity. This may be partially related to the root development and alteration of the root morphological traits (Peng *et al*., 2005). The root/shoot ratio response to elevated levels of CO_2 differed among species and growth conditions (Ferris and Taylor, 1993). From the viewpoint of phytoextraction, our finding that CO_2 level elevation increased the biomass of the two *Lolium* species grown in hydroponic solutions spiked with various levels of Cd, may also have implications for improvement of phytoextraction efficiency as well. When compared the two *Lolium* species, greater reduction in plant growth, due to spiked Cd in growth media, was observed in *L. perenne* than in *L. multiflorum*, suggesting that the latter has higher metal tolerance than the former. Similar reports were documented in Sabreen and Sugiyama (2008) who showed that *L. perenne* had a higher growth inhibition than *L. multiflorum* under Cd stress.

4.2 Combined effect of Cd concentrations and elevated CO_2 levels on root morphological traits

Root length, surface area, volume, and tip number are important parameters for understanding how root systems respond to environmental changes, such as increases in CO_2 levels and Cd stress. Both *Lolium* species had higher values of the root morphological parameters (including root length, surface area, volume, and tip numbers) at elevated levels of CO_2 when compared to the ambient CO_2 control, showing that elevated levels of CO_2 increased root elongation and root branching. Corresponding to the response of plant growth to elevated levels of CO_2, Cd treatments affected root tip number, root length, surface area, volume, and number of fine roots as reported in Daud *et al*. (2009) and Li *et al*. (2009). Generally, root length, surface area, and volume are more sensitive to Cd than root tip number (Ci *et al*., 2009). Fine roots were more affected than the coarse ones when exposed to Cd (Cosio *et al*., 2006). The exposure of plants to Cd stress reduced the nutrition uptake from growth media by affecting root growth,

elongation, and absorption zones (Peng *et al*., 2005), and thus inhibited the growth of plants. Our results showed that *L. perenne* was more sensitive to Cd than *L. multiflorum* as shown by their differences in the root parameters (Tables 3 and 4). For *L. multiflorum*, the root tip number was less influenced than the root length, surface area, and volume. A more substantial decrease of root tip number was observed in *L. perenne* than in *L. multiflorum*. Since the root tip number partly reflects the lateral root emergence, the higher inhibition of lateral root emergence in *L. perenne* may be used to explain why it had more inhibited root growth than *L. multiflorum*. For both *Lolium* species, we observed a more substantial decrease in fine roots than coarse roots although the latter was also strongly inhibited.

For both *Lolium* species, elevated levels of CO_2 triggered a more substantial increase in root length, volume, and tip number under Cd stress than under non-Cd treatments when compared to the ambient CO_2 control. Elevated levels of CO_2 caused a significant increase of root length in all three root size-categories, but the magnitude of increase varied to different degrees. It is widely documented that elevated levels of CO_2 increased fine root numbers (Pritchard and Rogers, 1999; Matamala and Schlesinger, 2000; Pritchard *et al*., 2001). Our study showed that the fine root proportion was not affected by elevated levels of CO_2 under the Cd control treatment, but it increased when plants were exposed to Cd. This suggested that under the Cd stress condition, elevated levels of CO_2 stimulated more fine roots than coarse ones, indicating that alleviation of Cd toxicity in response to elevated levels of CO_2 may be related to an increase in fine root numbers. The increased fine roots aid the nutrient and water uptake as they are the most active parts of the roots. This can explain why elevated levels of CO_2 triggered more increase of plant biomass under Cd stress than under non-Cd treatment compared to the ambient CO_2 control. It was more likely that plants obtained additional energy from the photosynthesis to combat with the stress under elevated levels of CO_2 by altering root morphological traits (Tables 1 and 2).

