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Effect of elevated atmospheric CO$_2$ concentration on growth and physiology of wheat and sorghum under cadmium stress

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ABSTRACT
Elevated concentrations of carbon dioxide (e[CO$_2$]) affect plant growth and physiological characteristics, including metal accumulation, and the activity of anti-oxidant enzymes. These effects were investigated in cadmium (Cd) tolerant wheat (Triticum aestivum L.) and sorghum (Sorghum bicolor (L.) Moench.) cultivars. Plants were grown at the ambient and elevated CO$_2$ levels, with four concentrations of Cd (0, 10, 20 and 40 mg kg$^{-1}$) added to the soil. After 60 days, subsamples were tested for chlorophylls and carotenoids, protein, enzyme activities and morphological characteristics. Results showed that e[CO$_2$] increased plant height, leaf area, and the dry weight of shoots and roots ($P < 0.01$). In addition, it decreased the Cd concentration in the shoots and roots of wheat, and increased the same concentrations for sorghum. With increasing Cd, the activities of the anti-oxidants, SOD and GSH-px increased in wheat. The differences in enzyme activity parallel the changes in Cd concentration in the plants of both species.

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KEYWORDS
Elevated [CO$_2$]; anti-oxidant enzyme; cadmium; wheat; soil contamination

Introduction
Industrialization and human activities increased the concentration of atmospheric carbon dioxide [CO$_2$] from 280 µL L$^{-1}$ before the industrial revolution to nearly 400 µL L$^{-1}$ in 2013, and the increase is expected to continue until the end of the 21st Century (IPCC 2013). Many researchers have shown that elevated [CO$_2$] (e[CO$_2$]) increases plant growth in uncontaminated soils (Erda et al. 2005; Kimball, Kobayashi, and Bindi 2002). e[CO$_2$] increases the net photosynthetic rate in C$_3$ plants, stimulates the growth of both well-watered and water-stressed C$_3$ plants (Ghannoum et al. 2000), increases carbon assimilation (Li et al. 2013), decreases photorespiration, and decreases antioxidant stress caused by heavy metals (Rogers et al. 2004). Although e[CO$_2$] can increase plant growth, this positive effect may be lessened by environmental factors and as a result of biotic or abiotic stress (Ainsworth and Long, 2005; Benloch-Gonzalez et al. 2014; Cheng et al. 2009).

In recent decades, increased heavy metal concentrations in soils have emerged as an important abiotic stress on ecological systems (Liu, Zhang, and Zhang 2007). Among the heavy metals, cadmium (Cd) is notable for its toxic effects on biota even at low concentrations (Das, Samantaray, and Rout 1997; Pinto et al. 2004), and excessive Cd endangers human health via contamination of the food chain (Liu et al. 2011). High Cd concentrations in plants cause changes in water and mineral uptake and reductions in biomass (Singh and Tewari 2003). Photosynthesis is sensitive to Cd stress (Ci et al. 2010), and high concentrations of Cd inhibit chlorophyll synthesis (Jain et al. 2007) and reduce the amount of chlorophyll in leaves (Laspina...
et al. 2005), interfere with steps in the Calvin cycle (Siedlecka et al. 1997) and decrease electron transport activity (Van Assche and Clijsters 1990). Additionally, Cd stimulates free radical formation and oxidative stress (Ekmeckci, Tanyolac, and Ayhan 2008), membrane damage and enzyme inactivation (Qiu et al. 2008). As a consequence, plant cells have enzymatic and non-enzymatic defense mechanisms to scavenge free radicals (Shah et al. 2001). These mechanisms include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px), well known antioxidant enzymes that directly catalyze the transformation of peroxides and superoxide to nontoxic species (Karuppanapandian et al. 2011).

Numerous studies have investigated the effects of e\(^\text{CO}_2\) on the growth and development of plants in relation to the uptake of heavy metals from contaminated soils (Guo et al. 2015, 2011; Li et al. 2010, 2013; Pietrini et al. 2016). In many of these studies, e\(^\text{CO}_2\) increased plant growth and metal uptake. Tang et al. (2003) reported that e\(^\text{CO}_2\) increased the biomass of Indian mustard (Brassica juncea (L.) Czern.) and sunflower (Helianthus annuus L.) and enhanced copper (Cu) accumulation in both plants. Wu et al. (2009) found that e\(^\text{CO}_2\) not only increased the aboveground biomass of sorghum (Sorghum bicolor (L.) Moench.) and Trifolium species, but also increased cesium hyper accumulation in these species. Zheng et al. (2008) reported that e\(^\text{CO}_2\) enhanced both biomass and Cu accumulation in Pteridium revolutum (Blume) Nakai and Pteris vittata L. In some of these studies, the increase in metal uptake has been related to a decrease in rhizosphere pH (Li et al. 2013) or an increase in microbial activity (Wu et al. 2009). In contrast, Li et al. (2010) showed that although the total biomass of six rice (Oryza sativa L.) varieties increased under e\(^\text{CO}_2\), the concentration of Cu decreased. Similarly, Jia et al. (2010) reported that e\(^\text{CO}_2\) reduced Cd concentrations in the shoots and roots of Lolium species. Pietrini et al. (2016) indicated that e\(^\text{CO}_2\) did not affect the ability of L. minor to accumulate Cd. They also reported that e\(^\text{CO}_2\) reduced Cd toxicity in duckweed by enhancing the antioxidant system. Jia et al. (2017) showed that e\(^\text{CO}_2\) decreased Cd uptake by seedlings of R. pseudo acacia. These contrasting results show that the effect of e\(^\text{CO}_2\) on heavy metal uptake may vary between metal contaminants, plant species and environments. It is essential to characterize these effects on crops to improve food safety in the future with e\(^\text{CO}_2\).

