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Original Research

Effects of Elevated [CO_2] on Photosynthetic Performance and Water Use Efficiency of Maize (*Zea mays* L.) under Cadmium Stress

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Abstract

The responses of maize plants to rising atmospheric concentration ([CO_2]) and cadmium (Cd) stress are crucial for accurately estimating corn production under future climate change. Here, we investigated the effects of CO_2 enrichment on leaf photosynthesis and water use efficiency of maize grown under four levels of Cd stress. We found that the net photosynthetic rates of maize plants at control and mild Cd stress were substantially increased by elevated [CO_2], which indicated a strong CO_2 fertilization effect on maize plants. However, this CO_2 fertilization effect on leaf photosynthesis disappeared when maize plants were subjected to moderate and severe Cd stress. The down-regulation of the high [CO_2] fertilization effect on leaf photosynthesis by Cd stress was primarily driven by reduced stomatal diffusional efficiency. This decline in stomatal diffusional efficiency was further caused by decreased regularity in stomatal distribution patterns under Cd stress. Moreover, the down-regulation of these positive fertilization effects was associated with decreases in photosynthetic pigments and peroxidase activity, which might also contribute to the down-regulation of the CO_2 fertilization effect. In addition, our results also showed that elevated [CO_2] enhanced the leaf-level intrinsic water-use efficiency (WUEI). Overall, our results demonstrated that Cd stress dampens the positive fertilization effect of leaf photosynthesis to elevated [CO_2] by altering stomatal distribution, photosynthetic pigments, and antioxidant enzyme activity. Therefore, our results suggest that current ecological-process models based on earlier results from doubling CO_2 experiments may overestimate the positive effects of elevated [CO_2], and meanwhile underestimate the risk of global change on maize yield and agricultural production.

Keywords: elevated [CO_2], Cd stress, leaf photosynthesis, stomatal morphology, water use efficiency

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Introduction

Human activities have imposed profound perturbations on the ecological environment, with carbon dioxide emissions intensified and heavy metal pollution increasingly accentuated since the Industrial Revolution. Pre-industrially, the CO_2 flux balance among the atmosphere, ocean, and terrestrial biosphere stood at 278 μmol mol^{-1}, whereas atmospheric greenhouse gas concentrations, especially CO_2, have increased due to extensive fossil fuel combustion, large-scale deforestation, and substantial land-use transformations, with the global average [CO_2] increasing at a rate of 2.4 μmol mol^{-1} per year over the past decade and reaching a historic record of 420 μmol mol^{-1} in 2023 [1]. Concurrently, Cd, a highly toxic non-essential heavy metal ranking 7th among the 20 most hazardous metals and classified as a Group 1 carcinogen [2], is widely distributed in the natural environment as geological weathering accelerates, industrialization advances, and the application of Cd-containing fertilizers and pesticides in agriculture expands, leading to continuously increasing Cd content in ecological media such as soil and water bodies [2, 3].

It is well known that Cd not only causes genetic damage in plants, leading to cellular dysfunction and hindering normal growth and development [4-8], but also accumulates at high levels in organisms along the food chain, affecting human health [3], especially in severely Cd-contaminated regions of southern China [2]. Conversely, rising atmospheric [CO_2] may benefit plants through the CO_2 fertilization effect, which is associated with promoting leaf photosynthesis and plant growth [9], inhibiting leaf dark respiration and photorespiration [10], and accumulating soluble sugars and starch [11]. Thus, under future climate change, plants may face combined impacts of elevated [CO_2] and Cd stress, which could exert interactive effects on plant growth and physiological processes, thereby drastically altering the structure and function of natural and managed ecosystems.

Plant growth responses to elevated [CO_2] and Cd stress are fundamentally adjusted by physiological and biochemical processes such as leaf photosynthesis [11], leaf respiration [10], and accumulation of nonstructural carbohydrates [9]. However, the roles of Cd stress in plant responses to elevated [CO_2] remain a critical unresolved issue [12].

Cd^{2+} stress is a major abiotic stressor affecting plant photosynthesis, whose inhibition of photosynthesis primarily leads to decreased carbohydrate availability [6, 13-17]. Specifically, the down-regulation of leaf photosynthesis under Cd stress may also relate to reduced chlorophyll fluorescence and photosynthetic pigment contents [18], decreased ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) activity and protein contents [19]. Notably, reduced expression levels of Cd-tolerance coding genes may also indirectly lead to photosynthetic down-regulation by affecting related metabolic pathways [8]. Additionally, many studies found that Cd-induced down-regulation of leaf photosynthesis may be linked to plant allocation of photosynthates to roots [20] as a strategy to resist root Cd^{2+} absorption.

In order to alleviate Cd stress, plants require more carbohydrates, which are produced by photosynthesis, to increase osmotic pressure for stress resistance. It is known that CO_2 is an indispensable substrate for plant photosynthesis, and the current atmospheric [CO_2] remains significantly below the saturation threshold for plant photosynthesis, which indicates crops can potentially gain growth advantages from the “fertilization effect” of high [CO_2] [21]. Moreover, elevated [CO_2] can provide a sustainable environment for microorganisms to alleviate Cd stress, which not only increases soil dissolved organic carbon and organic compound levels [22], but also enhances microbial metabolic activity and enzymatic reaction efficiency, and promotes heavy metal removal in rhizosphere soil [8, 23]. Most of all, elevated [CO_2] is able to increase leaf net photosynthetic rate, producing more carbohydrates to mitigate Cd stress. Although current research on leaf photosynthesis under Cd stress remains limited, existing studies demonstrate that elevated [CO_2] enhances net photosynthetic rate (P_n) and maximum photochemical quantum yield of PSII (F_v/F_m) while significantly reducing stomatal conductance (G_s) and transpiration rate (T_r) [6, 8]. Meanwhile, high-concentration CO_2 not only causes severe stomatal closure but also slows the tricarboxylic acid (TCA) cycle rate [24]. However, few studies have examined the effects of high-concentration CO_2 on stomatal distribution patterns related to photosynthesis under Cd stress [25, 26], especially regarding the expression of Cd-tolerance coding genes when high-concentration CO_2 alleviates Cd stress [27].

Maize is one of the most important cereal crops in the world; the global annual cultivation area for maize approximates 197 million hectares [28]. Meanwhile, 90% of global soil resources may be at risk of pollution by 2050 due to soil erosion, excessive fertilizer or pesticide use, and industrial pollution, with Cd showing the highest global exceedance rate at 9.0% among heavy metals [2]. Against the background of rising global atmospheric CO_2 concentration and aggravated soil Cd pollution, the plant growth of maize is confronted with dual environmental challenges. Unfortunately, it remains unclear whether this CO_2-induced stimulation occurs in maize yield under soil Cd stress, and whether soil Cd stress dampens the magnitude of the CO_2 fertilization effect on maize plants, mainly due to a lack of experimental evidence. These knowledge gaps create major uncertainties in future food security and production projections. Thus, fully understanding the underlying mechanisms and processes of maize responses to elevated [CO_2] and soil Cd stress is crucial for estimating the potential impacts of the CO_2 fertilization effect on the grain yield of maize under Cd stress in China.

To fill these knowledge gaps, we conducted a manipulation experiment using environmental growth chambers. The objectives of the current study were (1) to investigate the combined effects of elevated [CO_2] and Cd stress on plant growth and leaf photosynthesis of maize, and (2) to explore the potential photochemical and biochemical processes and mechanisms by which the CO_2 fertilization effect mitigates Cd stress on maize.

Specifically, we tested the following hypotheses:

(1) Elevated [CO_2] stimulates vegetative growth and leaf photosynthesis of maize under Cd stress (HY1);

(2) Down-regulation of the CO_2 fertilization effect under Cd stress is associated with changes in stomatal distribution, leaf functional traits, and biochemical processes (HY2);

(3) The CO_2 fertilization effect on leaf photosynthesis under Cd stress can be explained by enhanced expression levels of Cd-tolerance genes (HY3).

