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Elevated CO₂ can modify the response to a water status gradient in a steppe grass: from cell organelles to photosynthetic capacity to plant growth

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Abstract

Background: The atmospheric CO₂ concentration is rising continuously, and abnormal precipitation may occur more frequently in the future. Although the effects of elevated CO₂ and drought on plants have been well reported individually, little is known about their interaction, particularly over a water status gradient. Here, we aimed to characterize the effects of elevated CO₂ and a water status gradient on the growth, photosynthetic capacity, and mesophyll cell ultrastructure of a dominant grass from a degraded grassland.

Results: Elevated CO₂ stimulated plant biomass to a greater extent under moderate changes in water status than under either extreme drought or over-watering conditions. Photosynthetic capacity and stomatal conductance were also enhanced by elevated CO₂ under moderate drought, but inhibited with over-watering. Severe drought distorted mesophyll cell organelles, but CO₂ enrichment partly alleviated this effect. Intrinsic water use efficiency (WUE_i) and total biomass water use efficiency (WUE_t) were increased by elevated CO₂, regardless of water status. Plant structural traits were also found to be tightly associated with photosynthetic potentials.

Conclusion: The results indicated that CO₂ enrichment alleviated severe and moderate drought stress, and highlighted that CO₂ fertilization's dependency on water status should be considered when projecting key species' responses to climate change in dry ecosystems.

Keywords: Elevated CO₂, Grassland, Mesophyll cell organelle, Photosynthetic capacity, Plant growth analysis, Water status gradient

Background

The IPCC's Fifth Assessment Report (AR5) showed that atmospheric carbon dioxide concentration had increased by 40 % since the pre-industrial era, reaching ~390 μmol mol⁻¹ in 2011. It is predicted to rise to 500 μmol mol⁻¹, perhaps even above 900 μmol mol⁻¹, by the end of this century. Continued emissions of greenhouse gases will cause further warming and precipitation changes [1]. The effects of elevated CO₂ concentration (elevated CO₂) and climatic change on plants—from the molecular basis to physiological

processes, individual growth, and vegetation productivity aspects—have attracted considerable attention for several decades (e.g., [2–6]).

Many studies have reported the biological responses of plants to CO₂ enrichment and its interactions with other environmental factors (e.g., [3, 4, 6]). The effects of elevated CO₂ include enhanced net photosynthesis rate (A_{net}), down-regulated stomatal conductance (g_s) [3, 7–9], dilution of chemical elements [10], imbalance of sink–source relationships [11, 12], increased plant growth and vegetation productivity [2, 13], changes in species competition interactions and community structure [13–15], and lengthened growing seasons [16]. However, these elevated CO₂-induced changes might be mediated by other environmental factors, particularly

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changes of water availabilities. Severe drought adversely affects plant growth, gas exchange, and photosynthetic activity [17], but elevated CO₂ might partly alleviate the harmful impact of water deficit stress on these biological processes, and even survival [18–21]. Elevated CO₂ can enhance plant resistance to water deficit stress by mitigating oxidative damage, maintaining A_{net} and decreasing g_s , and improving the plant water status, thereby raising the water use efficiency (WUE) [4, 21–24]. Under moderate water stress, a marked stimulation occurs due to elevated CO₂ [22–26]. However, over-watering can reduce, or even reverse this stimulation [27–30]. Nevertheless, few studies on the combined effects of elevated CO₂ and a water status gradient have been conducted, particularly on multiple scales.

Agropyron cristatum (L.) Gaertn, or crested wheatgrass, a C₃ species, is a dominant species in the steppe regions of Central Asia, and is also widespread in western North America [31, 32]. It is a perennial herb native to North China with good palatability and high forage value. Moreover, its prosperity is recognized as an indicator of degradation of the steppe ecosystem in the context of overgrazing and adverse climate change. For instance, degradation might initially occur if crested wheatgrass thrives relative to other dominant species such as *Leymus chinensis* and *Stipa grandis* [33]. Thus, this species is crucial for assessing the vulnerability and restorative capacity of the semiarid region, as well as forage resource management. These temperate grasslands, which are dominated by several major grasses including *A. cristatum*, have been severely degraded during recent decades because of adverse climatic change and improper land use [34–37]. Although this arid region is projected to become drier and hotter, excessive precipitation events may occur more frequently [38, 39]. This would further threaten the ecosystem function including dominant species growth and survival [36, 37, 40]. The leaf-level instantaneous responses of A_{net} , g_s , and WUE to elevated CO₂ have been quite well investigated [4, 6, 8]. To our knowledge, however, few prior efforts have been made to examine the effects of elevated CO₂ under a wide-ranging water status gradient (seven watering treatments from extremely severe deficit to relative over-watering) (but see Manea and Leishman, [15]), particularly integrating multiple variables from organelle structure to physiological processes, individual morphology and structure, biomass allocation, and plant growth aspects. In this experiment, structural and physiological traits were examined to find sensitive indicators, and to summarize the adaptive strategy of *A. cristatum* to climatic changes. The objective of the current study was to test the hypotheses: (1) elevated CO₂ modifies the effects of soil water status on the dominant species, with stimulation in a moderate range of water status changes, no positive response with over-

watering, but an alleviation of damage from severe water deficit; and (2) associated responses co-occur at the mesophyll cell ultrastructure, photosynthetic physiological activity, plant growth and structure levels under elevated CO₂ and different water conditions.

Methods

Plant culture

Each PVC plastic pot (9.7 cm in diameter, 9.5 cm in height, 0.70 L) was filled with 0.68 ± 0.01 (\pm SE, $n = 60$) kg of dry soil, and planted with four plants per pot. The soil was retrieved from the local soil surface (0–30 cm), and *A. cristatum* seeds were collected the year before the experiment from the local steppe—a typical grassland ecosystem in Xilinhot (43°38'N, 116°42'E, 1100 m a.s.l.), Inner Mongolia, China. The soil was a castanozem type, with a medium texture containing 29.0 %, 31.2 %, and 39.8 % clay (<5 μm particle diameter), silt (5–50 μm), and sand (>50 μm), respectively. The soil organic carbon, total nitrogen, and available nitrogen concentrations were 12.31 ± 0.19 g kg⁻¹, 1.45 ± 0.02 g kg⁻¹, and 81.61 ± 0.71 mg kg⁻¹, respectively. The soil water field capacity (FC) and permanent wilting point were 25.8 and 6.0 % (w/w), respectively, and the gravimetric bulk density was 1.21 g cm⁻³. With a mean annual temperature of 2.95 °C and mean annual precipitation (MAP) of 266 mm over the past 30 years, this region is characterized by a continental temperate climate with a dry, frigid winter (−18.6 °C lowest mean month temperature in Jan), and a wet, hot summer (21.4 °C maximal mean month temperature in Jul). Most precipitation (88 %) occurs in the growth season from May to Sep. To obtain uniform seedlings [26, 41], all pots were initially placed in a naturally illuminated glasshouse (day/night temperatures of 26–28/18–20 °C) until the third leaf emerged (27 days after sowing), and then transferred into two open-top chambers (OTCs) to subject the plants to two-month watering and increased CO₂ concentration treatments. Water was provided by a sprayer at around 17:00 every 3 days according to previous similar experiments [26, 35].

The OTCs were established in a regular hexahedron shape (each side 85 cm wide, 150 cm high) with a top-opening rain shelter on the top and a space below for free exchange of gases between the OTC and ambient atmosphere. Pure CO₂ gas (99.999 %) was supplied from a cylinder (Chao Hong Ping Gas Co. Ltd, Beijing, China), and a CO₂ gas sensor (eSENSE-D, SenseAir, Delsbo, Sweden) was used to continuously monitor and control the CO₂ concentration over 24 h with a data logger (DAM-3058RTU, Altai Sci Tech Dev Co. Ltd., Shang Hai, China). The CO₂ concentrations had a ± 30 $\mu\text{mol mol}^{-1}$ change relative to the set points. The air temperature and relative humidity (RH) were monitored using thermocouples (HOBO S-TMB-M006, Onset

Computer Co., Bourne, MA, USA) and humidity transducers (HOBO S-SMA-M005) installed at 75 cm height in and out of the chambers, respectively. Climatic data were automatically recorded and collected by a logger (HOBO H21-002) every 30 min during the experiments; day/night temperatures were $28.3 \pm 5.7/22.7 \pm 4.2$ °C and RH was 62.4 ± 4.0 % in the OTCs, which were $1.3/0.5$ °C greater and 3.5 ± 2.3 % lower than outside the OTCs. The OTC system has been proven to have acceptable effectiveness, relative availability, and data comparability for assessing the effects of elevated CO₂ with climatic change at low cost [42, 43], although data interpretation needs to be cautious in various environmental contexts [44, 45].

