



CO₂ elevation modulates the growth and physiological responses of soybean (*Glycine max* L. Merr.) to progressive soil drying

Bingjing Cui¹ · Jie Liu¹ · Manyi Zhang¹ · Heng Wan¹ · Guiyu Wei¹ · Zhenhua Wei¹ · Fulai Liu^{2,3}

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Abstract

Understanding the impact of future climates on crop performance is essential for sustainable agricultural production. In the current research, the development and biological behavior of soybean plants during gradual desiccation of the soil (from the 100% of pot water holding capacity to the g_s of plant decreased to 10% of that of the control plants) at ambient [CO₂] (a [CO₂], 400 ppm) and elevated [CO₂] (e [CO₂], 800 ppm) were investigated. The results showed that plants grown under e [CO₂] conditions had remarkably higher photosynthetic rate (A_n) but lower stomatal conductance (g_s) and transpiration rate (E) compared to plants at a [CO₂] conditions, which led to an enhanced water use efficiency at both stomatal (WUE_i) and leaf levels (WUE_{leaf}). In addition, the e [CO₂]-grown soybeans showed a stunted g_s response to progressive soil drying, coinciding with a decrease in the susceptibility of g_s to the ABA signaling, though they tended to maintain a better leaf water status under drought than the a [CO₂]-grown plants. Although the leaf nitrogen concentration (N_{leaf}) and the total plant N content were notably lower at the e [CO₂] condition, the specific leaf N content (SLN) was similar at different [CO₂] conditions. Compared to soybean grown under e [CO₂], the greater number of nodules at e [CO₂] treatment would lead to an enhanced N-fixation, yet, it did not improve the N nutrition of the plants. Nevertheless, by sustaining the SLN, the soybean plants enhanced A_n when growing at e [CO₂], particularly under dry conditions. This knowledge is essential for sustaining soybean production in future climate change scenarios.

Keywords Climate change · Drought stress · Abscisic acid · Root nodules · Water use efficiency

Introduction

The average atmosphere CO₂ concentration has gone up from 280 ppm to 410 ppm over the past 250 years (Tausz-Posch et al. 2020). IPCC (2014) predicted atmospheric [CO₂] might exceed 700 ppm towards the late 21st generation. In

the future climate, rising ambient [CO₂], temperatures as well as changing precipitation patterns are predicted to have a farreaching impact on agricultural production (Shao et al. 2015). Plant growth and progression are directly influenced by elevated [CO₂] (e [CO₂]) (Wei et al. 2020). Likewise, drought stress can affect plant water relations (Bencze et al. 2014), cell membrane integrity (Hessini et al. 2009), photosynthesis and other physiological processes (Liu et al. 2018; Zheng et al. 2020). Therefore, a deeper knowledge of the integrated effect of plants to e [CO₂] environment combined with drought conditions could help to maximize crop yield in future climate scenarios.

Elevated [CO₂] often has a beneficial impact on plant development (Dong et al. 2020). Previous research shows that e [CO₂] increases the net photosynthetic rate (A_n) while reducing the stomatal conductance (g_s) (Ainsworth and Rogers 2007; Hu et al. 2022). The decrease in g_s curtails the transpiration rates (E), while the high A_n results in more photo-assimilates and biomass accumulating (Wei et al. 2020), thus improving water use efficiency (WUE) of plants

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✉ Fulai Liu
fl@plen.ku.dk

- ¹ Key Laboratory of Agricultural Soil and Water Engineering in Arid and Semiarid Areas, Ministry of Education, Northwest A&F University, Yangling, Yangling 712100, Shaanxi, China
- ² Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Højbakkegaard Alle 13, 2630 Taastrup, Denmark
- ³ Sino-Danish Center for Education and Research, University of Chinese Academy of Sciences, 380 Huaibeizhuang, Huairou, Beijing, China