Materials and Methods

Environmental Growth Chamber Experiment

The maize cultivar 'Zhengdan 958' (*Zea mays* L. cv. Zhengdan 958), which accounts for 9% of China's total maize planting area [29], was used as the test material. Seedlings were transplanted into plastic pots (top surface area: 272 cm^2, bottom surface area: 189 cm^2, height: 33 cm) filled with native soil sourced from the farm of Hebei University of Engineering in North China, which was mixed with organic nutrient soil at a 2:1 volume ratio (5.30 g/kg organic matter, 0.88 g/kg total nitrogen, 0.59 g/kg total phosphorus, 22 mg/kg available phosphorus, 60 mg/kg available potassium, pH 7.76) to sustain maize seedling growth and enhance relevance to field conditions. To reduce soil compaction and minimize the impacts of irrigation, small stones were spread over the bottom of the pots, acting as a filtration layer. Maize seeds were grown for 30 days in a greenhouse to develop canopies and roots under conditions of 30/25ºC day/night air temperature, 60%-65% relative humidity (RH), 400 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and a 12 h photoperiod (light from 8:00 to 20:00 daily). A split-plot experimental design was arranged, consisting of two factors ([CO_2] and Cd stress) with CO_2 concentration as the main plot (two CO_2 levels) and Cd stress (four different $CdCl_2$ dosages) as the subplot. Two large environmental growth chambers were employed for sustaining plant growth and controlling CO_2 concentrations, where the CO_2 concentration in one environmental growth chamber was supplied with 400 μmol mol^{-1} (a[CO_2]), and the target CO_2 concentration in the other growth chamber was maintained at 800 μmol mol^{-1} (e[CO_2]) and the [CO_2] values in these growth chambers for a[CO_2] or e[CO_2] were within 20 μmol mol^{-1} around the target [CO_2]. The space inside these environmental growth chambers (2.80 m high × 6.10 m long × 2.43 m wide) was large enough for maize plant growth. In addition to [CO_2], the other environmental variables in the two environmental growth chambers were maintained at 30/25ºC (day/night) air temperature, 65% RH, 1600 μmol m^{-2} s^{-1} PAR, and 12-h photoperiod from 8:00 to 20:00. In the current study, we applied four $CdCl_2$ levels: control (0 mg kg^{-1}), mild (40 mg kg^{-1}), moderate (200 mg kg^{-1}), and severe (400 mg kg^{-1}) [10, 30].

After the 30-day canopy establishment, 32 pots of maize seedlings were selected and randomly set up in each environmental growth chamber, representing four biological replicates (n = 4) of the four $CdCl_2$ levels. Before seedling transplantation, the dry weight of soil in each pot was measured to calculate the $CdCl_2$ application rate for each treatment. Then, during the treatment period, 1 L of $CdCl_2$ solution at the calculated dosages was watered to each pot every seven days. Throughout the entire growth period, maize plants were fertilized with half-strength Hoagland's solution (150 mL per pot) weekly [30, 31]. Additionally, maize plants were relocated among different growth chambers once per week during the vegetative growth of maize plants to minimize the confounding effect of environmental variation between different growth chambers [32].

Measuring Plant Biomass and Nonstructural Carbohydrates of Vegetative Organs

Plant biomass was obtained by harvesting and de-potting the leaves, stems, and roots with scissors after 90 days of treatment (maturity stage). Then, plant tissues were placed in paper bags and oven-dried at 85ºC for 72 h to a constant weight. The dry weight of maize plants was quantified with an electronic scale (FA2004, Shanghai Shangping Instrument Co., Ltd., China). For foliar nonstructural carbohydrate analysis, vegetative organ samples were oven-dried again at 75ºC for 72 h to a constant weight, milled into fine powder with a ball mill, and approximately 50 mg of dry powder per sample was extracted with 80% ethanol (v/v) at 85ºC for 60 min. Then, the extracts were centrifuged (12000 × g, 10 min, repeated three times), and the combined supernatants were analyzed for soluble sugars via spectrophotometry [32, 33], while residues were oven-dried (60ºC overnight) for starch analysis [34]. All data of soluble sugars and starch were expressed as a percentage of dry matter with four biological replicates in this study [10].

Measuring Leaf Gas Exchange

After 60 days of treatment (grain fill period), a new fully expanded leaf from each pot was randomly chosen to measure leaf gas exchange using a portable photosynthesis measurement system (LI-6800, LI-COR Biosciences, Lincoln, NE, USA) equipped with a leaf chamber fluorometer (LI-6800-01A with 90% red and 10% blue). We measured leaf net photosynthetic rates (P_n), leaf stomatal conductance (G_s), and leaf transpiration rates (T_r) at PPFD of 1600 μmol m^{-2} s^{-1}, growth temperature of 30°C, and growth [CO_2] of 400 or 800 μmol mol^{-1}. All of the measurements on leaf gas exchange were performed under leaf-to-air vapor pressure deficit (VPD) of 0.9 to 1.5 kPa to eliminate moisture limitation. The intrinsic water-use efficiency (WUEI) was calculated as the ratio of P_n to T_r [10]. After finishing the above measurements, the LED light source of the LI-6800 system was turned off, and the chamber was covered with a black cloth for 20 min to achieve dark adaptation, followed by the measurement of leaf dark respiration (R_d) at 30°C and each growth CO_2 level (400 or 800 μmol mol^{-1}).

Measuring Stomatal Traits

We selected one ear-leaf from each pot and used a widely employed method to characterize individual stomatal morphological traits [31, 35] at approximately 70 days after treatment (grain fill period), collecting stomatal imprints from the middle section of both adaxial and abaxial leaf surfaces with colorless nail polish. Then, we observed and photographed the sampled stomatal imprints in the laboratory using a light microscope (DM2500, Leica, Wetzlar, Germany), randomly capturing 5 images per leaf for each treatment, selecting 5 stomata from each image (5 stomata per image × 5 images per leaf × 4 leaves per treatment = 100 stomata total per treatment), and measuring stomatal length (SL), stomatal width (SW), stomatal aperture circumference (SAC), and stomatal area (SA) using the Autodesk program. Furthermore, following Liu et al. [30] and Xu [35], we captured 5 images per imprint, quantified stomatal density (SD), and used GIS software (ArcGIS 10.5, ESRI Inc., Redlands, CA) to perform point pattern analyses with the Ripley's K-function to evaluate two-dimensional distribution patterns at different scales, thereby evaluating the spatial distribution of stomata on each leaf surface.

Measuring Photosynthetic Pigments and Cd-tolerant Genes Expression Levels

We collected 0.2 g of fresh ear-leaf samples from maize plants and carefully immersed these samples in dimethyl sulfoxide (DMSO) in the dark for 72 h to determine the contents of chlorophyll (Chl) and carotenoids (Car). Subsequently, the concentrations of Chl a, Chl b, and Car were measured using a spectrophotometer (UV-1750, Shimadzu Corp., Tokyo, Japan) at the respective wavelengths of 663 nm, 645 nm, and 470 nm [10]. To further explore the underlying molecular processes and mechanisms of maize in response to [CO_2] and Cd stress, we determined the expression levels of Cd-ion tolerance-associated genes at the end of the 60-day treatment, and quantified shifts in the expression of coding genes *ZmNramp*1, *ZmHMA*2, *ZmHMA*3, and *ZmWRKY*64 using quantitative real-time PCR (qRT-PCR). For this qRT-PCR analysis, we first extracted total RNA using the TRNzol Universal Reagent (Sanshi, Hebei, China) and then reverse-transcribed roughly 1 μg of the extracted RNA to cDNA with the SanshiBio™ RT reagent Kit with gDNA Eraser (Sanshi, Hebei, China). Subsequently, we performed qRT-PCR on an Applied Biosystems 7300 Real-Time PCR System using the TB Green® Premix Ex Taq™ II kit (Foregene, Chengdu, China), following a program that included one cycle at 95ºC for 2 min and 40 subsequent cycles at 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s. Meanwhile, gene expression was quantified using the $2^{-\Delta Ct}$ method [16], with the β-actin gene serving as the reference standard (primers listed in Table 1) and three technical replicates performed for each biological replicate to ensure reliability.