Experimental design

The experiment was designed with two factors, two CO₂ concentrations (ambient CO₂, ~390; elevated CO₂, 550 μmol mol⁻¹) and a seven-level precipitation gradient: W₋₆₀ (-60 % relative to mean precipitation in the local site over 30 years, as an extreme drought treatment), W₋₃₀ (-30 % relative to local mean precipitation), W₋₁₅ (-15 % relative to local mean precipitation), W₀ (local mean precipitation, the control watering treatment), W₁₅ (+15 % relative to local mean precipitation), W₃₀ (+30 % relative to local mean precipitation, an over-watering treatment relative to the normal local precipitation), and W₆₀ (+60 % relative to local mean precipitation), roughly equaling 147.0, 257.3, 312.5, 367.6, 422.7, 477.9, and 588.1 mm of precipitation during the growth season, respectively. Two OTCs were used, with separate irrigation treatments within each one as a split plot and 3–5 replicates (pots/treatment). A total of 80 plots were included initially; some pots were kept in reserve in case of experimental or plant growth problems. Plants were randomly placed within each OTC initially, replaced every 3 days, and transferred between the two chambers weekly (CO₂ target points were switched simultaneously) to minimize any differences between growth chambers except for the desired treatments—CO₂ concentration and water status [26, 46, 47].

Soil water status and water use

The soil water content (SWC, g water g⁻¹ dry soil) during the experiment was determined by weighing pots. The soil dry weight (SDW) at sowing was calculated as $(TW - PW) \times (1 - SWC_0)$ at sowing (the SWC₀ was determined before sowing by oven-drying soil samples; there was no water drainage because the pots used had no holes, and plant weight was neglected). The SWC during the experiment was expressed as $(TW - SDW - PW)/SDW$, and the soil relative water content (SRWC) was expressed as $SWC/FC \times 100$. Water use, i.e., actual evapotranspiration during the entire experiment, was derived from a water balance equation; water use = TW at harvest

– TW at initial time + applied water amount. Thus, total biomass water use efficiency (WUE_t) could be estimated as total plant biomass/water use [48]. TW is the total weight of the pot plus soil at each measurement time; PW is the net pot weight determined before filling with soil; FC is the SWC measured 24 h after fully wetting the soil.

Plant biomass and leaf area

Each plant was separated into four parts, the stem, root, green leaves, and dead leaves, at both the start and end of the experiment, dried at 75 °C to a constant weight, and then weighed to get the biomass. Before drying, plant height, tiller and green leaf numbers were recorded; and green leaf area per plant, and the leaf parts used to determine gas exchange parameters were measured with a WinFOLIA system for root/leaf analysis (WinRhizo, Régent Instruments, Quebec, Canada).

Plant growth analysis

Plant growth analysis was performed following Poorter [49]. The relative growth rate of each individual (RGR, mg g⁻¹ day⁻¹) was expressed as $(\ln w_2 - \ln w_1)/(t_2 - t_1)$, where w_2 and w_1 are the biomass at final and initial harvest dates, respectively, and t_2 and t_1 indicate the two harvest times. The leaf mass ratio of total plant mass (LMR), stem mass ratio (SMR), and root mass ratio (RMR), as biomass allocation indicators, were expressed as percentages of leaf, stem, and root mass in the total plant mass, respectively. The plant/leaf morphological and structural indicators leaf area ratio (LAR, m² kg⁻¹; an indicator of the canopy size), leaf area and root mass ratio (LARMR, m² kg⁻¹; a proxy of the biomass balance between light-intercepting organs and resource element uptake organs), specific stem length (SSL, cm mg⁻¹; a marker of the investment of stem carbon into plant height), and specific leaf area (SLA, m² kg⁻¹; an indicator of leaf thickness and compactness) were expressed as ratios of leaf area to whole plant mass, leaf area to root mass, stem length to mass, and leaf area to mass, respectively [49].

Photosynthesis and chlorophyll *a* fluorescence

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously using an open gas exchange system (LI-6400 F, LI-COR, Inc., Lincoln, NE, USA) combined with a leaf chamber fluorometer (LI-6400-40, LI-COR). Illumination was supplied to the leaves from a red-blue LED light source and the data were initially analyzed with data acquisition software (OPEN 6.1.4, LI-COR). Before measurement, the leaves were acclimated in the chamber for at least 10 min at 26–28 °C with a CO₂ concentration of 390 μmol mol⁻¹ and a photosynthetic photon flux density of 1500 μmol m⁻² s⁻¹, under which photosynthesis is nearly saturated, to obtain gas

exchange parameters such as net light-saturated maximum photosynthetic rate (A_{sat}), g_s , transpiration rate (E), and intrinsic water use efficiency (WUE_i , A_{sat}/E). We measured at least three each of the youngest and fully expanded leaves from different individuals (one plant per pot) for all replicates, from 9:00 to 16:30 h daily. The vapor pressure deficit (VPD) in the cuvette was maintained at 1.7–2.7 kPa (2.39 ± 0.04 , $n = 492$), possibly reflecting the actual conditions within the OTCs [26, 50]. The steady-state value of fluorescence (F_s) was determined, and a second saturating pulse at $8000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was imposed to determine the maximal light-adapted fluorescence (F'_m). The actinic light was removed and the minimal fluorescence in the light-adapted state (F'_0) was determined after 3 s of far-red illumination. The maximum photochemical efficiency of photosystem II (F_v/F_m) was determined midday–pre-dawn in completely dark-adapted leaves with a leaf fluorometer (LI-6400-40) linked to a LI-6400 F gas exchange system. The minimal fluorescence yield (F_0) was determined under low modulated light of $1.0 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the maximal fluorescence yield (F_m) was obtained with a 0.8 s saturating pulse at $\sim 8000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The fluorescence parameters were calculated from the following formulae [51, 52]; the maximal efficiency of photosystem II (PSII) photochemistry is $F_v/F_m = (F_m - F_0)/F_m$, and the efficiency of excitation energy captured by open PSII reaction centers is $F'_v/F'_m = (F'_m - F'_0)/F'_m$.

Estimation of A/C_i response curves

To analyze the A/C_i response curve to obtain key photosynthetic capacity parameters, a stepwise CO_2 concentration gradient was implemented (390, 300, 200, 100, 50, 390, 390, 550, 800, and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$). Note, the third 390 value is not an error, but a trick to easily recover the ambient CO_2 level from the lowest point of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. At each CO_2 level, the leaves needed 2–3 min to equilibrate, and a match was also run to balance the CO_2 and water vapor concentrations between the reference and leaf chambers. Furthermore, to estimate maximum rate of carboxylation ($V_{c,\text{max}}$), maximum rate of electron transport (J_{max}), and rate of those phosphate utilization (TPU), a curve-fitting software tool by Sharkey et al. [53] based on the method of Farquhar et al. [54] was run to analyze the A/C_i response data.

Electron microscopy

For transmission electron microscopy, 2-mm² pieces from the middle sections of the youngest and fully expanded leaves were dissected and immediately fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) overnight at 4 °C. The samples were then washed three times with the same buffer and post-fixed

in 1 % osmium tetroxide overnight at 4 °C. After being washed in the same buffer, the leaf tissues were passed through an ethanol dehydration series, and then infiltrated and embedded in Spurr's resin (Agar Scientific, Essex, UK). Sections were cut using an Ultracut R ultramicrotome (Leica, Wetzlar, Germany). The thin sections were stained with 2 % uranyl acetate and lead citrate [55], and then observed and photographed under a transmission electron microscope (JEM-1230, JEOL Ltd, Tokyo, Japan). For each treatment, three leaf samples were examined, and approximately 130 mesophyll cells were randomly chosen for the observations.