(Tausz-Posch et al. 2015; Yan et al. 2017). Therefore, it is essential to understand how $e[\text{CO}_2]$ affects stomatal behavior and leaf gas exchange. In tomato plants, the decrease of g_s under $e[\text{CO}_2]$ was closely related to the endogenous ABA level (Fang et al. 2019). Ethylene compensates for induced stomatal closure during gradual desiccation of the soil (Liang et al. 2023). Hsu et al. (2018) discovered that $e[\text{CO}_2]$ triggered stomatal closure by regulating OST1/SnRK2 kinases. On the other hand, hydraulic signals also modulate plants' g_s response to $e[\text{CO}_2]$. Reduced g_s and E may help to maintain a higher leaf water potential (Ψ_l) at $e[\text{CO}_2]$ (Wullschleger et al. 2010). Bunce (1996) found that although g_s and E of plants at $e[\text{CO}_2]$ were lower, hydraulic conductance could also be reduced, and therefore Ψ_l was not always higher. Some reports have indicated that $e[\text{CO}_2]$ decreases hydraulic conductance by down-regulating aquaporin expression (Fang et al. 2019), while others suggest that $e[\text{CO}_2]$ may enhance hydraulic conductance (Li et al. 2004).

Water deficit reduces g_s hereby increase stomatal limitation to CO_2 diffusion (Bousba et al. 2009). Research has demonstrated that $e[\text{CO}_2]$ maintains a high WUE mainly by enhancing A_n while reducing g_s and E, thereby mitigating the negative impacts of aridity (Tausz-Posch et al. 2015; Wall et al. 2001). Plant water use efficiency increases under the interaction of elevated CO_2 and drought stress (Silveira et al. 2023). Contrastingly, other studies have also shown that the effect of drought on g_s is more profound than that of $e[\text{CO}_2]$. Leakey et al. (2006) conducted an experiment investigating the interaction impacts of drought and $e[\text{CO}_2]$, reporting that decreased g_s caused by drought tended to be greater than that caused by $e[\text{CO}_2]$. Li et al. (2013) pointed out that $e[\text{CO}_2]$ did not alleviate the suppressive effect of drought on soybean seed yield, although it increased A_n and WUE.

Soybeans are the 4th major agricultural crop worldwide and an important economic crop in China (Ainsworth et al. 2012). Shaanxi province belongs to Loess Plateau spring soybean production area. In the last few years, owing to the changing weather conditions, the temperature on the Loess Plateau is on the rise, precipitation is gradually decreasing, freshwater resources for irrigation are limited (Li et al. 2011), and droughts are probably persist in the coming climate. Wang et al. (2018) considered that $e[\text{CO}_2]$ enhances soybean stress resistance mainly by improving their photosynthetic capacity and water use efficiency. This mitigating effect was also seen in peanut, tomato and wheat crops (Laza et al. 2021, Li et al. 2019, Yang et al. 2020). Rhizomes in soybean roots are able to fix atmospheric nitrogen into ammonia for plant uptake (Minguillon et al. 2022). Due to its ability of biological nitrogen fixation, soybean responds to $e[\text{CO}_2]$ and drought may be differently from other C_3 plants. Rhizobia could consume 4–11% of the carbohydrates produced by photosynthesis (Kaschuk et al. 2009, Soba et al.

2021). On the one side, drought can limit photosynthesis and reduce carbohydrate availability in legume rhizomes, thereby limiting N_2 fixation (Parvin et al. 2019). On the other side, $e[\text{CO}_2]$ may promote the growth in soybeans under drought by providing more photo assimilates for nodule growth and hence N fixation. It is well understood that the impact of $e[\text{CO}_2]$ on plant performance is very much dependent on the N availability, and $e[\text{CO}_2]$ grown environment often leads to a reduced N concentration in most plant species, restricting the CO_2 -fertilization effect on plants (Aranjuelo et al. 2014; Gojon et al. 2023), particularly under stress environment. While this is true in most of the C_3 species, it could be different for legumes such as soybean due to its ability to fix N. Therefore, it is important to research the interaction of $e[\text{CO}_2]$ and aridity stimuli in soybean growth and physiology as such knowledge is essential for the future production of soybeans in a sustainable climate.