Measuring SOD and POD Activity, MDA Levels

We collected fresh ear-leaf samples (0.1 g) from maize plants, washed these samples, and homogenized them with a mortar and pestle in ice-cold phosphate buffer (0.1 M PBS, pH 7.2) at 0-4°C using a 1:9 (w/v, tissue: buffer) ratio. After centrifugation at 3500×g for 10 min at 4ºC, the supernatant was transferred to 1.5 mL cryotubes and stored at -80ºC for subsequent enzyme activity and malondialdehyde (MDA) content assays. MDA, peroxidase (POD), and superoxide dismutase (SOD) were analyzed using commercial kits (Jiancheng Bioengineering, Nanjing, China), with absorbance measured at 532 nm, 420 nm, and 550 nm, respectively, to calculate the content of MDA and the activities of POD and SOD.

Table 1. Primers information for qRT-PCR.

Gene name	The upstream primer (5'→3')	The downstream primer (5'→3')
*ZmNramp*1	ATCGTGTTCTCGTGGCTG	GAAGGTGAGGTAGATGACGG
*ZmHMA*2	ACCATCAACTTAGACGGCTAC	ACATGCAAAAGACACAACCAC
*ZmHMA*3	TCGAGCATTGAGAGCAAGTC	TGCCATGATCCTTGTGTTCC
*ZmWRKY*64	ACGACAGCATCCCAAGAGA	GGAAACGGCGACTTAGTGATC

Statistical Analysis

Employing a split-plot design and subjecting the data to two-way ANOVA in SPSS 27.0 (SPSS Inc., Chicago, IL, United States), we evaluated the main and interactive effects of [CO_2] and Cd stress on plant biomass, stomatal traits, leaf gas exchange, photosynthetic pigments, and nonstructural carbohydrates in maize vegetative organs. For any physiological, biochemical, or stomatal variables that exhibited significant [CO_2] or Cd stress effects, pairwise differences among treatments were identified with the Student-Newman-Keuls method at $P<0.05$.

Results and Discussion

Plant Biomass and Allocation

Elevated [CO_2] substantially increased leaf fresh weight by 31.2% ($P = 0.010$), stem fresh weight by 22.0% ($P = 0.033$), and aboveground fresh weight by 34.0% ($P<0.001$) compared to *a*[CO_2] in the control treatment. However, Cd stress exerted strong suppression on stem fresh weight ($P = 0.010$) and aboveground fresh weight ($P<0.001$), with decreasing trends observed as Cd concentrations increased under both [CO_2] levels, demonstrating a pronounced CO_2 fertilization effect on maize fresh biomass, which disappeared when maize plants were exposed to Cd stress. Notably, mild Cd stress under *a*[CO_2] increased the fresh weights of leaves, stems, and aboveground parts compared to the control treatment. In addition, an interactive effect of [CO_2] and Cd stress was detected on the leaf fresh weight ($P = 0.010$) and aboveground fresh weight ($P = 0.010$) in maize (Table 2).

Similarly, the CO_2 fertilization effect was observed for the ratio of shoot-to-root dry weight ($P<0.001$) and leaf dry weight ($P = 0.004$), while for dry grain yield ($P = 0.010$), this effect was only detected under mild and moderate Cd treatment levels but not observed under severe Cd stress. Specifically, severe Cd stress reduced leaf dry weight by 10.8% (*a*[CO_2]) and 34.1% (*e*[CO_2]), and grain yield by 2.6% (*a*[CO_2]) and 62.6% (*e*[CO_2]). Moreover, we also found interactive effects of CO_2 concentration and Cd stress on leaf dry weight ($P<0.001$, Table 2).

Few reports indicate that there may be a biomass enhancement at low Cd concentrations [36], but many references show that higher Cd stress can decrease photosynthetic efficiency, enzyme-catalyzed reactions, and gene expression, resulting in a decrease in maize biomass [4, 6, 8, 12]. However, elevated [CO_2] can increase the carbon assimilatory rate of maize leaves [37], and as the content of carbohydrates produced by photosynthesis increased, the tolerance of maize to Cd stress was enhanced. However, the CO_2 fertilization effect was so limited that Cd stress could still reduce the biomass of different organs in maize, thereby indicating

Table 2. Effects of elevated CO_2 concentration and Cadmium stress on plant biomass.

CO_2 concentration µmol mol^{-1}	Cd stress	Leaf fresh weight g plant^{-1}	Stem fresh weight g plant^{-1}	Aboveground fresh weight g plant^{-1}	Root dry weight g plant^{-1}	Leaf dry weight g plant^{-1}	Stem dry weight g plant^{-1}	Yield dry weight g plant^{-1}	Aboveground dry weight g plant^{-1}	Shoot-to-root dry weight ratio %
a[CO_2]	Control	35.9(2.3)	186.7(10.9)	366.5(11.5)	20.7(1.4)	20.4(0.4)	51.4(1.8)	25.0(2.6)	99.8(8.7)	480.8(20.6)
	Mild	45.0(2.4)	188.2(11.4)	420.0(14.2)	28.5(3.2)	24.1(0.7)	52.1(4.0)	60.6(3.5)	129.8(12.9)	470.0(60.3)
	Moderate	39.7(3.3)	174.5(11.1)	393.3(17.5)	23.1(0.4)	21.1(0.3)	50.4(4.1)	36.6(6.8)	124.0(2.0)	538.0(17.1)
	Severe	34.0(1.7)	157.7(14.1)	339.4(17.1)	20.6(2.0)	18.2(1.3)	48.6(3.6)	31.4(10.7)	100.8(11.5)	484.5(24.2)
e[CO_2]	Control	47.1(5.0)	227.7(14.2)	491.1(31.9)	20.1(2.6)	27.3(1.0)	43.3(4.1)	65.5(4.6)	128.3(14.5)	690.8(121.5)
	Mild	35.6(1.9)	168.5(7.4)	400.5(10.2)	20.2(2.9)	24.1(1.2)	34.3(1.4)	68.8(3.2)	138.0(5.0)	719.3(72.5)
	Moderate	33.7(3.0)	168.2(18.4)	377.6(18.3)	15.8(1.4)	22.1(0.3)	31.9(1.1)	58.5(3.5)	122.5(4.4)	794.5(67.2)
	Severe	35.5(1.5)	154.3(11.9)	340.3(22.3)	19.9(5.5)	18.0(1.0)	32.3(4.0)	24.5(10.0)	83.5(5.5)	491.5(79.9)
CO_2		$P = 0.750$	$P = 0.750$	$P = 0.160$	$P = 0.050$	$P<0.001$	$P<0.001$	$P = 0.010$	$P = 0.560$	$P<0.001$
Cd		$P = 0.090$	$P = 0.010$	$P<0.001$	$P = 0.330$	$P<0.001$	$P = 0.170$	$P<0.001$	$P<0.001$	$P = 0.170$
CO_2×Cd		$P = 0.010$	$P = 0.120$	$P = 0.010$	$P = 0.380$	$P<0.001$	$P = 0.390$	$P = 0.040$	$P = 0.200$	$P = 0.350$

that it could not fully offset Cd toxicity in maize, especially under severe Cd stress.

Stomatal Morphology and Spatial Distribution Pattern

Our results showed that stomata were generally distributed on both the adaxial and abaxial leaf surfaces of maize (Table 3). However, stomatal density (SD), defined as the number of stomata per unit leaf area, was obviously higher on the abaxial surface than on the adaxial surface. Elevated [CO_2] had a significant effect increasing SD under the same Cd stress on both adaxial ($P<0.001$) and abaxial ($P = 0.001$) surfaces; however, Cd only had a significant effect on the adaxial surface ($P = 0.017$), and the interactive effects of CO_2 concentration and Cd stress were also exclusively observed on the adaxial surface ($P<0.001$). Previous studies reported that cadmium toxicity causes a reduction in stomatal density [38], which is consistent with our results. We found that severe Cd stress distinctly reduced SD on both the adaxial (19.0%) and abaxial (15.5%) surfaces compared to the control under ambient a[CO_2], however, when [CO_2] was elevated, the control group showed increased SD on both the adaxial (55.6%) and abaxial (4.4%) surfaces relative to a[CO_2] conditions. This implies that the CO_2 fertilization effect can increase leaf stomatal density, thereby inducing higher initial P_n and faster photosynthetic induction, and may ultimately lead to greater biomass production [25].