Most of the research on plant responses to e\(^\text{CO}_2\) has been carried out with C\(_3\) species, with C\(_4\) plants receiving little attention, because it was assumed that the CO\(_2\) concentrating mechanism used by C\(_4\) species would limit the response to e\(^\text{CO}_2\) (Prior and Runion 2011). The response of C\(_3\) species to e\(^\text{CO}_2\) may be greater than that of C\(_4\) species due to increases in the rate of photosynthesis by <58% (Drake et al. 1997). Wheat (Triticum aestivum L.) is the dominant C\(_3\) grain crop worldwide with an important role in the supply of energy and protein (Hogy and Fangmeier 2008). Wheat is the main cereal cultivated in Iran for human food. Sorghum is a C\(_4\) grass and fifth-most important cereal in the world for use as food for animals (Angelova et al. 2011). Sorghum is tolerant to metal pollution and able to produce high biomass, even in the presence of heavy metals (Pinto et al. 2004). The e\(^\text{CO}_2\) increases the growth of wheat and sorghum in both Cd contaminated and uncontaminated soils (Hogy and Fangmeier 2008; Hogy et al. 2009), and in soils contaminated with other metals (Tian et al. 2014; Wu et al. 2009). However, there is little information regarding how these plants grow in Cd contaminated soil and on the degree of Cd accumulation. Moreover, the tolerance mechanisms to Cd stress under e\(^\text{CO}_2\) are not well understood for wheat and sorghum.

We hypothesize that e\(^\text{CO}_2\) will enhance the growth and Cd tolerance of both wheat and sorghum due to improvements in parameters such as chlorophyll content and antioxidant enzyme activity. The hypothesis is tested in a controlled environment using Cd-tolerant lines of both species.

### Materials and methods

#### Soil preparation and conditions of plant growth

Surface soil (0–300 mm) was collected from an uncontaminated site located at 36°30′N, 50°41′E, southwest of Karaj, the capital of Alborz Province, Iran. The main properties of the soil (Table 1) were determined as follows: texture, using the hydrometric method of Bouyoucos (1962); pH, in
soil: solution of distilled water shaken for 1 h (Sparks 1996); organic matter, using chromate oxidation (Nelson and Summers, 1982); cation exchange capacity (CEC), according to Sumner and Miller (1996); total N, by the Kjeldahl method (Bremner 1996); available P, by the Olsen method (Olsen et al. 1954); and available K, extracted using 1 M NH₄OAC (pH 7) and measured using a flame photometer. Total Cd was determined by acid digestion (HNO₃, HCl and H₂O₂; US EPA, 3050B, 1996), and available Cd was extracted by DTPA (Lindsay and Norvell, 1978).

Soil samples were contaminated by mixing Cd (NO₃)₂ solutions to the soil in each pot (3.5 kg) to obtain four concentrations above the natural level: 0, 10, 20 and 40 mg Cd kg⁻¹ soil. There were six replications at each Cd level. The spiked soils were watered to field capacity and kept in darkness and at 25°C for four months to equilibrate. During this period, soil moisture was maintained by periodic re-watering to field capacity.

Seeds of wheat and sorghum cultivars were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. These cultivars were chosen based on their high Cd-tolerance (Marvdasht cvv. for wheat and Sepideh cvv. for sorghum) among three cvv. of both species (data not shown). The seeds were surface-sterilized using 10% NaOCl and ethanol, washed with distilled water and germinated on wet filter paper in Petri dishes at 28°C for 48 h; ten uniform seedlings were transferred to each pot. At the two-leaf stage, five uniform plants were retained in each pot. A complete set of the Cd (4) × species (2) treatments x 3 replicates (i.e., 24 pots) was transferred to one growth chamber at 450 ± 50 µL CO₂ L⁻¹ (ambient CO₂, a[CO₂]) and the other 24 pots to a second chamber with similar conditions in which the air was enriched to 900 ± 50µL CO₂ L⁻¹ (e[CO₂]). The CO₂ enrichment was supplied from a gas cylinder and the concentration in the chamber controlled by mass flow and a CO₂ sensor (Jumo, GmbH. Co. Germany). Plants were exposed to the different CO₂ concentrations from the two-leaf stage during daylight hours only. Both growth chambers had 26/20°C day/night temperatures, a 12 h light period at a photosynthetic photon flux density of 480 µmole m⁻² s⁻¹, and ~ 60% relative humidity. To minimize the effect of pot position within a chamber, the pot locations were re-randomized every week. Watering was based on plant demand and was supplied up to 80% of field capacity. The temperature and [CO₂] inside the chambers was monitored and controlled daily.