This research aims to explore the impacts of $e[\text{CO}_2]$ on leaf water relations and gas exchange, and plant N nutrition of soybean subjected to gradual desiccation of the soil. It was hypothesized that, soybean plants subjected to the fertilization effect of elevated CO_2 would have increased rhizomatous nitrogen fixation, which might have altered the plant's N status and mitigated the adverse effects of $e[\text{CO}_2]$ on leaf N concentrations. Furthermore biological nitrogen fixation in soybean may cause plants under $e[\text{CO}_2]$ to alter their response to drought.

Materials and methods

Experimental material and site

The study was conducted from 22 March to 28 May 2021 in two $[\text{CO}_2]$ controlled growth chambers with $a[\text{CO}_2]$ (400 ppm) and $e[\text{CO}_2]$ (800 ppm) $[\text{CO}_2]$, respectively, at the Northwest A&F University, Yangling, Shaanxi province. Each of the growth chambers had an area of 3.8 m × 2.6 m. Carbon dioxide concentration in the chambers was measured every 6 s with a CO_2 Transmitter Series GMT220 (Vaisala Group, Helsinki, Finland). Both chambers were maintained at 60% relative humidity and $25/18 \pm 2$ °C day/night temperature (T), the photosynthetic photon flux density was controlled at about $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ from 7:00 to 19:00 h. S1 (in the supplementary figures) shows the average of daily $[\text{CO}_2]$ and T in the chambers during this study.

Soybean seeds (variety “Shanning 17”) were sterilized and sown in 6 L pots with 6.5 kg of air dried clay loam soil per pot. Five seeds were sown in each pots. One week after emergence, thinning was done and plants with similar size (one plant per pot) were kept. In addition, 10 ml soybean rhizobia solution (strain: “HH103”) was inoculated in each pot when the first true leaf appeared. The soil had a pH of

8.2, total C and N content of 12.5 and 0.35 g kg⁻¹, total P and K content of 0.84 and 18.56 g kg⁻¹. To ensure adequate nutrient supply, additional 0.5 g N, 0.8 g P and 0.8 g K were added to each pot. Additionally, 1 cm layer of perlite was covered on the surface of soil before the start of drought treatment to reduce evaporation.

Treatments

All plants were supplied with sufficient water (i.e. 90% pot water-holding capacity) for their growth until 15 May, 2021. Pot water-holding capacity was determined according to Liu et al. (2019). In each growth chamber, four plants were randomly selected as controls and maintained with adequate water supply, other plants were subjected to progressive drought by stopping irrigation. The endpoint of the progressive drought was determined when the stomatal conductance (g_s) of the drought treatment decrease to 10% of the g_s of the well-watered treatment based on the daily measurement of the leaf gas exchange rates after onset of the drought treatment (see "Leaf gas exchange" Section). Harvests of the drought-treated plants were taken every other day during the progressive soil drying (five times in total with four replicates each).

Measurements

Soil water status

During the progressive drying treatment, the pots were weighed at 9:00 am every day to calculate the daily water consumption. The fraction of transpirable soil water (FTSW) is a measure of soil moisture status in the pot. It was calculated as:

$$FTSW = (WT_n - WT_f) / TTSW, \quad (1)$$

where TTSW is total transpirable soil water, which is obtained using the pot weight at 100% WHC (i.e., 8.45 kg) minus the weight at final harvest (i.e., 6.85 kg). WT_n and WT_f (i.e., 6.85 kg) are the pot weight of the drought stressed pots at a given date and at final the harvest, respectively. S2 (in the supplementary figures) shows the changes of FTSW under the two [CO₂] growth conditions during the experimental period.

Leaf gas exchange

A_n , g_s and E were measured using a portable photosynthetic system (LiCor-6800, LI-Cor, NE, USA) every day at 10:00–12:00 h during the progressive drying treatment (i.e., from 16 May to 28 May). Measurements were made by placing fully expanded upper canopy leaves in leaf chambers

(four replicates per treatment) with a leaf chamber temperature of 25 °C, a photon flux density of 1400 μmol m⁻² s⁻¹, CO₂ concentrations of 400 and 800 ppm, respectively. Based on the above data, the intrinsic water use efficiency (WUE_i) was calculated as A_n/g_s , the instantaneous water use efficiency (WUE_{leaf}) was calculated as A_n/E .