By comparing the control groups under a[CO_2] and e[CO_2], we found that elevated [CO_2] increased stomatal traits to different extents. On the abaxial surface, elevated [CO_2] increased SW by 17.7%, SA by 13.3%, and SAC by 3.6%, while on the adaxial surface, e[CO_2] increased SL by 0.6%, SW by 7.5%, SA by 14.6%, and SAC by 17.4%, respectively. In contrast, when maize was exposed to Cd stress, these stomatal traits (SL, SW, SA, and SAC) were different between the abaxial and adaxial surfaces. Specifically, on the adaxial surface, severe Cd stress increased SL, SW, SA, and SAC compared to the control

Table 3. Effects of CO_2 concentration and Cd stress on stomatal traits in maize leaves.

Stomatal traits	CO_2 concentration μmol mol^{-1}	Cd stress	SD mm^{-2}	SL μm	SW μm	SA μm^2	SAC μm
Adaxial surface	a[CO_2]	Control	63(1)	18.0(0.6)	9.3(0.5)	103(4)	42.5(0.8)
		Mild	57(2)	18.6(0.5)	9.6(0.5)	119(7)	45.7(1.2)
		Moderate	61(3)	16.5(0.4)	9.3(0.4)	97(4)	40.7(0.7)
		Severe	51(2)	18.5(0.5)	11.9(0.3)	126(4)	49.9(0.9)
	e[CO_2]	Control	54(5)	18.1(0.5)	10(0.5)	118(7)	45.4(1.2)
		Mild	65(5)	17.0(0.6)	9.8(0.2)	106(5)	44.2(1.3)
		Moderate	74(3)	19.1(0.6)	9.4(0.3)	123(2)	47.6(1)
		Severe	84(4)	17.7(0.5)	9.5(0.3)	115(4)	46(1.1)
Abaxial surface	a[CO_2]	Control	84(4)	16.3(0.4)	9.6(0.5)	98(5)	41.2(1.2)
		Mild	85(3)	16.9(0.4)	10.6(0.5)	104(4)	43.3(0.7)
		Moderate	84(4)	17.5(0.5)	11.5(0.4)	117(5)	46.3(1.1)
		Severe	71(4)	15.3(0.4)	9.5(0.5)	92(6)	39.2(1)
	e[CO_2]	Control	90(2)	16.3(0.5)	11.3(0.7)	111(6)	42.7(1.3)
		Mild	95(4)	16.0(0.5)	9.5(0.6)	91(5)	39.8(1)
		Moderate	86(2)	15.3(0.8)	8.1(0.6)	92(8)	38.8(1.9)
		Severe	94(3)	13.9(0.6)	6.9(0.5)	72(6)	34.3(1.6)
Adaxial surface	CO_2		$P<0.001$	$P = 0.848$	$P = 0.204$	$P = 0.142$	$P = 0.195$
	Cd		$P = 0.017$	$P = 0.927$	$P = 0.008$	$P = 0.001$	$P = 0.119$
	CO_2×Cd		$P<0.001$	$P = 0.001$	$P = 0.001$	$P<0.001$	$P<0.001$
Abaxial surface	CO_2		$P = 0.001$	$P = 0.004$	$P = 0.001$	$P = 0.012$	$P<0.001$
	Cd		$P = 0.315$	$P = 0.002$	$P<0.001$	$P = 0.001$	$P<0.001$
	CO_2×Cd		$P = 0.071$	$P = 0.280$	$P<0.001$	$P = 0.013$	$P = 0.009$

under a[CO_2], but decreased these four traits compared to the control under e[CO_2], while on the abaxial surface, severe Cd stress reduced SL, SW, SA, and SAC relative to the control regardless of CO_2 concentration. These results suggested that the observed alterations in stomatal traits under cadmium stress represent an adaptive response of the plants to heavy metal stress, which may enhance the photosynthetic compensation mechanism [39, 40]. Additionally, the interactive effects of CO_2 and Cd also showed differences between the adaxial and abaxial surfaces. On the adaxial surface, we found interactive effects of CO_2 concentration and Cd stress on all measured stomatal traits, whereas on the abaxial surface, we revealed this interaction on SW ($P = 0.001$), SA ($P = 0.013$), and SAC ($P = 0.009$).

Elevated CO_2 concentration and Cd stress not only affected the morphological properties of individual stomata but also modulated the spatial organization of stomata on maize leaves. Spatial pattern analysis with Ripley's K-function revealed a strong scale-dependence in maize stomatal distribution on both leaf surfaces, with significant regular patterns observed at small scales (below the lower 95% confidence envelope) transitioning to random patterns at larger scales (within the 95% confidence envelopes), a phenomenon that remained consistent across all CO_2 and Cd stress treatments [32] (Fig. 1). Moreover, we also found that elevated [CO_2] significantly enhanced the regularity of stomatal distribution on both adaxial (Fig. 1a) and b)) and abaxial (Fig. 1c) and d)) leaf surfaces under control, mild, and moderate Cd stress conditions. However, this effect was absent under severe Cd stress, implying that e[CO_2] enhanced the regularity of stomatal distribution on both surfaces, whereas Cd stress, particularly under severe Cd stress, specifically reduced regular patterns on the adaxial surfaces. Collectively, these results suggested that while Cd stress disrupted the regular distribution pattern of stomata, thereby negatively impacting gas exchange, this adverse effect was mitigated by e[CO_2]. The more regular spatial distribution pattern under e[CO_2] enhanced substrate supply and improved photosynthesis in maize [41], further implying that e[CO_2] can mitigate Cd stress to a certain degree.

Leaf Photosynthesis, Stomatal Conductance, Transpiration Rate, and WUEI

Many reports have demonstrated that exposure of plants to Cd stress reduces stomatal conductance [14, 17, 42, 43], blocks PSII photosynthetic electron transport, and inhibits carbon assimilation. Consistent with previous studies, our results show that severe Cd stress decreased P_n, G_s and T_r under both a[CO_2] and e[CO_2]. However, due to the CO_2 fertilization effect, e[CO_2] substantially increased P_n by 14.4% and 5.6% under control and mild Cd stress conditions, respectively,