Statistical analyses

A principal component analysis (PCA) was first conducted to test the relationships among the traits including the plant growth, structural, morphological, biomass allocation, and photosynthetic parameters, and the multivariate patterns of the effects of CO_2 concentration and watering treatments alone and in combination [26, 56, 57]. Thereafter, we conducted an analysis of variance (ANOVA) for the plant growth, structural, morphological, biomass allocation, and photosynthetic traits, and the anatomical changes in mesophyll cells and their organelles with GLM Full Factorial Mode to test the main effects of watering, elevated CO_2 , and their interactions. Where watering treatments had a significant effect based on ANOVA, a multiple comparison was done with Duncan's multiple range test. A one-way ANOVA was also conducted to test the differences between the two CO_2 levels within the same watering treatment. The multiple factorial ANOVA model can be used for unequal variances and data near a normal distribution. All statistical analyses were made using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Unless otherwise noted, $P < 0.05$ was considered statistically significant.

Results

Soil water status

The watering treatments produced a wide-ranging soil water gradient combined with either ambient or elevated CO_2 during the entire experimental period; when measured before watering at 17:00 every 3 days (the irrigation day) during a consecutive 10-day period, the SRWCs were 26.7 %, 39.4 %, 42.7 %, 45.6 %, 50.8 %, 53.3 %, and 61.7 % in the W_{-60} , W_{-30} , W_{-15} , W_0 , W_{+15} , W_{+30} , W_{+60} watering treatments at ambient CO_2 , and 25.4 %, 36.1 %, 38.4 %, 44.0 %, 45.5 %, 50.9 %, and 51.5 % at elevated CO_2 , respectively (Additional file 1: Figure S1), indicating that elevated CO_2 reduced the SRWC under every watering treatment (on average from 45.7 to 41.7 %, decreasing by 8.9 %). Watering and elevated CO_2 exerted significant effects almost every day over the continuous 10-day period except for elevated CO_2 concentration on

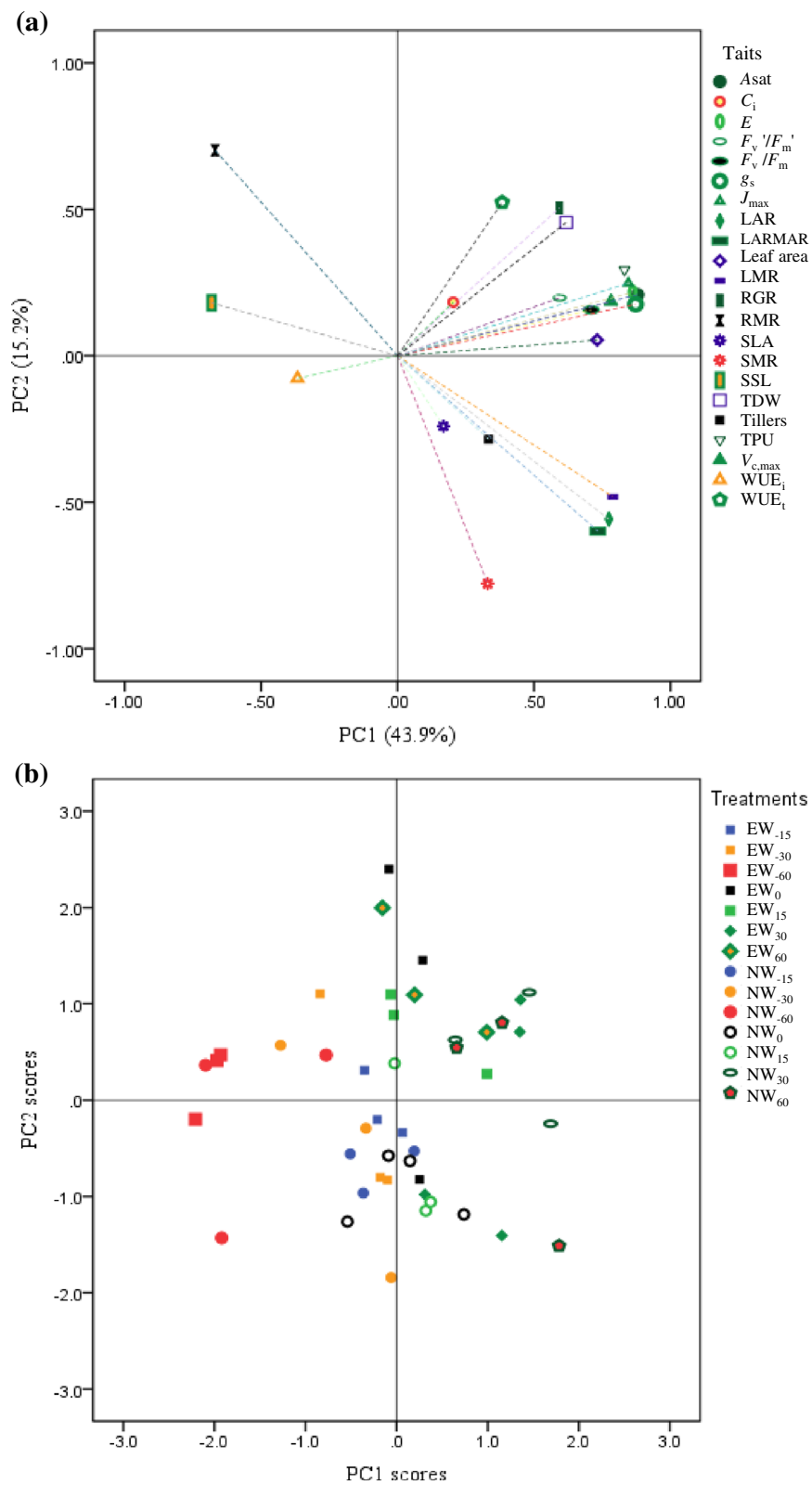


Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Principal component analysis on plant functional traits of *Agropyron cristatum* under elevated CO₂ with water status gradient. The first two principal components (PCs) were shown (a), and projections of the two PCs were sorted by the combined treatments (b). A_{sat} , light-saturated maximum photosynthetic rate; C_i , intercellular CO₂ concentration; E , transpiration rate; g_s , stomatal conductance; $V_{c,\text{max}}$, maximum rate of carboxylation; J_{max} , maximum rate of electron transport; TPU, rate of those phosphate utilization; F_v/F_m , photochemical efficiency of open reaction centers of photosystem (PS) II; F_v/F_m , maximal photochemistry efficiency; LAR, leaf area ratio; LARMR, leaf area and root mass ratio; LMR, leaf mass ratio; RGR, relative growth rate; RMR, root mass ratio; SLA, specific leaf area; SMR, stem mass ratio; SSL, specific stem length; TDW, total plant biomass weight; WUE_i , intrinsic water use efficiency; WUE_t , total biomass water use efficiency. NW₋₆₀, NW₋₃₀, NW₋₁₅, NW₀, NW₁₅, NW₃₀, NW₆₀ denote -60 %, -30 %, -15 %, 0 %, 15 %, 30 %, and 60 % of watering relative to mean precipitation in the local site over 30 years with normal/ambient CO₂ concentration; while EW₋₆₀, EW₋₃₀, EW₋₁₅, EW₀, EW₁₅, EW₃₀, EW₆₀ are those with elevated CO₂

the 57th and 62nd days after sowing according to a GLM ANOVA (Additional file 2: Table S1).

Multiple plant traits and environmental effects

PCA on multiple traits showed that the first and second principal components (PCs) explained 43.9 % and 15.2 % of the total variance, respectively (Fig. 1a). Most of the traits related to plant growth and photosynthetic activities were closely and positively correlated with PC1, and their loadings were mostly located in quadrant I. Those related to canopy size had similar correlations with PC1, and were mostly located in quadrant IV. Root and shoot biomass allocation traits were closely correlated with PC2, but separated conversely in quadrants II and IV, respectively. Projections for the multivariate traits and the effects of the two factors showed a complex pattern (Fig. 1b). However, the projections with extreme water deficit, including W₋₆₀ with ambient CO₂ (NW₋₆₀) and W₋₆₀ with elevated CO₂ (EW₋₆₀), and severe water deficit (NW₋₃₀, and EW₋₃₀) showed a distinct pattern, mostly appearing in the left part relative to the vertical line of origin, being opposed to those under relatively ample water conditions.