**Harvest and analyses**

Plants tops were harvested after 60 days of growth. The tops and roots were rinsed with distilled water and blotted dry with tissue paper. Fresh weight (FW) and leaf area (CL-202, CID Inc.) were measured. Specific leaf area (SLA) was calculated by dividing the leaf area by the leaf dry weight. Two or three young upper leaves were cut from each plant, and subsamples of (0.1 g) were homogenized in a chilled ethanol solution (ethanol: water 19:1 v/v), and the homogenate filtered and diluted to 25 mL. The absorbances of chlorophyll a and b, and carotenoids were measured at 665, 649 and 470 nm and the respective concentrations were calculated (Lichtenthaler and Wellburn 1983). The remainder was wrapped in aluminum foil, frozen in liquid nitrogen, and then stored at −80°C. Otherwise, the tops and roots were oven dried at 70°C for 72 h for dry weight (DW) determination. Dried samples were pulverized before Cd analysis. Plant samples (1.0 g) were soaked in 15 mL of a mixture of HNO₃, HClO₄ and H₂SO₄ (40:4:1 v/v) over night. Digestion proceeded by gradual heating from 70 to 120°C until the digests became dark, after which the temperature was

<table>
<thead>
<tr>
<th>pH</th>
<th>Organic matter (%)</th>
<th>CEC (cmol, kg⁻¹)</th>
<th>Total Cd (mg kg⁻¹)</th>
<th>Available Cd (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1.70</td>
<td>12.67</td>
<td>2.02</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Available N (%)</td>
<td>Sand (%)</td>
<td></td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>Silt (%)</td>
<td></td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td>Available P (mg kg⁻¹)</td>
<td>Clay (%)</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>2.34</td>
<td>271.2</td>
<td></td>
<td>Textural class</td>
</tr>
<tr>
<td></td>
<td>Available K (mg kg⁻¹)</td>
<td></td>
<td></td>
<td>Loam</td>
</tr>
</tbody>
</table>

Table 1. Some properties of the soil used in this study.
raised to 220°C until the formation of white fumes. After cooling, 10 mL of distilled water was added; the mixture was warmed for 10 min, filtered and diluted to 25 mL with distilled water (Gupta 2000). Cadmium concentrations in the digests were determined using flame atomic absorption spectrometry (Shimadzu 670, Japan). For quality control, reagent blanks and a commercial standard solution of 1000 mg Cd L$^{-1}$ (Sigma) were used.

The previously frozen plant samples were used for protein, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px) activities. First, the protein content was measured by the Bradford method (1976) using bovine serum albumin (Sigma-Aldrich) as a standard. Second, the activity of SOD was assayed by the method of Dhindsa and Matowe (1981). Briefly, ~ 0.5 g of leaf sample was homogenized in 5 mL of 100 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 1% (w/v) polyvinyl pyrrolidone. The samples were centrifuged at 20,000 $\times$ g for 15 min at ~ 4°C and the supernatants assayed. The assay mixture contained 50 mM phosphate buffer, 13 mM methionine, 75 $\mu$M nitro blue tetrazolium (NBT), 100 $\mu$M EDTA, 2 $\mu$M riboflavin and 200 $\mu$L of enzyme extract. Absorbance was recorded at 560 nm. The amount of enzyme that caused 50% inhibition of the reduction of NBT was defined as one unit of enzyme and activity was expressed as unit mg$^{-1}$ protein.

Third, CAT activity was assayed according to Aebi (1984). Briefly, 0.5 g of leaf sample was homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA and centrifuged at 12000 $\times$ g for 15 min at ~ 4°C. The reaction mixture contained 3 mL of 50 mM phosphate buffer, 12.5 $\mu$L of H$_2$O$_2$ and 50 $\mu$L of enzyme extract. The decrease in absorbance was recorded at 240 nm for 1 min. The activity was calculated using an extinction coefficient of 39.4 mM$^{-1}$ cm$^{-1}$. The amount of enzyme that decomposed 1 mM of H$_2$O$_2$ min$^{-1}$ was defined as one unit of enzyme and activity was expressed as unit mg$^{-1}$ protein. Fourth, the GSH-px activity was assayed using the method of Paglia and Valentine (1987). Briefly, 0.5 g of leaf sample was homogenized in 5 mL of 50 mM phosphate buffer (pH 7.8). The reaction mixture contained 0.56 mM KH$_2$PO$_4$, 1.2 mM EDTA and 0.2 mM NADPH, 0.2 mL reduced glutathione, 0.1 mM H$_2$O$_2$ and enzyme extract. The change in the absorbance was recorded at 340 nm. The amount of enzyme that catalyzed 1 $\mu$mol of NADPH per min was defined as one unit of enzyme, and activity was expressed as unit mg$^{-1}$ protein.

The rhizosphere soil was collected by shaking the root system gently over a sheet of paper after a short period of drying in the air and most of soil attached to roots were removed as rhizosphere soil (Yanai, Majdi, and Park 2003). Dehydrogenase activity in the soil was determined by the method of Ohlinger (1996). Five g of soil was placed in three different test tubes; two of these replicates were test samples and the third acted as a control. 5 mL of triphenyltetrazolium chloride solution was added to the test samples and 5 mL 0.1M TRIS buffer (pH 7.4) was added to the control tube. Samples were mixed thoroughly and incubated for 24 h at 25°C. After this time, to extract the triphenyl formazan (TPF) produced, 25 mL acetone was added to the three tubes and the tubes shaken for 2 h in the dark. Then, the samples were filtered in a semi-dark room and the absorbance determined at 546 nm. Soil dehydrogenase activity was expressed as mg TPF kg$^{-1}$ dry soil 24 h$^{-1}$. The spectrophotometer was calibrated using standard solutions of 0, 100, 200, 500 and 1000 $\mu$g TPF in acetone.