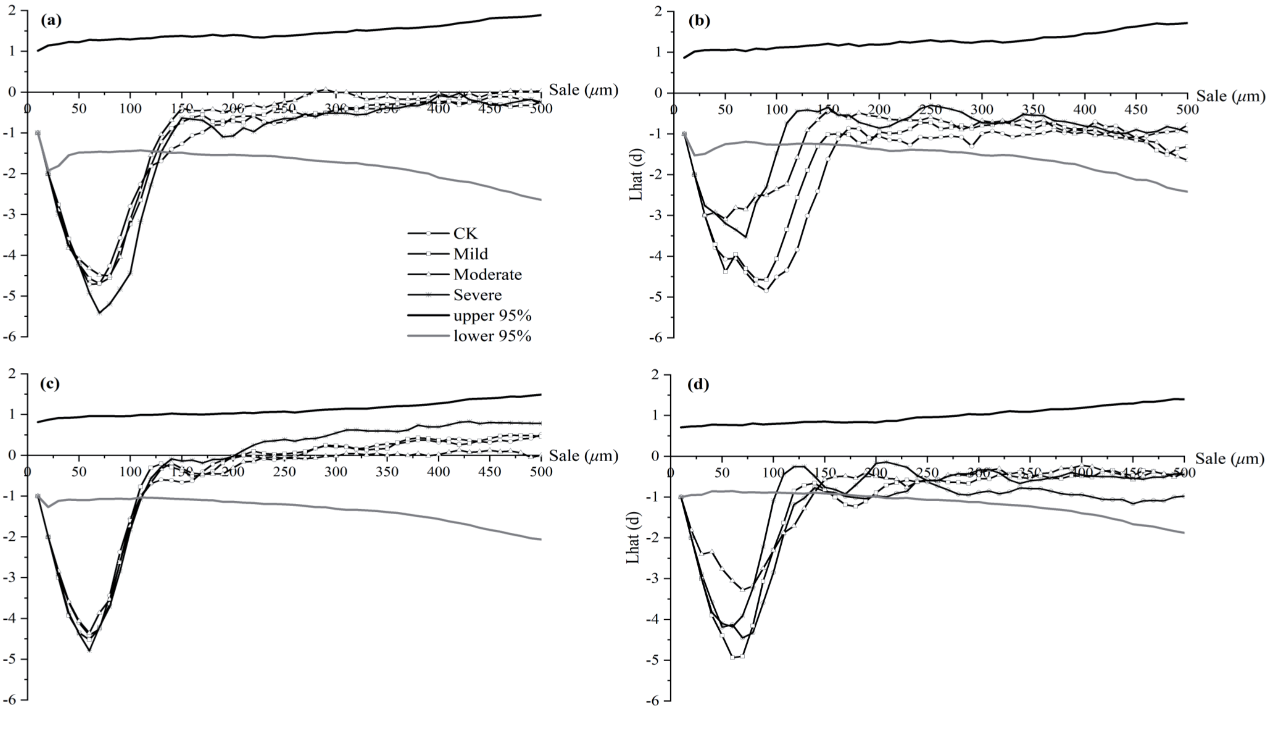

Fig. 1. Effects of CO_2 concentration and Cd stress on stomatal distribution of maize.
Note: Spatial distribution pattern of stomata on the leaves of maize grown at (a, c) a[CO_2] and (b, d) e[CO_2] along a soil $CdCl_2$ gradient. Note that elevated [CO_2] made the stomatal distribution pattern more regular under the control treatment. If the Lhat (d) value is lower than the lower 95% boundary, the stomata follow a regular distribution at that scale. Otherwise, the stomata will follow a random distribution pattern at that scale if the Lhat (d) value is between the lower and upper 95% boundaries.

although no significant differences in P_n were observed between the two [CO_2] treatments (Fig. 2a)).

Conversely, *e*[CO_2] also decreased the T_r ($P<0.001$) by 50.0%, 53.7%, 59.8%, and 55.5% under control, mild Cd stress, moderate Cd stress and severe Cd stress conditions (Fig. 2b)). Similarly, *e*[CO_2] significantly decreased G_s ($P<0.001$) by 49.4%, 53.7%, 59.8%, and 55.5% in maize plants grown under control, mild Cd stress, moderate Cd stress, and severe Cd stress conditions, respectively (Fig. 2c)). Meanwhile, increasing WUE is widely recognized as an important strategy for responding to Cd stress [43]. Consistently, we observed that the CO_2 fertilization effect induced a significant rise in WUEI ($P<0.001$), specifically, when [CO_2] increased from *a*[CO_2] to *e*[CO_2], leaf WUEI increased significantly ($P<0.001$) by 129.3%, 122.9%, 138.2% and 108.9% under control, mild Cd stress, moderate Cd stress and severe Cd stress conditions (Fig. 2d)), respectively, indicating a greater amount of CO_2 assimilation or dry matter production per unit mass of water consumed when the [CO_2] was elevated.

Foliar Nonstructural Carbohydrates and Photosynthetic Pigments

Our results revealed that, except for moderate Cd stress, elevated [CO_2] increased soluble sugars in stems by 4.4%, 37.5%, and 7.9% under control, mild Cd stress, and severe Cd stress. The increased soluble sugars induced by *e*[CO_2] in stems may be due to increased carbohydrate supply to maintain the pressure gradient for sap flow and alleviate Cd-induced stress [44]. Contrary to soluble sugars in stems, *e*[CO_2] significantly reduced soluble sugar content in roots ($P<0.001$), where soluble sugar content decreased by 26.2%, 10.7%, 32.7%, and 26.7% under control, mild Cd stress, moderate Cd stress, and severe Cd stress, respectively. Similarly, soluble sugars in leaves were significantly affected by *e*[CO_2] ($P = 0.006$), decreasing by 0.7%, 0.2%, and 11.3% under control, mild Cd stress, and moderate Cd stress, respectively. These results are consistent with Higuchi et al. [45] showing that when the [CO_2] was elevated, plants may preferentially allocate assimilated carbon as the carbon source for the

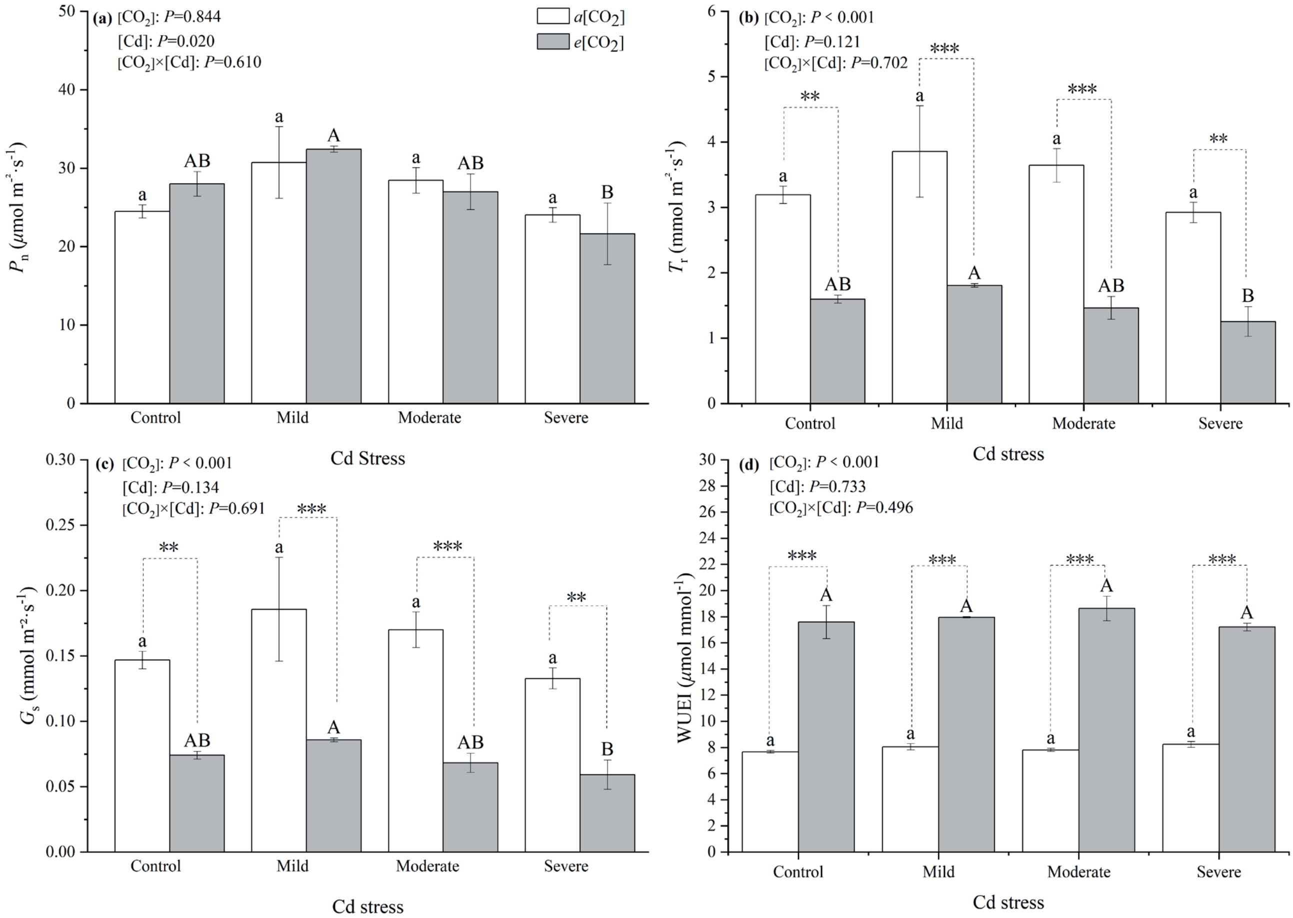

Fig. 2. Effects of CO_2 concentration and Cd stress on photosynthetic parameters of maize.
Note: Lowercase letter such as "a" is employed to signify the significance analysis conducted among the four Cd stress under *a*[CO_2]; uppercase letter such as "A" is utilized to denote the significance analysis performed among the four cadmium concentrations under *e*[CO_2]; "*" is used to indicate the significance analysis between the same cadmium concentration under *a*[CO_2] and *e*[CO_2], the "*" represents significance at the 0.05 level, "**" indicates significance at the 0.01 level, and "***" signifies significance at the 0.001 level and the line segments represent the standard errors.

formation of α-glucan complexes in their stems in order to grow under Cd stress conditions, thereby preventing Cd transfer to photosynthetic leaves.