Plant growth analysis

Plant growth was increased significantly by applying water under both ambient and elevated CO₂ (Fig. 2a). Elevated CO₂ increased plant biomass by 21.6 %, 30.6 %, 32.3 %, 49.7 %, 52.1 %, 18.3 %, and 13.2 % in the W₋₆₀, W₋₃₀, W₋₁₅, W₀, W₁₅, W₃₀, W₆₀ watering treatments, respectively, indicating that the relationship between stimulation by CO₂ enrichment and water status was a well-fitted quadratic function with higher points under moderate water change but declining under both water deficit and well-watered conditions. According to GLM ANOVA, CO₂ concentration and watering treatment alone each significantly affected plant growth ($P < 0.01$, Additional file 3: Table S2). Plant individual leaf area significantly decreased with water deficit, whereas CO₂ had no significant or systematic effect in GLM ANOVA (Fig. 2b; Additional file 3: Table S2).

From W₋₆₀ to W₀, an increase in LAR with improving water status was observed under the ambient CO₂ level; however, this increase trend seemed to be partly offset

by elevated CO₂ (Fig. 2c). LARMR had a similar response to the water status gradient, and CO₂ enrichment-induced attenuation of the response to water status change was also observed (Fig. 2d). Both watering and CO₂ concentration had significant effects on these two parameters ($P < 0.05$, Additional file 3: Table S2).

Watering had a significant effect on SLA, with a maximum under moderate water status and a reduction under water deficit or increased watering (Fig. 3a). SSL was also significantly affected by watering treatment, decreasing linearly with increased water (Fig. 3b). However, ANOVA on SLA and SSL suggested elevated CO₂ and the interaction of watering and CO₂ had no significant effect (Additional file 3: Table S2). LMR increased, while RMR decreased, with increasing water; in contrast, elevated CO₂ seemed to reduce LMR and increase RMR in most cases except under W₃₀ treatment (Fig. 3c, d). LMR and RMR were both significantly affected by watering, and LMR was also significantly affected by elevated CO₂ (Additional file 3: Table S2).

Photosynthetic capacity, stomatal conductance, and intrinsic WUE

Watering had significant effects on the photosynthetic capacity parameters ($V_{c,\text{max}}$, J_{max} , TPU, A_{sat}) with a maximum under W₃₀ treatment, above or below which the values declined (Fig. 4). With elevated CO₂, the photosynthetic capacity showed an increasing trend under relative water deficit, but a decrease under water surplus conditions (W₃₀ and W₆₀) (Fig. 4a–c, e). Stomatal conductance (g_s) increased with increasing water, but showed a decreasing trend under W₆₀, and was stimulated by elevated CO₂, except under extreme drought and excess water conditions (W₃₀ and W₆₀) (Fig. 4f). WUE_i was increased only under extreme drought with ambient CO₂, but was generally elevated under the high CO₂ concentration except in the W₆₀ treatment (Fig. 4g). Watering had significant effects on $V_{c,\text{max}}$, J_{max} , TPU, A_{sat} , and g_s , but no significant effect on WUE_i (Additional file 3: Table S2).

The two chlorophyll *a* fluorescence parameters— F_v/F_m and F'_v/F'_m —were only significantly decreased by severe water deficit. Elevated CO₂ did not have a significant effect on either parameter, except a slight

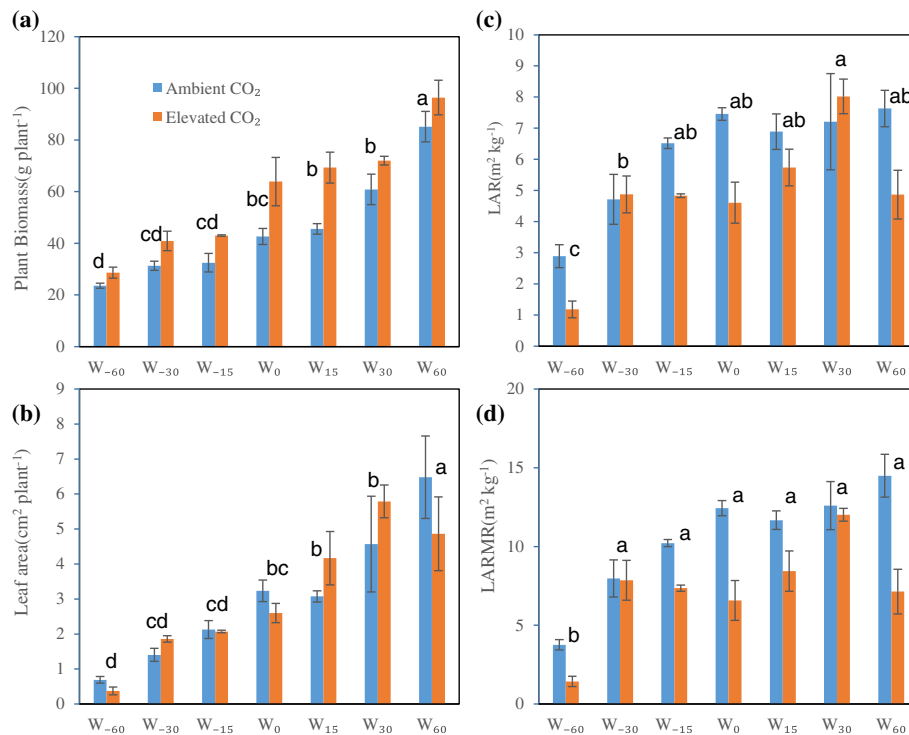


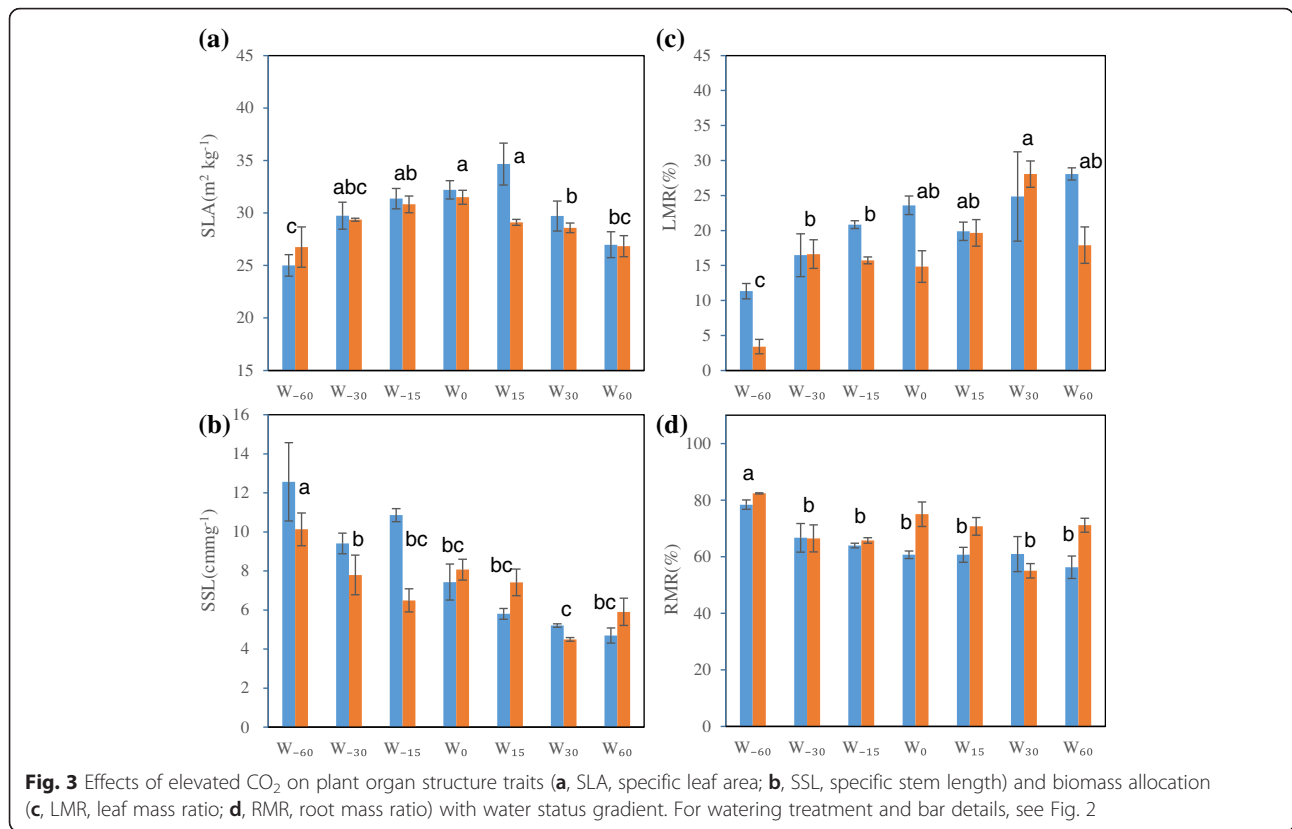
Fig. 2 Effects of elevated CO₂ on plant growth and its structure with water status gradient. **a** plant biomass; **b** leaf area; **c** LAR, leaf area and plant mass ratio; **d** LARMR, leaf area and root mass ratio. W₋₆₀, W₋₃₀, W₋₁₅, W₀, W₁₅, W₃₀, and W₆₀ represent -60 %, -30 %, -15 %, 0 %, 15 %, 30 %, and 60 % of watering relative to mean precipitation in the local site over 30 years. A GLM ANOVA between temperature and watering, and their interaction was show in Additional file 3: Table S2. Different lower case letters indicate differences between watering treatments across the two CO₂ levels at *P* < 0.05 according to Duncan multiple range test. Vertical bars denote SE of the mean (*n* = 3–4)