**Statistical methods**

All experiments were conducted using 3 replicates. Data were analyzed using ANOVA and treatment means were compared using Duncan’s test at $P = 0.05$. These analyses were performed using SAS 9.2 (SAS INC., USA).

**Results**

**Plant growth**

For wheat and sorghum at both CO$_2$ levels, increases in Cd concentration ([Cd]) decreased all the indices of plant growth, i.e. leaf area, and shoot and root DW, whereas at the same [Cd], e[CO$_2$]
significantly increased the growth indices (Table 2). e[CO₂] had less effect on the DW of the roots and, as a consequence, caused the ratio of the shoot to the root DW to increase. The magnitude of the positive effect of e[CO₂] differed at different concentrations of Cd, i.e. there were significant interactions between [CO₂] and Cd application for leaf area, specific leaf area, and shoot and root dry weight ($P < 0.05$, Table 3), but not for the shoot/root ratio ($P < 0.05$). For wheat, the increases in leaf area due to e[CO₂] were 16.20%, 15.40%, 13.50% and 15.10%, in shoot DW were 26.30%, 23.70%, 18.85% and 17.02% and in root DW were 13.60%, 17.04%, 12.30% and 11.50% for Cd treatments of 0, 10, 20 and 40 mg kg$^{-1}$, respectively. For sorghum, e[CO₂] caused 25.70%, 25.81%, 20.12% and 18.42% increases in leaf area, 13.84%, 16.41%, 18.40% and 10.93% increases in shoot dry weight, and 10.54%, 8.41%, 13.10% and 8.22% increases in root dry weight under 0, 10, 20 and 40 mg Cd kg$^{-1}$ soil treatments, respectively. With the exception of leaf area, e[CO₂] increased the growth of wheat more than sorghum. There was also a trend for e[CO₂] to have the least effect on growth at the highest Cd addition.

**Cadmium concentration in shoots and roots**

In both species and for both CO₂ treatments, as the applied soil [Cd] increased, the [Cd] in the shoots and roots also increased. The [Cd] in the roots was higher than that in the shoots. For both plant species, the greatest increase in [Cd] occurred between the 10 and 20 mg kg$^{-1}$ Cd treatment, which was particularly notable in the roots. e[CO₂] increased the [Cd] in the shoots and roots of sorghum, whereas the reverse was true for wheat (Figure 1). The effect of e[CO₂] differed for the various Cd treatments.

### Table 2. Effect of [CO₂] and [Cd] treatments on leaf area, shoot and root DW of wheat and sorghum plants.

<table>
<thead>
<tr>
<th>Shoot/Rootratio</th>
<th>RDW (g)</th>
<th>SDW (g)</th>
<th>SLA</th>
<th>LA (cm²)</th>
<th>C_Cd (mg/kg)</th>
<th>[CO₂] Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.20b</td>
<td>1.62j</td>
<td>3.54f</td>
<td>228.2h</td>
<td>435.8g</td>
<td>0</td>
<td>Ambient Wheat</td>
</tr>
<tr>
<td>1.94e</td>
<td>1.76b</td>
<td>3.42g</td>
<td>214.1l</td>
<td>383.2l</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.00de</td>
<td>1.22m</td>
<td>2.44k</td>
<td>226.1l</td>
<td>343.7k</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.66f</td>
<td>1.13n</td>
<td>1.90o</td>
<td>234.8f</td>
<td>284.1n</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>2.43a</td>
<td>1.84h</td>
<td>4.50d</td>
<td>210.1m</td>
<td>506.3e</td>
<td>0</td>
<td>Elevated</td>
</tr>
<tr>
<td>2.05cd</td>
<td>2.06f</td>
<td>4.23e</td>
<td>200.1n</td>
<td>442.1f</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.11bc</td>
<td>1.40l</td>
<td>2.90i</td>
<td>216.7k</td>
<td>390.1h</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.74f</td>
<td>1.26m</td>
<td>2.20l</td>
<td>231.9g</td>
<td>326.9l</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>1.97de</td>
<td>2.75b</td>
<td>5.42b</td>
<td>214.8l</td>
<td>569.4c</td>
<td>0</td>
<td>Ambient Sorghum</td>
</tr>
<tr>
<td>2.04cd</td>
<td>2.26d</td>
<td>4.63c</td>
<td>246.2d</td>
<td>551.6d</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>1.42g</td>
<td>1.91g</td>
<td>2.72j</td>
<td>217.9k</td>
<td>300.7m</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.25h</td>
<td>1.46k</td>
<td>1.83n</td>
<td>256.6c</td>
<td>241.2o</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>2.02cde</td>
<td>3.04a</td>
<td>6.17a</td>
<td>237.7e</td>
<td>715.5a</td>
<td>0</td>
<td>Elevated</td>
</tr>
<tr>
<td>2.20b</td>
<td>2.45c</td>
<td>5.40b</td>
<td>265.8b</td>
<td>693.9b</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>1.50g</td>
<td>2.16e</td>
<td>3.22h</td>
<td>221.6j</td>
<td>361.2j</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.30h</td>
<td>1.60j</td>
<td>2.03m</td>
<td>274.7a</td>
<td>285.7n</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