Different from the results for soluble sugars, we found that even under Cd stress, *e*[CO$_2$] could significantly enhance starch content in roots ($P<0.001$) and stems ($P<0.001$). Specifically, when [CO$_2$] was elevated from *a*[CO$_2$] to *e*[CO$_2$], starch content in roots increased by 72.2%, 23.7%, 47.2%, and 29.0% under control, mild Cd stress, moderate Cd stress and severe Cd stress, respectively, and starch content in stems increased by 82.6%, 38.2%, 63.1%, and 62.8% under the same treatments. Meanwhile, Cd had no significant effect on starch content in roots, stems, and leaves; additionally, we found that leaf starch content was affected by the interaction effect of [CO$_2$] and Cd stress ($P = 0.007$; Table 4). The difference between the soluble sugars and the starch content of maize under Cd stress to *e*[CO$_2$] implied that the effect of *e*[CO$_2$] on soluble sugar and starch contents in maize indicates that *e*[CO$_2$] mainly enhances the energy storage of maize to resist Cd stress, and the soluble sugars produced by starch decomposition also provide the possibility for increasing cellular osmotic pressure under Cd stress [45, 46].

In this study, chlorophyll content decreased with CO$_2$ enrichment, which is consistent with the findings of Yang et al. [12] and Hao et al. [10]. For photosynthetic pigments, elevated [CO$_2$] led to a 4.9% reduction in Chl a content under the control treatment and it significantly decreased ($P<0.001$) Chl a content by 16.2%, 50.4%, and 25.9% when the maize plants were exposed to mild, moderate, and severe Cd stress, respectively (Fig. 3a)). Similarly, *e*[CO$_2$] reduced Chl b content ($P = 0.016$) by 48.2% and 18.4% under moderate and severe Cd stress, respectively, whereas Chl b increased by 5.9% under mild Cd stress (Fig. 3b)). As a result, total Chl a+b content decreased ($P<0.001$) by 4.3%, 13.8%, 50.2%, and 25.1% under control, mild Cd stress, moderate Cd stress, and severe Cd stress conditions by enriching [CO$_2$] from *a*[CO$_2$] to *e*[CO$_2$] which was attributed to the decreased Chl a and Chl b contents when maize plants were exposed to *e*[CO$_2$] (Fig. 3c)). While elevated [CO$_2$] had a significant effect on Car content ($P = 0.001$), *e*[CO$_2$] increased Car content by 5.2% under the control treatment and decreased Car content by 18.3%, 15.3%, and 36.0% under mild, moderate, and severe Cd stress, respectively (Fig. 3d)). This decrease in foliar photosynthetic pigments may result from elevated [CO$_2$] inducing sugar accumulation and decreasing nitrogen content, which imbalances the C/N ratio in mature leaves and promotes premature senescence and pigment degradation [47]. Similarly, Cd could cause a significant decrease in Chl a ($P<0.001$), Chl b ($P<0.001$), Car ($P<0.001$), and total Chl a+b ($P<0.001$), because Cd primary sites of action were the photosynthetic apparatus, pigments, carotenoid synthesis, and chlorophyll [38]. Additionally, we found that the Chl a ($P = 0.001$), Chl b ($P = 0.007$), total Chl a+b ($P<0.001$), and Car ($P = 0.031$) contents were affected by the interaction effects of [CO$_2$] and Cd stress (Fig. 3).

Foliar SOD and POD Activities, MDA Contents, and Cd-tolerant Genes Expression

Our results revealed that when [CO$_2$] was elevated from *a*[CO$_2$] to *e*[CO$_2$], POD activity increased by 13.3% in the control treatments, whereas Cd stress decreased POD activity by 29.7%, 17.7%, and 17.6% under mild, moderate, and severe conditions, respectively (Table 5). Similarly, SOD activity exhibited little difference between *a*[CO$_2$] and *e*[CO$_2$] in the control

Table 4. Effects of CO$_2$ concentration and Cd stress on soluble sugar and starch content in different organs of maize.

CO$_2$ concentration μmol mol^{-1}	Cd stress	Soluble sugars mg g^{-1}			Starch mg g^{-1}		
		Roots	Stems	Leaves	Roots	Stems	Leaves
a[CO$_2$]	Control	58.9(1.7)	168.6(16.0)	79.6(3.6)	14.6(0.9)	18.9(1.1)	29.6(0.3)
	Mild	51.8(1.2)	129.0(19.0)	77.5(3.3)	20.0(1.6)	25.2(2.5)	30.8(1.9)
	Moderate	61.6(2.9)	142.4(22.5)	77.5(3.4)	18.2(1.9)	20.2(1.9)	36.3(2.6)
	Severe	68.4(5.0)	215.3(17.5)	78.8(2.7)	18.2(1.0)	18.2(1.4)	27.4(0.5)
e[CO$_2$]	Control	43.5(0.8)	175.9(21.5)	79.1(0.9)	25.1(1.9)	34.5(2.9)	31.1(1.8)
	Mild	46.3(3.4)	177.3(10.4)	59.5(2.4)	24.7(1.7)	34.9(3.1)	30.1(1.1)
	Moderate	41.5(1.3)	136.1(2.4)	68.7(1.7)	26.8(1.6)	32.9(1.3)	31.2(0.8)
	Severe	50.2(3.1)	232.3(9.3)	83.5(1.8)	23.5(1.12)	29.6(1.8)	33.3(0.9)
CO$_2$		$P<0.001$	$P = 0.159$	$P = 0.006$	$P<0.001$	$P<0.001$	$P = 0.702$
Cd		$P = 0.006$	$P<0.001$	$P<0.001$	$P = 0.268$	$P = 0.061$	$P = 0.064$
CO$_2$×Cd		$P = 0.062$	$P = 0.396$	$P = 0.001$	$P = 0.201$	$P = 0.568$	$P = 0.007$