Leaf water relation and ABA concentration

Gas exchange parameters were measured at the beginning of each destructive sampling, then the leaf was removed and frozen in liquid nitrogen, then kept in a refrigerator at – 80 °C for later analysis of abscisic acid (ABA). The method of ABA determination was as follows: Grinding of fresh leaves in liquid nitrogen, 40 mg of the sample was admitted into a 1.5 ml Eppendorf tube. And 1.0 ml milli-Q water was admitted into the test tube and shaken overnight at 4 °C for the extraction of ABA, extracts were then centrifuged for 5 min. After centrifugation, 0.7 ml supernatants were taken as the test solution and $[ABA]_{leaf}$ was determined by the ELISA method (Enzyme Linked Immuno Sorbent Assay). ABA-ELISA kits (The cross-reactivity of anti-ABA McAb with ABA analogs is < 3.5%) were purchased from China Agricultural University.

Before the final harvest, gas exchange parameters were measured, followed by leaf water potential (Ψ_l , MPa) and RWC and ABA measurements on leaves selected at the same leaf position as those used for photosynthesis measurements. The Ψ_l was determined by pressure chamber at noon (Soil Moisture Equipment, Santa Barbara, CA, USA), the relative water content (RWC) of the leaves were calculated following the protocol of Liu et al. (2019). In detail, one leaflet was excised from the plant and its fresh weight (FW) was determined immediately. Then, the leaflet was put on distilled water with the cutting ending submerged in the water at 20 °C for 2 h under dim illumination to avoid respiratory losses. After blotting the turgid weight (TW) was determined. Dry weight (DW) was measured after drying the leaflet to constant weight at 70 °C. Then the RWC was calculated as:

$$RWC = (FW - DW) / (TW - DW). \quad (2)$$

Plant morphological and physiological traits

Since the evaporation from the soil was negligible, the sum of the change in soil moisture and the amount of irrigation during the experiment were used to calculate the water consumption of the potted plants (WU, cm³). At each sampling time, the plant samples were divided into leaves, stems, and roots, then they were dried at 70 °C to constant weight (about 48 h). Plant water use efficiency (WUE_p, kg m⁻³) was calculated as:

$$WUE_p = \text{biomass}/E_p, \quad (3)$$

Where the biomass refers to the total biomass produced by a plant during the experimental treatment, which was obtained by subtracting the dry mass at the beginning from the dry mass at the end of the processing. E_p refers to the total amount of water transpired by the plant in the same period, which was WU in this study.

The dried plant samples were ground into fine powder in a mortar and pestle. Total carbon (C) and total nitrogen (N) in the samples (%) were determined by elemental analyzer (Vario Max CN; Elementar Corp., Germany) and the amount of N per plant (Plant N content, g plant⁻¹) is determined by multiplying the plant N concentration by the dry weight. Leaf N concentration ($[N]_{\text{leaf}}$, g kg⁻¹) converted from proportional N (%). The specific leaf nitrogen content (SLN, g N m⁻²) was calculated as the ratio of leaf N concentration to the specific leaf area (SLA, cm² g⁻¹) at different growth stages.

The leaf area (LA, cm²) of plants was determined by leaf area meter (LICOR 3100). The specific leaf area (SLA, cm² g⁻¹) was calculated as the ratio of LA to leaf dry mass. The nitrogen balance index (NBI) was determined by Plant polyphenol chlorophyll optical leaf clip meter (DUALEX SCIENTIFIC TM).