C_Cd: Cd concentration, LA: leaf area, SLA: specific leaf area, SDW: shoot dry weight, RDW: root dry weight in each column different letters show significant differences ($P < 0.05$) between treatments

### Table 3. Results of ANOVA for leaf area (LA), specific leaf area (SLA), shoot dry weight (DWS) and root dry weight (DWR) and their ratio for wheat and sorghum plants. ns: no significant differences, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

<table>
<thead>
<tr>
<th>source</th>
<th>LA</th>
<th>SLA</th>
<th>DWS</th>
<th>DWR</th>
<th>Shoot/Root ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>32818***</td>
<td>93.5***</td>
<td>1250.8***</td>
<td>515.4***</td>
<td>50.1***</td>
</tr>
<tr>
<td>Cd</td>
<td>96177***</td>
<td>2637.7***</td>
<td>6626.6***</td>
<td>2257.3***</td>
<td>407.1***</td>
</tr>
<tr>
<td>CO₂× Cd</td>
<td>1499***</td>
<td>69.2***</td>
<td>66.0***</td>
<td>10.4***</td>
<td>ns</td>
</tr>
<tr>
<td>Plant</td>
<td>32274***</td>
<td>7170.4***</td>
<td>2240.2***</td>
<td>538.4***</td>
<td>412.4***</td>
</tr>
<tr>
<td>CO₂× Plant</td>
<td>2674***</td>
<td>2806.7***</td>
<td>5.4*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Cd× Plant</td>
<td>25011***</td>
<td>2047.9***</td>
<td>657.8***</td>
<td>417.8***</td>
<td>107.7***</td>
</tr>
<tr>
<td>CO₂× Cd× Plant</td>
<td>611***</td>
<td>147.9***</td>
<td>ns</td>
<td>7.2**</td>
<td>ns</td>
</tr>
</tbody>
</table>
Soil pH

In general, the pH values in the rhizosphere soil of wheat and sorghum trended down with increasing soil [Cd] at both ambient and e[CO₂], and e[CO₂] also tended to decrease pH; however the effects were small and were significant only at 40 mg Cd kg⁻¹ for wheat, and at 10, 20 and 40 mg Cd kg⁻¹ for sorghum (Table 4).

Soil dehydrogenase activity

At both ambient and e[CO₂], and for the two species, soil dehydrogenase activity generally decreased with increasing Cd concentration (Figure 2). Independent of Cd treatment, the dehydrogenase activity was enhanced under e[CO₂] on average by ~ 18% for wheat and ~ 28% for sorghum, respectively.

Chlorophylls and carotenoids

Concentrations of all pigments were higher in sorghum than in wheat (Figure 3). There was a negative relationship between Cd treatments and the concentrations of chlorophyll a and b, and of carotenoids, at both CO₂ levels and for both species (with the exception of sorghum at 10 mg Cd kg⁻¹). Regardless of the effect of Cd, under e[CO₂] the concentration of chlorophyll increased in

Table 4. Effect of CO₂ and Cd treatments on rhizosphere pH of wheat and sorghum.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Cd (mg/kg)</th>
<th>Ambient CO₂</th>
<th>Elevated CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>0</td>
<td>7.90 ± 0.04ab</td>
<td>7.84 ± 0.08bcd</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.92 ± 0.06ab</td>
<td>7.87 ± 0.12abc</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.89 ± 0.02abc</td>
<td>7.81 ± 0.06cd</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>7.85 ± 0.04bc</td>
<td>7.76 ± 0.03e</td>
</tr>
<tr>
<td>Sorghum</td>
<td>0</td>
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<td>7.83 ± 0.03bcd</td>
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<tr>
<td></td>
<td>10</td>
<td>7.93 ± 0.06a</td>
<td>7.81 ± 0.06cd</td>
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<td>20</td>
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<td>7.77 ± 0.09de</td>
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<td>40</td>
<td>7.81 ± 0.04cd</td>
<td>7.66 ± 0.10f</td>
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Different letters within the same row show significant differences (P < 0.05) between treatments. Each value represents the mean ± SD.
Figure 2. Effects of CO₂ (400 and 900 µL L⁻¹) and Cd (0, 10, 20 and 40 mg kg⁻¹) treatments on soil dehydrogenase activity of wheat and sorghum. Different letters show significant differences (p < 0.05) between treatments. Bars represent the standard deviation of the means. For ANOVA, ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Effects of CO₂ (400 and 900 µL L⁻¹) and Cd (0, 10, 20 and 40 mg kg⁻¹) treatments on chlorophyll a (a), chlorophyll b (b), carotenoid (c) and chlorophyll a/b ratio (d) of wheat and sorghum. Different letters show significant differences (P < 0.05) between treatments. Bars represent the standard deviation of the means. For ANOVA, ns: no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001.
wheat, while in sorghum, an increase was only observed at higher Cd treatments. Under e\(\text{CO}_2\) compared to ambient, the amount of chlorophyll b decreased in sorghum grown in Cd-amended soils, but for wheat there was no significant difference. e\(\text{CO}_2\) caused the chlorophyll a/b ratio to increase in the plants grown in Cd amended soils. e\(\text{CO}_2\) had no significant effect on carotenoid concentrations in either species (\(P > 0.05\)).