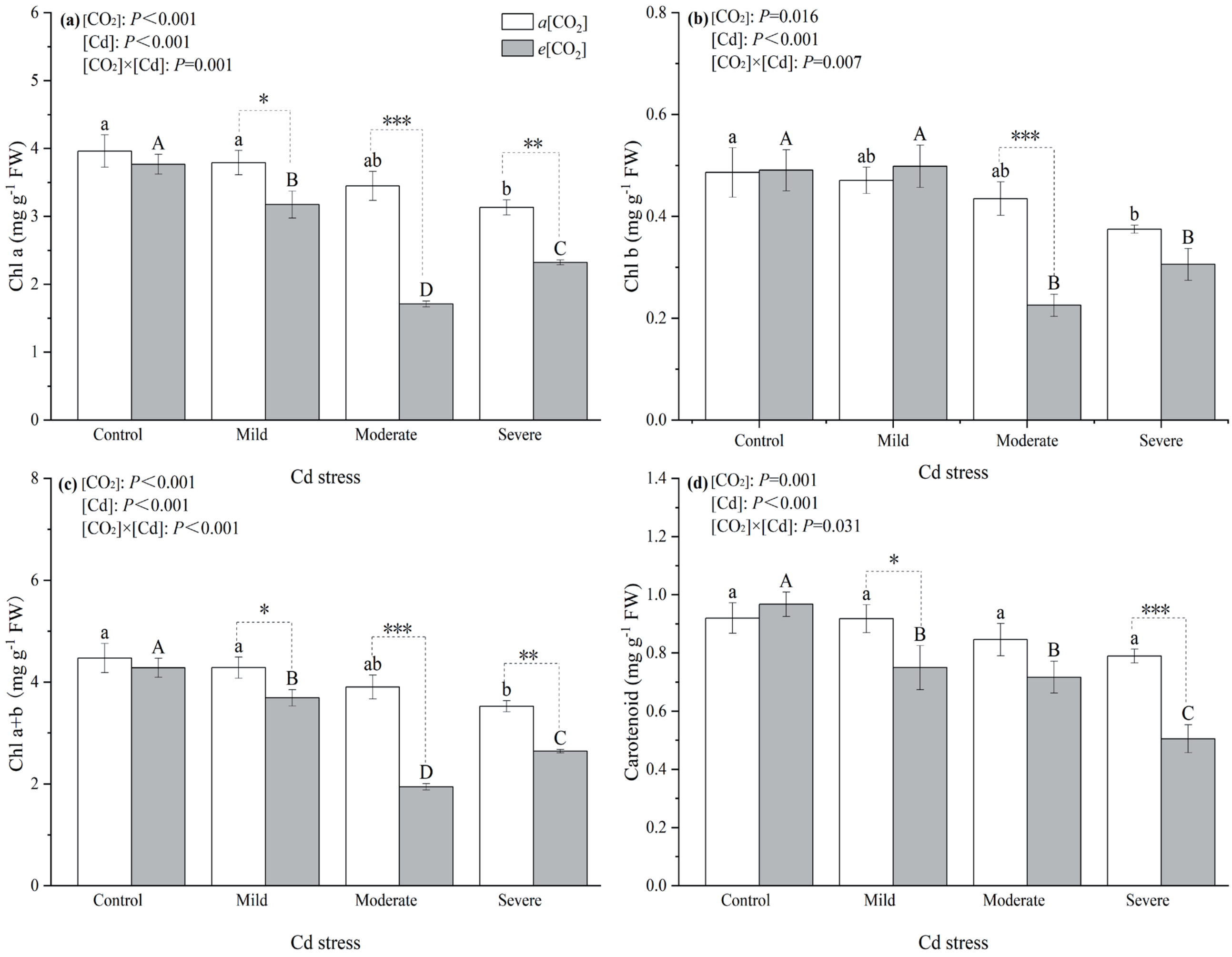

Fig. 3. Effects of CO_2 concentration and Cd stress on photosynthetic pigments of maize.

Note: Lowercase letter such as "a" is employed to signify the significance analysis conducted among the four Cd stress levels under *a*[CO_2]; uppercase letter such as "A" is utilized to denote the significance analysis performed among the four cadmium concentrations under *e*[CO_2]; "*" is used to indicate the significance analysis between the same cadmium concentration under *a*[CO_2] and *e*[CO_2], the "*" represents significance at the 0.05 level, "**" indicates significance at the 0.01 level, and "***" signifies significance at the 0.001 level and the line segments represent the standard errors.

Table 5. Effects of CO_2 concentration and Cd stress on oxidative stress in maize leaves.

CO_2 concentration μmol mol⁻¹	Cd stress	MDA μmol g⁻¹ FW	SOD U g⁻¹ FW	POD U g⁻¹ FW
a[CO_2]	Control	0.57(0.08)	617.2(3.0)	77.8(4.2)
	Mild	0.59(0.03)	614.2(32.4)	91.9(9.2)
	Moderate	0.66(0.09)	607.1(3.0)	101.7(5.1)
	Severe	0.75(0.32)	520.9(4.1)	107.4(7.9)
e[CO_2]	Control	0.53(0.07)	615.2(11.2)	88.2(12.4)
	Mild	0.58(0.12)	434.8(3.0)	64.6(24.2)
	Moderate	0.49(0.09)	618.2(38.5)	83.6(9.1)
	Severe	0.62(0.14)	542.2(90.2)	88.5(14.5)
CO_2		$P = 0.390$	$P = 0.190$	$P = 0.143$
Cd		$P = 0.798$	$P = 0.062$	$P = 0.397$
CO_2×Cd		$P = 0.933$	$P = 0.076$	$P = 0.470$

treatments, whereas SOD activity under *e*[CO_2] decreased by 29.2% under mild Cd treatment and increased by 1.8% and 4.1% under moderate and severe Cd stress, respectively. These results indicated that SOD was likely the main protective enzyme of the ROS removal system in maize under elevated [CO_2], which may be attributed to the fact that *e*[CO_2] inhibits photorespiration, thereby reducing the production of O_2^- in peroxisomes, and meanwhile, the reduction in O_2^- also resulted in a decrease in H_2O_2 content, which may account for the decrease in POD activity [48], implying that elevated [CO_2] weakly promoted SOD activity under moderate Cd stress and severe Cd stress.

Meanwhile, at *a*[CO_2], MDA content increased significantly with rising soil Cd concentration, with increases of 3.3%, 16.5%, and 32.2% under mild Cd stress, moderate Cd stress, and severe Cd stress, respectively, compared with the control group. To summarize, the increase in reactive oxygen species (ROS) content caused by Cd further elevated MDA content. However, under *e*[CO_2], MDA content decreased regardless of soil Cd levels, with reductions of 7.4% in the control and 1.6%, 26.2%, and 18.1% under mild Cd stress, moderate Cd stress, and severe Cd stress, respectively, indicating that elevated [CO_2] mitigated oxidative damage caused by Cd stress.

At ambient [CO_2] conditions, when maize plants were exposed to Cd stress, the expression levels of *ZmHMA*2 under mild, moderate, and severe Cd stress increased by 121.0%, 578.1%, and 435.8%, respectively, compared with the control group. In contrast, when [CO_2] was elevated from *a*[CO_2] to *e*[CO_2], *ZmHMA*2 expression decreased by 76.8%, 81.9%, and 60.0% under mild, moderate, and severe Cd stress, respectively, while in the control group, it increased by 4.4%. Similarly, at ambient [CO_2], the expression levels of *ZmNramp*1 under mild, moderate, and severe Cd stress increased by 41.2%, 121.6%, and 61.0%, respectively, compared with the control group, while *ZmN-ramp*1 expression was decreased regardless of soil Cd status under elevated [CO_2]. Elevated [CO_2] also reduced *ZmHMA*3 expression levels by 54.3%, 33.6%, and 34.7% in the control, mild Cd stress, and moderate Cd stress treatments, respectively, but *e*[CO_2] promoted *ZmHMA*3 expression by 212.8% under severe Cd stress. In addition, elevated [CO_2] decreased *ZmWRKY*64 expression levels in the control, mild Cd stress, and severe Cd stress groups. In conclusion, elevated [CO_2] decreased expression of three Cd-tolerance genes, specifically *ZmNramp*1, *ZmHMA*3, and *ZmWRKY*64 (Table 6), possibly because CO_2 enrichment inhibits a pathway wherein the encoded proteins cooperate with membrane-localized carbonic anhydrase to co-transport bicarbonate and protons for osmotic regulation [42]. More interestingly, the relative expression levels of *ZmNramp*1, *ZmHMA*2, and *ZmWRKY*64 under severe Cd treatment remained higher under both [CO_2] conditions than the control, indicating that these three genes respond stably to severe Cd stress independent of [CO_2] (Table 6).

Table 6. Effects of CO_2 concentration and Cd stress on gene relative expression levels.