stimulation under relative water deficit. F_v/F_m was unchanged but F'_v/F'_m was decreased by elevated CO₂ under relatively sufficient water conditions (Fig. 4d, h). Watering alone had significant effects on F_v/F_m and F'_v/F'_m , while CO₂ concentration and the interaction of CO₂ and water had no effect (Additional file 3: Table S2).

Mesophyll cell ultrastructure

No significant changes in mesophyll cell length were observed under changes in the two treatment factors (Additional file 4: Table S3). Cell width increased with elevated CO₂, but decreased with reducing water except in the W₃₀ and W₆₀ treatments. There was a linear increase in cell area with increasing water under ambient CO₂; under elevated CO₂, cell area was often increased with improving water status, but decreased with excess water. Cell wall thickness (CWT) was decreased only under extreme drought (W₋₆₀), but was drastically increased by CO₂ enrichment by 22.2 % across all watering treatments. The chloroplast number per cell was decreased by extreme drought, but markedly increased by CO₂ enrichment in all watering treatments. Although the three chloroplast size parameters (length, width, and

profile area) showed no systematic responses to the water status gradient, they were decreased under elevated CO₂ in plants subjected to extreme and severe drought (W₋₆₀ and W₋₃₀) and increased in the excess water treatments (W₃₀ and W₆₀). The number of grana thylakoid membranes (TMN) was unaffected by watering treatment, but was decreased by elevated CO₂ by an average of 22.9 % across all watering treatments. Water deficit and relative water surplus caused declines in the starch grain number per chloroplast profile (SGN), and no intact starch grains were found at W₋₆₀ with ambient CO₂. Elevated CO₂ led to decreases in SGN in the W₋₁₅ and W₋₃₀ treatments, but increases in the other water treatments, indicating that the effect of elevated CO₂ strongly depended on water status. The plastoglobuli number per chloroplast (PGN) tended to decrease under ample watering at ambient CO₂; elevated CO₂ seemed to decrease PGN under severe drought (W₋₃₀ and W₋₆₀), but increase it under excess water treatments. Based on ANOVA, CO₂ concentration, watering, and their interaction all significantly affected CWT and PGN. Elevated CO₂ and watering both had significant effects on the chloroplast number, but their interaction did not. Cell



width, cell area, and SGN was significantly affected only by watering, and TMN only by elevated CO₂. Finally, watering, and its interaction with CO₂ concentration significantly affected the chloroplast length (Additional file 4: Table S3).

Furthermore, as directly seen from the transmission electron micrographs of mesophyll cells at different magnification scales (Fig. 5), the cells tended to become more circular under normal watering conditions and produced more chloroplasts under elevated CO₂ (Fig. 5a, d). The starch grains in chloroplasts were more numerous and larger under elevated CO₂ than under ambient CO₂, and less grana thylakoid membranes were observed at the high CO₂ level (Fig. 5b, c, e, f). An abnormally swollen grana thylakoid possibly extruded by the greater starch grains was also observed (Fig. 5f). A cell wall with distinct layers appeared (Fig. 5c, f). In plants exposed to extremely severe water deficit (Fig. 5g–l), very few chloroplasts were observed (Fig. 5g), the chloroplast envelope seemed to be broken, most of the chloroplast grana were swollen, grana thylakoids were unclear and appeared to have disintegrated, the cell wall was abnormal with uneven layers, and a number of large plastoglobuli were observed (Fig. 5h, i). However, under severe drought accompanied by elevated CO₂, partial recovery seemed to have occurred, i.e., the damage was partly alleviated (Fig. 5j–l).

Total water use and biomass water use efficiency

Total water use, i.e., the actual evapotranspiration amount, significantly and linearly increased with increasing irrigation, from 304.6 to 654.5 g pot⁻¹, a total increase of 114.9 % (Fig. 6a). However, elevated CO₂ did not affect the total water use during the experimental period. WUE_t tended to increase with increasing water, particularly at the high CO₂ concentration (Fig. 6b). WUE_t showed significant and strong relationships with total water use (Fig. 6c) and total biomass (Fig. 6d), particularly at the higher CO₂ level, indicating that WUE_t is often higher in plants with a faster growth rate, even greater water consumption and under an increased CO₂ concentration.

Discussion

Our experiment on the effects of elevated CO₂ on plants under various watering regimes showed that plant growth and photosynthetic capacity were stimulated by elevated CO₂ under moderate water changes relative to normal precipitation; however, over-watering or extreme water deficit diminished or even eliminated this stimulation. The damage from severe drought, i.e., chloroplast and grana thylakoid damage, was partially ameliorated under the high CO₂ level. This mostly confirmed our first hypothesis. This response to water status gradient at a high CO₂ concentration was reflected by combined