**Enzyme activities**

Enzyme activities in sorghum were higher than those of wheat in most cases (Figure 4). For wheat at ambient and e\(\text{CO}_2\), SOD activity increased as the [Cd] increased in the soil, whereas for sorghum, SOD activity decreased significantly until the 20 mg kg\(^{-1}\) Cd treatment and then rose at higher Cd treatments. For wheat in all Cd treatments, SOD activity was lower at e\(\text{CO}_2\) than ambient [CO\(_2\)]. In contrast, in sorghum, e\(\text{CO}_2\) caused the SOD activity to increase by 16.7%, 16.1%, 11.5% and 9.5% in the 0, 10, 20 and 40 mg Cd kg\(^{-1}\) treatments, respectively.

The CAT activity in wheat grown on Cd amended soils declined at both CO\(_2\) levels. The tendency of CAT activity in sorghum was similar to that of wheat, but the activity was somewhat higher than control in the 20 mg kg\(^{-1}\) Cd treatment. For the same level of Cd, the CAT activity in sorghum was higher under e\(\text{CO}_2\) than ambient CO\(_2\). The increases due to e\(\text{CO}_2\) were 13.3%, 9.5%, 15.5% and 7.2% in the 0, 10, 20 and 40 mg Cd kg\(^{-1}\) treatments, respectively. For wheat, enhanced CAT activity under e\(\text{CO}_2\) occurred only at the 0 and 10 mg kg\(^{-1}\) Cd additions.

Increasing soil [Cd] increased GSH-px activity regardless of [CO\(_2\)] and plant species. For sorghum, GSH-px activity was 20.5%, 22.6%, 27.2% and 31.4% higher in 0, 10, 20 and 40 mg Cd kg\(^{-1}\) treatments, respectively, under e\(\text{CO}_2\) than ambient whereas, in contrast, it was lower at e\(\text{CO}_2\) for wheat.

**Discussion**

Irrespective of [CO\(_2\)], increases in applied soil [Cd] caused significant decreases in plant leaf areas, and shoot and root DW, i.e., decreased plant growth. This result is in accordance with reports by Thamayanthi, Sharavanan, and Vijayaragavan (2011) and Maria, Puschenreiter, and Rivelli (2013). e\(\text{CO}_2\) had a positive effect on shoot and root DW of both species at all Cd treatments, i.e. CO\(_2\) acted as carbon fertilizer. Enhanced plant growth under e\(\text{CO}_2\) has been shown in previous studies; however, the magnitude of increase was dependent on plant species (Hogy and Fangmeier 2009, Kimball, Kobayashi, and Bindi 2002, Tian et al. 2014). Poorter (1993) reported an average increase in the DW of 41% for C\(_3\) plants and 22% for C\(_4\) plants, and Morgan et al. (2001) found that doubling the [CO\(_2\)] caused a 54% and 44% increase in the DW of shoots of C\(_3\) and C\(_4\) plants, respectively. Kimball, Kobayashi, and Bindi (2002) showed that e\(\text{CO}_2\) significantly increased biomass and yield in C\(_3\) species, such as wheat, rice, potato, but had little effect on C\(_4\) species, such as sorghum. Our study also showed that e\(\text{CO}_2\) increased shoot DW of the C\(_3\) species wheat (17–26%) more than that of the C\(_4\) species sorghum (10–18%). Similarly, e\(\text{CO}_2\) increased the root DW by 11–17% and by 8–13% for wheat and sorghum (Table 2), respectively, i.e. e\(\text{CO}_2\) had more effect on shoot than root dry weight. In all Cd treatments, as a consequence of CO\(_2\) elevation, wheat and sorghum produced larger leaf areas; therefore, e\(\text{CO}_2\) increased the shoot/root ratio. e\(\text{CO}_2\) can influence the vegetative growth of plants due to increasing leaf area thus increasing light capture (Bowes 1993). Wand, Midgley, and Jones (1999) reviewed the responses of C\(_4\) and C\(_3\) grass species to e\(\text{CO}_2\) and concluded that C\(_4\) species showed a greater increase in leaf area than C\(_3\) species. We also found that the leaf area in sorghum was more affected by e\(\text{CO}_2\) than wheat—by 18–25% for sorghum and by 13–16% for wheat—thereby reducing SLA in wheat and increasing it in sorghum.

As expected, as the soil Cd concentration increased, so too did the Cd concentration in wheat and sorghum tissues; however, in our study, plant [Cd] differed with [CO\(_2\)], and there were different Cd accumulation patterns in wheat and sorghum. A significant decrease in shoot and root [Cd] was
Figure 4. Effects of CO$_2$ (400 and 900 µL L$^{-1}$) and Cd (0, 10, 20 and 40 mg kg$^{-1}$) treatments on the activity of SOD (a), CAT (b) and GSH-px (c) in wheat and sorghum. Different letters show significant differences ($P < 0.05$) between treatments. Bars represent the standard deviation of the means. For ANOVA, ns: no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
observed in wheat, while sorghum showed a significant increase in shoot and root [Cd]. These responses can be important in terms of food safety and phytoremediation. Other studies looking at the effect of e[CO$_2$] on plants have reported both reductions and accumulations of metals. Hogy and Fangmeier (2008) showed that e[CO$_2$] caused the concentration of microelements to decrease in wheat. Jia et al. (2010) found that [Cd] decreased in Lolium species under e[CO$_2$]. Guo et al. (2006) reported a decrease in [Cd] in the grains of rice at e[CO$_2$], and Tian et al. (2014) showed a significant decrease in shoot and root Cu concentrations in wheat. Guo et al. (2015) found that e[CO$_2$] led to lower concentrations of Cu and higher [Cd] in wheat and rice. Jia et al. (2017) showed that e[CO$_2$] decreased Cd uptake by R. pseudo acacia seedlings. Li et al. (2010) showed either an increase or decrease in [Cd] for rice, depending on variety, and Wu et al. (2009) reported cesium accumulation in Sorghum vulgare var. Sudanese and Trifolium pretense under e[CO$_2$]. In our study, reduced [Cd] in wheat can be related to the dilution effect resulting from the greater enhancement of growth in wheat than sorghum. Loladze (2002) found that dilution often occurs in pot experiments. This phenomenon may lower Cd toxicity and transport in the food chain.