Gene	Cd stress	*a*[CO_2]	*e*[CO_2]
*ZmNramp*1	Control	100.8(9.0)	19.2(0.6)
	Mild	142.3(6.0)	12.8(0.2)
	Moderate	223.3(7.0)	35.2(9.1)
	Severe	162.2(7.7)	39.7(5.7)
*ZmHMA*2	Control	9.2 (1.0)	9.6(2.3)
	Mild	20.4(0.9)	4.7(1.4)
	Moderate	62.6(0.0)	11.4(2.7)
	Severe	49.5(8.4)	19.8(3.2)
*ZmHMA*3	Control	139.3(16.5)	63.7(2.7)
	Mild	134.8(7.2)	89.6(6.4)
	Moderate	134.6(15.1)	88.0(1.9)
	Severe	30.8(9.5)	96.3(3.5)
*ZmWRKY*64	Control	165.9(25.9)	45.4(1.0)
	Mild	530.1(6.4)	27.1(1.3)
	Moderate	69.2(24.8)	99.1(6.6)
	Severe	433.3(212.4)	151.9(10.0)

Relationships among WUEI, Leaf Photosynthesis, Stomatal Traits, Nonstructural Carbohydrates, and Plant Allocation

The intrinsic water-use efficiency (WUEI), which is defined as the ratio of P_n to $T_{r,}$ represents an important adaptive mechanism for plants under Cd stress, and the elevation of WUEI in turn, partially alleviates Cd^{2+} stress by regulating root absorption, root-to-leaf substance transport, and biomass allocation [43]. In the current study, we found that starch accumulation in roots might enhance leaf WUEI, as evidenced by the positive correlation between WUEI and starch accumulation (R^2 = 0.873, P<0.001, Fig. 4a)), but WUEI showed a negative correlation with soluble sugars (R^2 = 0.719, P = 0.008, Fig. 4b)), which might be explained by reduced cadmium absorption, as starch accumulation in roots counteracts Cd^{2+} uptake by strengthening root sinks [49] and restricting Cd^{2+} transport to aerial tissues through the formation of Cd-α-glucan complexes [45]. Similarly, stems required more starch for water and nutrient elements uptake, transport, partitioning, and utilization [45], leading to linear increases in starch concentration (R^2 = 0.875, P<0.001, Fig. 4c)) and decreases in dry weight with WUEI (R^2 = 0.848, P = 0.001, Fig. 4d)), and elevated WUEI also facilitated photosynthate translocation from leaves and stems to grains [13], enhancing crop yield as confirmed by the positive correlation between shoot-to-root dry weight ratio and WUEI (R^2 = 0.612, P = 0.022, Fig. 4e)).

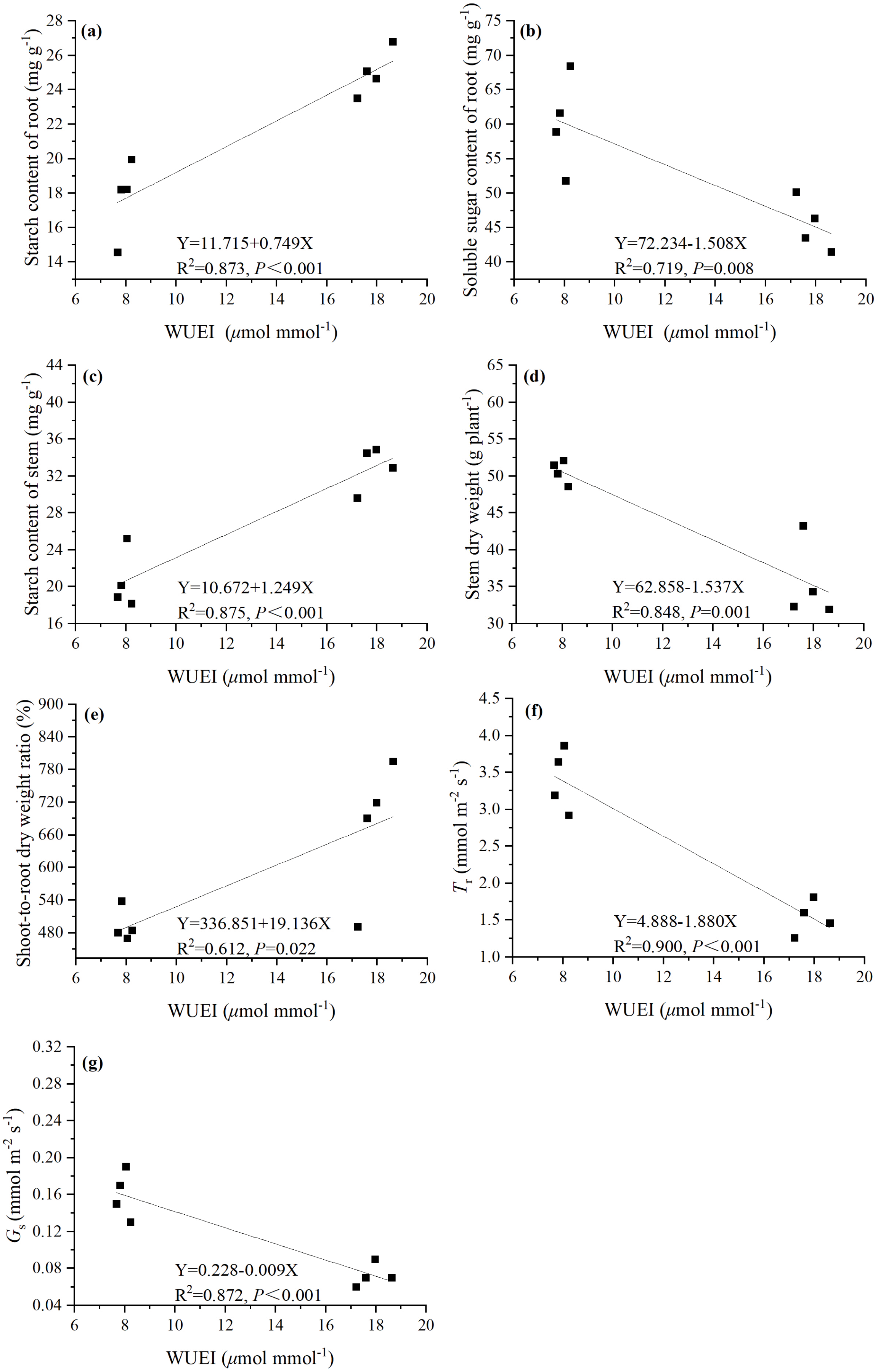

Fig. 4. The relationships between WUEI and starch content of root, soluble sugar content of root, starch content of stem, stem dry weight, shoot-to-root dry weight ratio, T_r, and G_s.

Meanwhile, regarding Cd^{2+} translocation, maize can reduce transpiration by regulating stomatal aperture, which not only improves WUEI [50] but also decreases Cd^{2+} transport [43]; this is consistent with the negative linear correlations between WUEI and both T_r and G_s, where WUEI increased linearly as T_r decreased ($R^2 = 0.900$, $P<0.001$, Fig. 4f)) and as G_s decreased ($R^2 = 0.872$, $P<0.001$, Fig. 4g)).

Conclusions

This study was one of the first to explore the interactive effects of elevated [CO_2] and Cd stress on maize, with a focus on stomatal traits, stomatal distribution, leaf physiological and biochemical characteristics, and Cd-tolerance gene expression. Our results indicated that under non-Cd-contaminated soil conditions, there was an initially strong CO_2-induced fertilization effect on maize leaf photosynthesis, but this effect was drastically reduced by soil Cd stress, coupled with impaired stomatal diffusion efficiency, reduced photosynthetic pigments, and down-regulated POD activity. In summary, our findings suggested that the initial strong CO_2 fertilization effect on maize might partially be mitigated or even offset by soil Cd stress, which was generally accompanied by rising atmospheric [CO_2] under climate change. Therefore, this study provides evidence that existing model projections, based on strong CO_2 fertilization effects from earlier elevated [CO_2] experiments, may overestimate yield responses to elevated [CO_2] and that coupled climate-carbon models should incorporate constraints imposed by Cd stress on maize productivity for climate-change assessments.

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Conflict of Interest

The authors declare no conflict of interest.

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