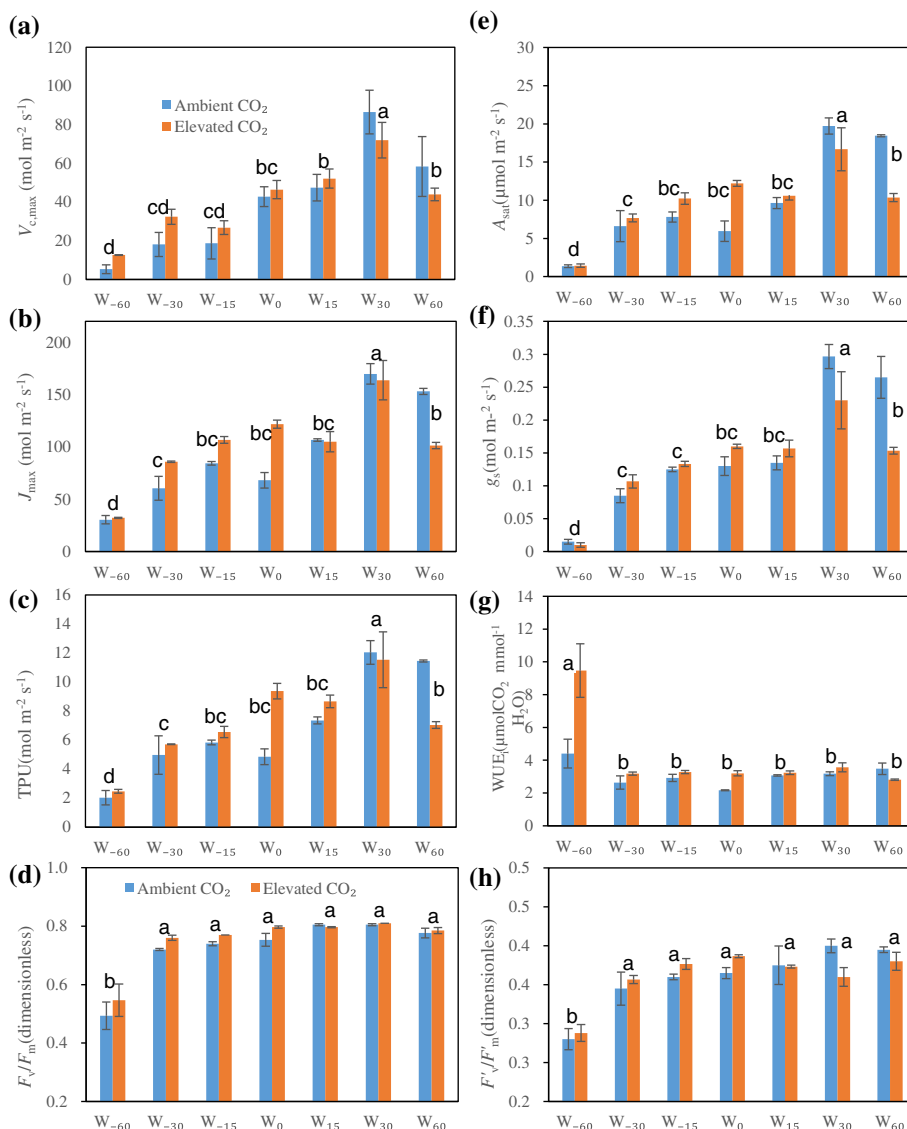


Fig. 4 Effects of elevated CO₂ on photosynthetic capacity (**a**, $V_{c,max}$; **b**, J_{max} ; **c**, TPU; **e**, A_{sat}), stomatal conductance (**f**, g_s), intrinsic water use efficiency (**g**, WUE), maximal photochemistry efficiency (**d**, F_v/F_m) and photochemical efficiency of open reaction centers of PSII (**h**, F_v'/F_m) with water status gradient. $V_{c,max}$, maximum rate of carboxylation; J_{max} , maximum rate of electron transport, TPU, rate of those phosphate utilization; A_{sat} , light-saturated maximum photosynthetic rate. For watering treatment and bar details, see Fig. 2

changes in plant architecture, biomass allocation, stomatal behavior, CO₂ assimilation, PSII photochemical process, cell organelle structure, and water use efficiency (WUE), demonstrating highly synergistic changes at multiple scales. This coordinated response pattern may partly support our second hypothesis. Our results provide a deeper insight into the effects of varying water status on the response to CO₂ enrichment, from cell ultrastructure to in vivo photosynthetic physiology and whole plant growth, highlighting that various aspects of the comprehensive responses of the dominant species need to be considered when assessing and projecting terrestrial ecosystem responses to climatic change, particularly in arid regions.

Stomatal conductance

A reduction in g_s under enhanced CO₂ can improve plant water status, thereby ameliorating the adverse effects of soil water deficiency on plant growth and physiological activity [8, 58]. As reported by Easlon et al. [59], a low g_s coupled with high photosynthetic capacity in *A. thaliana* plants growing under elevated CO₂ might result from more conservative N investment in the photosynthetic apparatus. In the present experiment, the marked g_s decline due to water deficit seemed to be alleviated by elevated CO₂, implying that increased CO₂ has a protective role in drought-stressed leaves (Fig. 4f). In *Liquidambar styraciflua* plants, however, severe drought

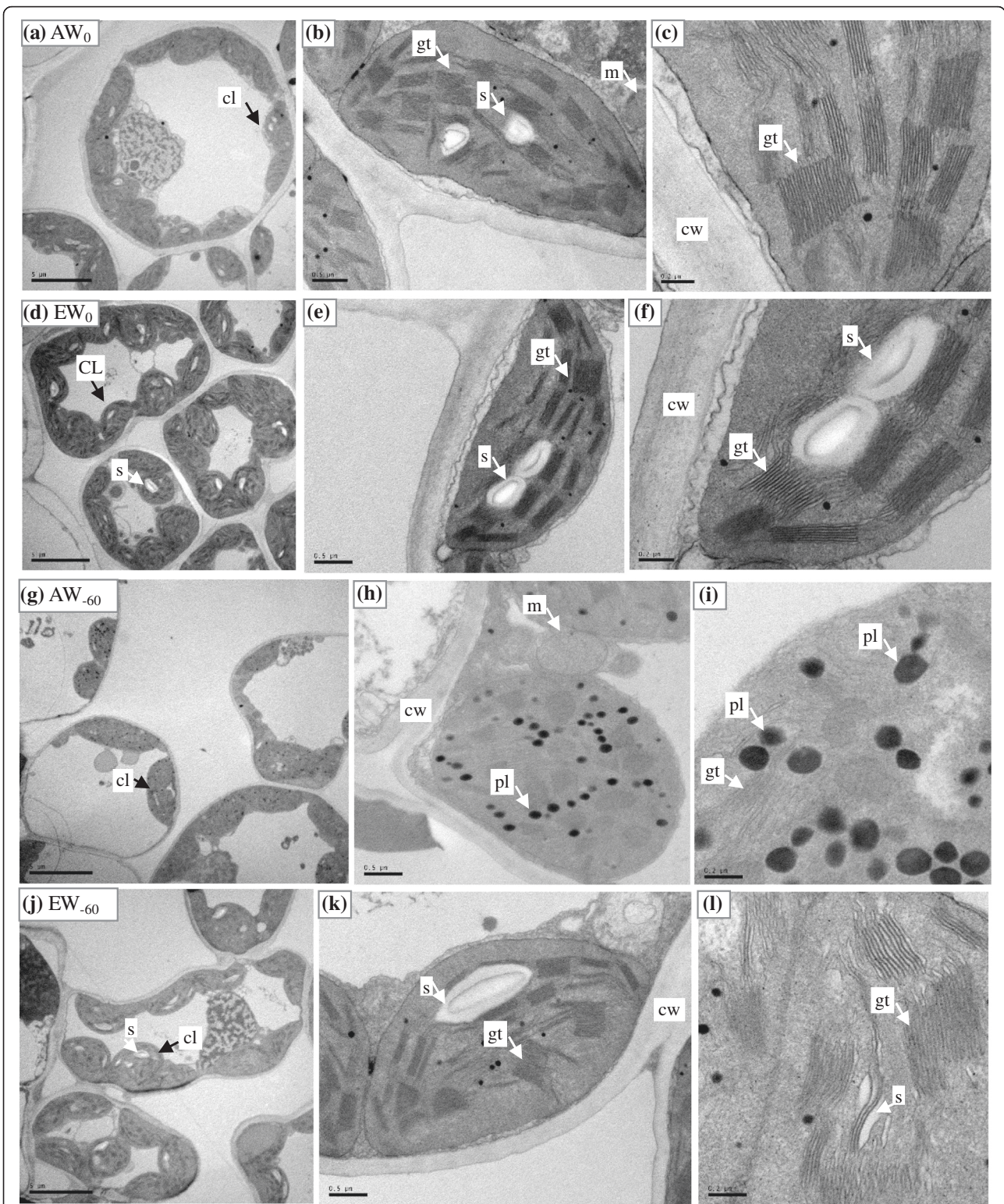
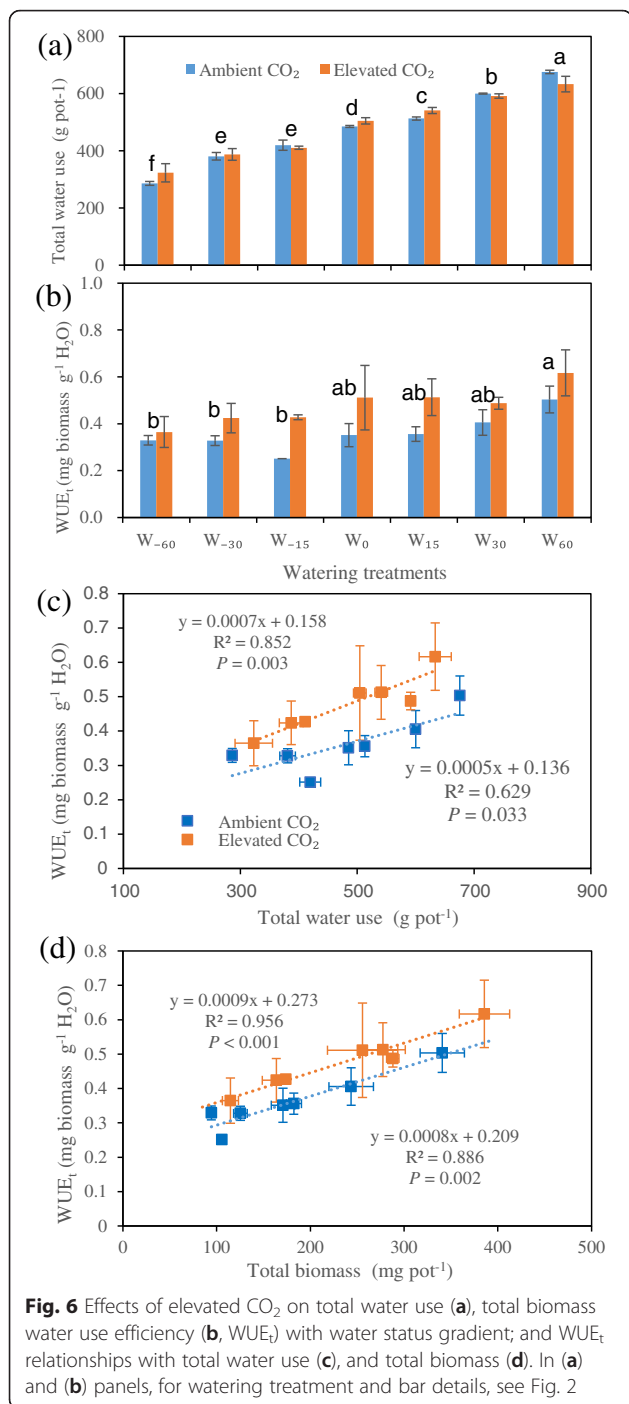