4.3 Effects of elevated CO_2 levels on Cd uptake and implication for food safety and phytoremediation

Both of the two *Lolium* species showed potential materials for phytoremediation due to their high Cd uptakes. Our study showed a reduction of Cd concentration in the two tested plant species by the lower BCF, especially in the roots, at elevated levels of CO_2. The lower Cd concentration under elevated levels of CO_2 will benefit plants grown under Cd stress. This reduction in Cd concentration might be related to the so-called dilution effect induced by fast plant growth as reported in Loladze (2002). Lieffering *et al*. (2004) hypothesized that if the root production response under elevated levels of CO_2 was much higher than the above-ground biomass response, the elemental uptake may match the increase in above-ground biomass. In our case, the relatively lower biomass increase in roots of the two *Lolium* species under elevated levels of CO_2 (as showed by root/shoot ratio) may suggest a Cd dilution mechanism. Further research is required to determine whether elevated levels of CO_2 interact with the Cd influence on membrane permeability and the activity of the transport protein, thus altering the allocation of elements within the plant.

Despite a reduction of Cd concentration in plant species at elevated levels of CO_2, we observed higher TI at elevated levels of CO_2 than at ambient CO_2, suggesting that elevated levels of CO_2 improved Cd transportation from roots to shoots (Tables 5 and 6). The increased TI may be due to the lower Cd concentration in plants under elevated levels of CO_2, as the higher TI was observed in lower Cd stress. Taking into consideration the reduction of Cd concentration in plant tissues under elevated levels of CO_2, we speculate that the improvement in Cd transportation from roots to shoots may be insufficient to compensate the dilution effect due to increased biomass under elevated levels of CO_2.

A survey of literature shows that dilution phenomena are well documented for some staple food crops in the pot studies where nutrient supplies are generally limited. Högy and Fangmeier (2009) observed a 3.7%–18.3% reduction in all micro-elements in wheat grown in the different exposure systems. Guo *et al*. (2006) reported decreased Cd accumulation in leaves, stems, roots and grains of rice at elevated CO_2. Zheng *et al*. (2008) showed that *Pteridium revolutum* and *Pteridium aquilinum* grown on Cu-contaminated soils accumulated less Cu in plant tissues at elevated levels of CO_2 than at ambient

CO_2. Li *et al*. (2010) found that elevated levels of CO_2 diluted grain Cd concentration. In view of the potential benefits of CO_2-triggered "metal dilution" and the very limited available data on the subject, we speculate that this might have an important positive implication for the food safety regarding the contaminated soil from which crops are harvested.

Calculation of total Cd uptake in each plant tissue from each pot showed that there was a much higher total Cd uptake at elevated levels of CO_2 than at the ambient CO_2 control (an increase of 42.2% to 73.4% for shoots). Taking into account our present results and previously obtained results (Wu *et al*., 2009; Jia *et al*., 2010; Li *et al*., 2010), we proposed that enriching CO_2 in growth chambers can help plants remove more metals from growth media as indicated by their enhanced metal total uptake. This might have positive implications for improving phytoremediation efficiency if enriching CO_2 in growth chambers is used to assist phytoextraction of the contaminated soil.

5 Conclusions

Root morphological parameters, including root length, surface area, volume, tip number, and fine roots, all decreased under Cd exposure. By contrast, elevated levels of CO_2 significantly increased all those parameters in the presence of Cd, compared to the CO_2 control, suggesting that elevated levels of CO_2 had an ameliorating effect on Cd-induced stress. The significantly higher total Cd uptake per pot, calculated on the basis of biomass under elevated levels of CO_2 in association with increased biomass production, may have implications for food safety and phytoextraction. The conclusions of increased biomass, total Cd uptake, and changes in root morphological traits with CO_2 fertilisation were based on plants grown under growth chamber and Cd spiked hydroponic conditions, and were probably exaggerated. The potential for elevated levels of CO_2 to trigger changes in biomass, root morphological traits, and Cd uptake might be overestimated. There is a need to carry out more researches that focus on plants grown under field conditions under elevated levels of CO_2 in order to make reasonable predictions on the combined effects of elevated levels of CO_2 and metal-contaminated media on metal uptake by plants.

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