Heavy metal bioavailability and uptake is affected by several factors such as soil pH and microbial activity (Cornelis 2008). In previous studies, e[CO$_2$] has been shown to reduce rhizosphere soil pH and cause plants to take up more metals. Tang et al. (2003) reported higher Cu concentrations in sunflower and Indian mustard are related to lower rhizosphere soil pH under e[CO$_2$]. Wu et al. (2009) reported that e[CO$_2$] decreased soil pH by 0.2–0.4 units compared to ambient CO$_2$ and helped the plants take up more cesium. Li et al. (2010) reported a decrease in pH of 0.04–0.15 units in the soil of rice under e[CO$_2$]. Our results showed that e[CO$_2$] decreased the pH of the rhizosphere by 0.06–0.15 units for sorghum compared to ambient CO$_2$ in all soil Cd treatments (Table 4). For wheat, e[CO$_2$] caused a significant decrease of pH only in the 40 mg kg$^{-1}$ Cd treatment. The decrease in rhizosphere soil pH of sorghum compared to wheat, under e[CO$_2$], may be due to enhanced root exudation by the roots of sorghum. A reduction in pH would reduce the negative charge on clay particles and soil organic molecules (Yanai et al. 2006), and thus result in a greater release of Cd into the soil solution, and allowing greater uptake of Cd by sorghum.

Microorganisms play an important role in organic matter decomposition and uptake of metals. Microbial activity may be affected by plant species and variety and by root exudations in contaminated and uncontaminated soils (Chen et al. 2006). The effect of e[CO$_2$] on the roots of plants can change the environmental conditions for microorganisms and thus changes in soil microbial communities and their activities (Jongen and Jones 1998). The activity of dehydrogenase, an enzyme that plays an important role in the oxidation of organic matter, can be used as an indicator of microbial activity. In the present study, in all Cd treatments, dehydrogenase activity increased at e[CO$_2$] compared to ambient CO$_2$, and the percentage increase for sorghum (23–32%) was more than for wheat (16–25%). Song et al. (2012) showed that microbial biomass was increased in cesium-polluted soil by e[CO$_2$]. Blagodatskaya et al. (2010) reported that microbial biomass C increased by 30% in the rhizosphere of Populus deltoides Bartram ex Marshall due to a doubling of atmospheric [CO$_2$]. However, changes in dehydrogenase activity under e[CO$_2$] and Cd contamination can depend on plant species. Li et al. (2013) found that microbial biomass in the rhizosphere of a hyperaccumulator grown in contaminated soil increased due to e[CO$_2$], but no significant increase was seen for a non-hyperaccumulator.

In our study, at both elevated and ambient CO$_2$ levels, increases in cadmium concentration caused a significant decrease in dehydrogenase activity in the rhizosphere soils. This is in agreement with Hassan et al. (2013), who incubated three different textured soils with increasing concentrations of Cd, and with Malley, Nair, and Ho (2005), who examined the effect of Cd on phosphatase activity during vermicomposting. However, Huang et al. (2017) found that dehydrogenase activity increased in Cd- and Pb-contaminated soils under e[CO$_2$], and Kim and Kang (2011) also reported that e[CO$_2$] increased dehydrogenase activity in Pb contaminated soil. Previous results and those of this study show that the interactions between [CO$_2$] and heavy metal contaminants in soil are complex.
and depend on the plant species and characteristics of metal. Our study used a pot experiment in a growth chamber, so the results may differ from those in the field.

Chlorophyll synthesis is the first step in photosynthesis, and our results show that at the two CO₂ levels, the chlorophyll content decreased with the increasing Cd concentration in the soil (Figure 3), which is consistent with the findings of Thamayanthi, Sharavanan, and Vijayaragavan (2011) and Amani (2008). Compared to ambient CO₂, e[CO₂] increased the concentration of Chl a in wheat at all Cd treatments, while in sorghum increases were restricted to the two highest Cd treatments. The increased chlorophyll content would improve photosynthetic capacity of the species and help to maintain growth under e[CO₂]. Increased chlorophyll contents under Cd stress at e[CO₂] were also reported in Lolium spp. (Jia et al. 2010). Looking at fractions of chlorophyll, contrary to the increase in Chl a, Chl b decreased significantly under e[CO₂] in sorghum grown in Cd-amended soils; however, in wheat, there was no significant difference in Chl b contents compared to ambient CO₂. The reduction in Chl b in sorghum resulted in an increase in Chl a/b ratio under [eCO₂]. The effects of Cd and e[CO₂] on the Chl a/b ratio are variable with increases, decreases or no change in this ratio reported for both factors (Gadallah 1995, Jia et al. 2010), and Gomes et al. (2011) suggested that heavy metal contamination may affect the production of Chl a more than Chl b.