Fig. 5 Transmission electron micrograph (TEM) of mesophyll cell in *Agropyron cristatum* leaves under control watering (W_0 , **a-f**) and severe water deficit (W_{-60} , **g-l**) with ambient (**a-c, g-i**) and elevated CO_2 (**d-f, j-l**) for whole mesophyll cell (**a, d, g, j**), whole chloroplast (**b, e, h, k**), and granum thylakoids (**c, f, i, l**). cl, chloroplast; cw, cell wall; gt, granum thylakoids; m, mitochondria; pl, plastoglobuli; s, starch grains. Bars, 5 μm (**a, d, g, j**), 0.5 μm (**b, e, h, k**), and 0.2 μm (**c, f, i, l**)



can lead to excessive stomatal closure and accelerated leaf senescence that may offset the benefits of CO₂ enrichment for saving water [60]. The lack of benefit from increased CO₂ to drought-stricken plants may be attributable to a lack of stomatal regulation [61]. Thus, the effect of elevated CO₂ on the stomatal response to water change may depend on the species and extent of water deficit [58], but this needs to be explored further.

A marked decline in g_s under severe water deficit in close parallel with photosynthetic capacity may reflect the coexistence of stomatal and mesophyll limitations on photosynthesis (Fig. 4), in agreement with Flexas and Medrano [62]. Their results indicated that non-stomatal limitations including decreased photochemistry and Ru-bisco activity corresponded with decreases in g_s , particularly below $100 \text{ mmol m}^{-2} \text{ s}^{-1}$. Photosynthetic mesophyll limitations, such as decreases in both photosynthetic activity and cell size, may play a major role in photosynthetic depression [63], which is almost in agreement with the present results. A recent report showed that *Ramonda nathaliae* plants with smaller stomata had higher resistance to drought than *R. serbica* [64], in line with the reductions in mesophyll cell size and g_s under severe drought in the present experiment (Additional file 4: Table S3). Thus, an associated change in cell size and stomatal behavior might confer a highly adaptive response to climate change.

In vivo physiological capacity

Photosynthetic capacity parameters such as A_{sat} , V_{cmax} , J_{max} , and TPU are inhibited under extreme and severe drought, but elevated CO₂ can partly alleviate this inhibition [4, 24, 26, 65, 66]. However, in the present results, although elevated CO₂ increased the photosynthetic capacity under severe and moderate drought and control treatments, it had no significant effect, or caused a decrease, under surplus watering conditions (Fig. 4). As reported previously, the photosynthetic capacity was decreased under elevated CO₂ in *Eucalyptus* seedlings grown under well-watered but not water-stressed conditions [46]. Thus, the positive effects of elevated CO₂ on photosynthesis may favor plants exposed to a moderate range of water statuses—from severe to moderate water deficit stress.

The effect of elevated CO₂ on in vivo chlorophyll *a* fluorescence is also uncertain. For example, the maximal photochemical efficiency of PSII (F_v/F_m) was increased by elevated CO₂ in poplar seedlings [67]. However, chlorophyll fluorescence showed no changes under elevated CO₂ in Scots pine needles [68] and some grasses [26]. Roden and Ball [46] showed that elevated CO₂ led to a reduction of F_v/F_m in amply watered *Eucalyptus* seedlings, but no effect was found under drought. No significant differences in F_v/F_m were found in control and water-deprived *Phaseolus vulgaris* plants, although plant fresh weight decreased approximately 30 % in water-stressed conditions [69], suggesting that other metabolic processes related to growth, rather than PSII photochemical activity, might play a critical role in the response to water deficit stress. Our findings indicated that CO₂ enrichment had no effect on F_v/F_m , and even inhibited the photochemical efficiency of open reaction

centers of PSII (F'_v/F'_m) in relatively well-watered plants, but had a stimulatory effect under relative water deficit (Fig. 4h). Moreover, we found that accelerated accumulation in starch grains might damage the chloroplast structure under well-watered conditions, which might partly explain the depression of PSII activity.

In Rakić et al. [64], a reduction in F_v/F_m occurred only in severely drought-stressed resurrection plants, consistent with our results in which a dramatic decline in F_v/F_m and F'_v/F'_m appeared only under extreme water deficit. Therefore, a drastic decline occurs only in plants subjected to extreme environmental stress, suggesting that these two parameters might not be good indicators of moderate water status changes. Elevated CO_2 might not affect or might decrease these parameters, possibly because of decreased leaf thickness (greater SLA) or down-regulation of photosynthetic potential [26].

Organelle structure changes

The chloroplast, a compulsory light-harvesting organelle, can be easily and seriously affected by elevated CO_2 [70, 71]. Many previous studies have shown that elevated CO_2 could increase the number of chloroplasts in mesophyll cells [47, 70, 72, 73], which was also confirmed by the current study (Additional file 4: Table S3, Fig. 5). However, the mechanism by which elevated CO_2 positively regulates chloroplast numbers still remains unclear [47, 72]. Some reports have suggested it results from stimulation of chloroplast biogenesis processes [47, 74]. Additionally, there is other evidence for this abnormal change induced by increased CO_2 : a drastic increase in the amount of chloroplast stroma thylakoid membranes has been found relative to those in lower CO_2 levels [70]. Damage to chloroplast ultrastructure can also occur under elevated CO_2 , partly as a result of increased starch grain size and numbers through accelerated starch accumulation in chloroplasts [47]. Enhanced accumulation of starch grains within chloroplasts by elevated CO_2 can induce distortion of grana thylakoids, with plants exposed to a high CO_2 level often having a low A_{net} [71]. In our current results, the increase in photosynthetic capacity was in agreement with the more numerous and larger starch grains in the chloroplasts of well-watered plants under the high CO_2 level. It could be reasoned that starch accumulation might not be enough to limit the increase in photosynthesis induced by CO_2 enrichment. However, this phenomenon would disappear under water deficit stress. Moreover, extremely severe water deficit can damage mesophyll cells, resulting in abnormal and disorganized cell organelles including chloroplasts and their grana [75], which was confirmed by the current experiment. However, this damage was partly ameliorated by elevated CO_2 , implying that plants have a strong dependence on the combination of CO_2 concentration and water status.

Plant structural traits and associations with physiological activities

Interestingly, LARMR increased with increasing water supply but decreased at the high CO_2 level (Fig. 2d), reflecting different effects on the biomass investment balance between the light trapping organs and resource element-deriving organs from the two climatic factors [49, 76]. This indicates that elevated CO_2 might negate the enhanced investment in light-intercepting organs under an over-watering regime in this species, in line with our earlier study [26]. PCA can not only unveil the extent and directions of correlations among plant structural and functional traits, but also distinguish the effects of treatment factors or their combinations from the projections, highlighting the importance of this useful analysis tool [23, 26, 57]. Here, for example, RMR and SMR had opposite distributions in the PC loadings (Fig. 1a), possibly reflecting the carbon allocation trade-off between root and stem organs [27]. Moreover, we found positive close relationships between plant structural traits and functional traits such as photosynthetic activities and g_s ; they were all positively related with PC1, which might highlight their coordinated changes under different climatic change factors (Fig. 1a). Close associations between morphological/structural and functional traits have been widely reported, depending on the species and environment [26, 77–79]. Our results again highlight that multiple variables at different scales might together play a critical role in the adaptive response to global change by balancing or offsetting each other.

Water use efficiency

Elevated CO_2 can improve plant water status by reducing g_s and thereby increasing WUE to ameliorate the adverse effects on plant growth and physiological processes from stress factors alone and in combination [4, 8, 58]. Water status also mediated the effectiveness of rising CO_2 by coupling the processes of gas exchange and leaf enlargement. Nevertheless, the pros and cons of acclimation to changes in water conditions might coexist in plant responses to elevated CO_2 ; leaf area enlargement induced by CO_2 might exaggerate water use, while decreased g_s would promote WUE_i [15, 58]. However, WUE_i might decline under severe drought in some relict plant species exposed to elevated CO_2 [61]. In the present experiment, both WUE_i and WUE_t were increased by elevated CO_2 , implying that the promotion of A_{net} and plant biomass by elevated CO_2 might play a dominant role.

In the same steppe, conflicting results can occur because of different data types. For example, field rain use efficiency (RUE) increased with increasing MAP across different vegetation types, but decreased across different years in a given site, particularly in drier areas

[40, 80]. Decreased RUE with increasing precipitation can be due to low productivity or other resource limitations such as N deficit [80]. WUE_t and RMR increased significantly with decreasing precipitation, but decreased with elevated CO_2 [27]. In the present experiment, however, we found that the increase of WUE_t with increases in both water use and plant biomass (Fig. 6), particularly at a high CO_2 level, might explain the resource limitation to WUE ; both a water use increase and CO_2 enrichment, as increases in available resources, might promote WUE by stimulating photosynthetic capacity and plant growth. Furthermore, our results indicated that although WUE_t and WUE_i showed a similar response to elevated CO_2 , the former was more sensitive, implying that WUE_t might be a better indicator than WUE_i for assessing responses to climate change [81].

Elevated CO_2 mitigation of severe drought

Elevated CO_2 can ameliorate the negative effects of environmental stresses including severe water deficit in many different plant functional types or species such as the desert shrubs *Caragana intermedia* and *Caragana microphylla* [26, 82], the C_3 perennial grasses *L. chinensis* and *Stipa grandis* [26, 27], and the C_4 grass species *Cleistogenes squarrosa* [26]. The current experiment confirmed this amelioration due to CO_2 enrichment in a C_3 perennial grass from the same steppe as earlier experiments [26, 27, 82]. However, this amelioration was not observed in other reports on species such as some relict species [61], *Populus deltoides* [83], *L. styraciflua* [60], and *Eucalyptus radiata* [81]. Moreover, CO_2 enrichment had a negligible effect on the response of *E. radiata* seedlings to drought, and did not alleviate the deleterious effects of drought due to rising temperature [81]. Thus, high CO_2 may protect against or exacerbate stress effects, depending on different plant functional types and species.

A previous report showed that the growth of a dominant perennial shrub in a Mojave Desert ecosystem was doubled by a 50 % increase in CO_2 only in a drier year [13]. In the present study, although elevated CO_2 stimulated plant growth and photosynthetic activity in water deficit-treated plants, it had an inhibitory effect on the amply watered plants. This again indicates that CO_2 enrichment may be more beneficial in drought conditions, implying that elevated CO_2 may eliminate drought-induced negative plant responses. Thus, the allocation response to rising CO_2 may also depend on water status. However, in a recent report, elevated CO_2 productivity did not significantly modify the effects from soil water status in mesic grassland, semi-arid grassland, and xeric shrubland [84]. Taken together, these results suggest the integrated effects of elevated CO_2 and water status on plants may be highly species- and habitat-specific.

Conclusions

Elevated CO_2 can improve plant water status and therefore stimulate various physiological and ecological processes from the organelle to cell, organ and plant individual level. However, this stimulation is strongly dependent on water status. Elevated CO_2 generally increased the growth and photosynthetic physiological parameters of *A. cristatum* such as V_{cmax} , A_{sat} , J_{max} , g_s , TPU, and F_v/F_m at severe to moderate water status, but had no effect on or even decreased these parameters in over-watering conditions. This indicates that CO_2 enrichment can often ameliorate deleterious drought effects under moderate water deficit, but not extreme drought or over-watering conditions, and that plant morphological and structural alterations, and carbon allocation may be involved in this adaptive regulation. Our results for a dominant species from a degraded steppe suggest that water status changes such as severe drought or over-watering events might fundamentally contribute to the effects of CO_2 enrichment on key physiological activities, cell structure, plant growth and even survival in a future climatic context, even completely reversing the direction of the effect. Our results highlight that CO_2 fertilization's dependency on water status should be considered when projecting plant responses to climate change. These findings contribute to our understanding of plant responses to global climate change, and may be useful in vulnerable ecosystem management.

Additional files

Additional file 1: Figure S1. Changes in soil relative water contents (SRWC) at ambient and elevated CO_2 concentrations with a water status gradient during a given period. Measured at 17:00 every three days, the watering day, but before watering during a consecutive 10-day period. W_{-60} , W_{-30} , W_{-15} , W_0 , W_{15} , W_{30} , and W_{60} represent -60 %, -30 %, -15 %, 0, 15 %, 30 %, and 60 % of watering relative to mean precipitation in the local site over 30 years. Vertical bars denote SE of the mean ($n = 3-4$). GLM ANOVA refers to Additional file 2: Table S1. (DOCX 51 kb)

Additional file 2: Table S1. Tests of between-subjects effects of CO_2 concentration and watering on soil relative water content (SRWC) from GLM ANOVA. Bold font for P values indicates significance at $P < 0.05$. (DOCX 17 kb)

Additional file 3: Table S2. Tests of between-subjects effects of CO_2 concentration and watering on plant functional traits, physiological activity parameters from GLM ANOVA. Bold font for P values indicates significance at $P < 0.05$. (DOCX 24 kb)

Additional file 4: Table S3. Mesophyll cell ultrastructure in *Agropyron cristatum* grown under ambient and elevated CO_2 concentrations with water status gradient. (DOCX 21 kb)

Abbreviations

A_{sat} , light-saturated maximum photosynthetic rate; F_v/F_m , photochemical efficiency of open reaction centers of PSII; F_v/F_m , maximal PSII photochemical efficiency; g_s , stomatal conductance; J_{max} , maximum rate of electron transport; LAR, leaf area and plant total mass ratio; LARMR, leaf area and root mass ratio; LMR, leaf mass ratio; PCA, principal component analysis; RGR, relative growth rate; RMR, root mass ratio; SLA, specific leaf area; SMR, stem mass ratio; SSL, specific stem length; TPU, rate of thiose phosphate utilization;

$V_{c,max}$ maximum rate of carboxylation; WUE_i intrinsic water use efficiency; WUE_p total biomass water use efficiency.

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

Authors' contributions

JY, XZ and ZG conceived and designed the study; JY, XZ and LT conducted the experiment and performed the data analysis; JY and XZ drafted the manuscript. All authors approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent to publish

Not applicable.

Ethics approval and consent to participate

The soil and seed collections from the field in our experiments are conducted in and permitted by Inner Mongolia Grassland Ecosystem Research Station, Institute of Botany, Chinese Academy of Sciences, Beijing. No species are involved at risk of extinction in the present experiment. Others are not applicable.

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