Heavy metals such as Cd induce reactive oxygen species and cause oxidative stress to plants (Ekmeekci, Tanyolac, and Ayhan 2008, Shah et al. 2001). One of the reasons for reductions in chlorophyll content due to Cd has been ascribed to oxidative damage (Chaoui et al. 1997), and plants use enzymatic and non-enzymatic defense systems to minimize oxidative stress (Mittler et al. 2004). In this study, regardless of [CO₂], SOD activities increased with increasing Cd concentration in wheat. In contrast, in sorghum, SOD activity reduced at the two lower Cd concentrations and was enhanced at the higher Cd treatment. For both species, as [Cd] increased, CAT activity decreased (with the exception of the 20 mg Cd kg⁻¹ treatment) and GSH-px activity increased (Figure 4). Increases and decreases in antioxidants activity in different plants have been detected in previous studies (Posmyk, Kontek, and Janas 2009, Qiu et al. 2008, Shafi et al. 2009, Soudek et al. 2014). The variation in the activities of these enzymes both between species and at different Cd treatments may be attributed to relative changes in oxidative stress, the induction of genes, levels of cellular damage and the unbound [Cd] in leaf tissues. SOD activity varies with stress intensity, plant part and species (Piquery et al., 2000). Increasing SOD activity can be due to increased production of free radicals leading to the expression of genes encoding SOD (Bowler, Van Montagu, and Inze 1992). In sorghum, the variation in SOD activity may be due to these interactions in combination with SOD deactivation. With more stress, cells start to produce SOD again to eliminate free radicals. As the results show, for both species at the highest Cd treatment SOD activity increased, while CAT activity declined. The CAT enzyme is sensitive to the superoxide anion, and high concentrations of this radical can cause its deactivation (Cakmak, Strboe, and Marschner 1993). Work with a catalase-deficient mutant suggests that this enzyme does not play a crucial role in protecting against Cd toxicity (Iannone, Groppa, and Benavides 2015). It seems that the low activity of CAT was compensated by high activity of GSH-px, which is induced by stress, and uses reduced glutathione and the ascorbate-glutathione cycle to reduce H₂O₂ (Posmyk, Kontek, and Janas 2009). Plants’ antioxidant defense systems and glyoxalase systems both use GSH-dependent pathways to detoxify reactive oxygen species(ROS) and methylglyoxal (MG), respectively. Under stress conditions, MG concentration in plants can be increased 2- to 6-fold compared with normal conditions depending on the plant species.

A number of studies have examined the effect of e[CO₂] on the antioxidative capacity of plants and the activities of antioxidative enzymes (Schwanz et al. 1996; Jia et al. 2010, Lolium spp.; Gillespie, Rogers, and Ainsworth 2011, Glycine max; Guo et al. 2015, Oryza sativa; Pietrini et al. 2016, L. minor plants). In general, these studies have found that at e[CO₂] markers of antioxidant stress are lower, antioxidant capacity is raised and the antioxidant activities are lower, and it is suggested that plants grown at e[CO₂] are better protected from oxidative stresses (Polle et al.
In our study, this situation was found for wheat, as the activities of SOD and GSH-px were clearly reduced at e\([\text{CO}_2]\) and the activities of CAT marginally so. However, for sorghum, the opposite was found. Jia et al. (2010), working with two species of *Lolium*, also found that SOD activity was higher for a given Cd addition at e\([\text{CO}_2]\) and attributed the differences to increases in gene expression. There is little information about the combined effects of heavy metals and e\([\text{CO}_2]\) on the activity of antioxidant enzymes, and in our study the interspecific differences include effects on internal Cd concentration.

The latter effect encouraged us to examine the relation between GSH-px and the internal [Cd] with remarkable effect, i.e., although the GSH-px activity varied between plant species and with [CO\(_2\)], the overriding control was due to the internal Cd concentration: so much so that a single strong relation describes all the data (\(r^2 = 0.94\), Figure 5). Whether this finding is peculiar to our study or may apply more generally merits further investigation.

**Conclusions**

The effect of e\([\text{CO}_2]\) on the C\(_3\) (wheat) and C\(_4\) (sorghum) cvv was to increase chlorophyll concentration and leaf area and thus photosynthesis of both wheat and sorghum, leading to increased biomass production at different levels of Cd additions. Sorghum grown at e\([\text{CO}_2]\), accumulated a greater Cd concentration, which in part may be attributed to a lower pH and higher dehydrogenase activity in the rhizosphere. The activities of antioxidant enzymes were higher in sorghum than in wheat; however a single relation describes the GSH-px data for both species and all the treatments. Our data were obtained for a limited genetic range of C\(_3\) and C\(_4\) plants, under controlled conditions, and results for a wider array of genotypes in the field may differ. Such experiments will improve our understanding of the interactions between heavy metals and e\([\text{CO}_2]\): knowledge that is essential to managing effects of future climate change on plant productivity and on human and animal health.

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References