Statistical analysis

The responses of g_s , A_n , E , WUE_i and WUE_{leaf} to gradual desiccation of the soil were characterized by a linear-plateau model (Wei et al. 2020):

$$\text{If } FTSW > c; y = y_{\text{initial}}, \quad (3a)$$

$$\text{If } FTSW < c; y = y_{\text{initial}} + a \times (FTSW - c), \quad (3b)$$

where y represents g_s , A_n , E , WUE_i or WUE_{leaf} , respectively; y_{initial} means the initial values of g_s , A_n , E , WUE_i and WUE_{leaf} , respectively, at which the plants were unaffected by water deficit, c is the FTSW threshold at which y started to diverge from y_{initial} for g_s , A_n , E , WUE_i and WUE_{leaf} . The parameters y_{initial} , a and c were determined by PROC NLIN fitting using PC SAS 9.4 software (SAS Institute Inc., Cary, NC, USA, 2002–2012). T test was performed for each parameter determined by linear-plateau regression between two $[CO_2]$ conditions using Med Calc statistical software 19.0.7, and P value was obtained.

All the figures and PCA analysis were done by Origin 2022 software (OriginLab Corp., USA). All data were analyzed by SPSS 22 software (two-way ANOVA) (IBM Corp., 2021), the mean values of all treatments were compared using Duncan's multiple test ($P=0.05$). The quantitative

relationships between g_s and $[ABA]_{\text{leaf}} / \Psi_1$ were analyzed by linear regressions.

Results

Effects of CO_2 and soil water deficits on leaf gas exchange

Under the condition of sufficient water supply, A_n of soybean leaves at $e[CO_2]$ condition increased 85.7% remarkably compared to that at $a[CO_2]$ (Fig. 1a; Table 1). With the decrease of soil moisture, the FTSW decreased continuously, the A_n of plants at $e[CO_2]$ started to decrease when the FTSW was 0.43, while the A_n at $a[CO_2]$ declined when the FTSW was 0.42. Compared with $a[CO_2]$, A_n of $e[CO_2]$ decreased more sharply when $FTSW < 0.42$. At the initial stage of soil desiccation, leaf stomatal conductance was 38.4% inferior at $e[CO_2]$ than that at $a[CO_2]$ ($P < 0.05$, Fig. 1b; Table 1). With the decrease in FTSW, g_s of soybean leaves at $e[CO_2]$ tended to decline later than plants at $a[CO_2]$ (i.e., $FTSW = 0.44$ vs. 0.48, not significant). Likewise, the initial transpiration rate (E) of leaves at $a[CO_2]$ was 46.6% higher than those at $e[CO_2]$ ($P < 0.05$, Fig. 1c; Table 1). As FTSW further decreased, E of the plants grown at $e[CO_2]$ tended to decline later than those at $a[CO_2]$ (i.e., $FTSW = 0.44$ vs. 0.48, not significant).

Effects of CO_2 and water stress on plant water relations

Aridity treatment had remarkable effect on leaf RWC and Ψ_1 (Table 2). Under $a[CO_2]$, the RWC of drought-stressed soybean leaves was 32.2% lower than that of well-watered leaves. Under $e[CO_2]$, the RWC of the drought-stressed treatment was 23.6% lower than that of well-watered treatment. Compared to $a[CO_2]$ -treated plants, RWC in $e[CO_2]$ -treated plants decreased less pronouncedly by drought stress in relation to the well-watered controls, yet there were no obvious correlations between the CO_2 and water treatment. The level of CO_2 in the growth environment had no remarkable impact on plant water relation characteristics (Table 2). Plant water use (WU) under drought was significantly less than that under well-watered condition. WU of plants at $e[CO_2]$ was notably less than that at $a[CO_2]$ ($P = 0.02$).

Under well-watered condition, the ABA concentration of soybean leaf ($[ABA]_{\text{leaf}}$) was slightly higher at $e[CO_2]$ than that at $a[CO_2]$ (Fig. 2a). As the FTSW gradually decreases, the $[ABA]_{\text{leaf}}$ increased exponentially under both CO_2 concentrations, and at a same FTSW level the $[ABA]_{\text{leaf}}$ at $e[CO_2]$ was slightly greater than that at $a[CO_2]$ ($P > 0.05$) (Table 2; Fig. 2a). When $FTSW > 0.3$, the g_s of soybean leaves grown under the two different CO_2 concentrations

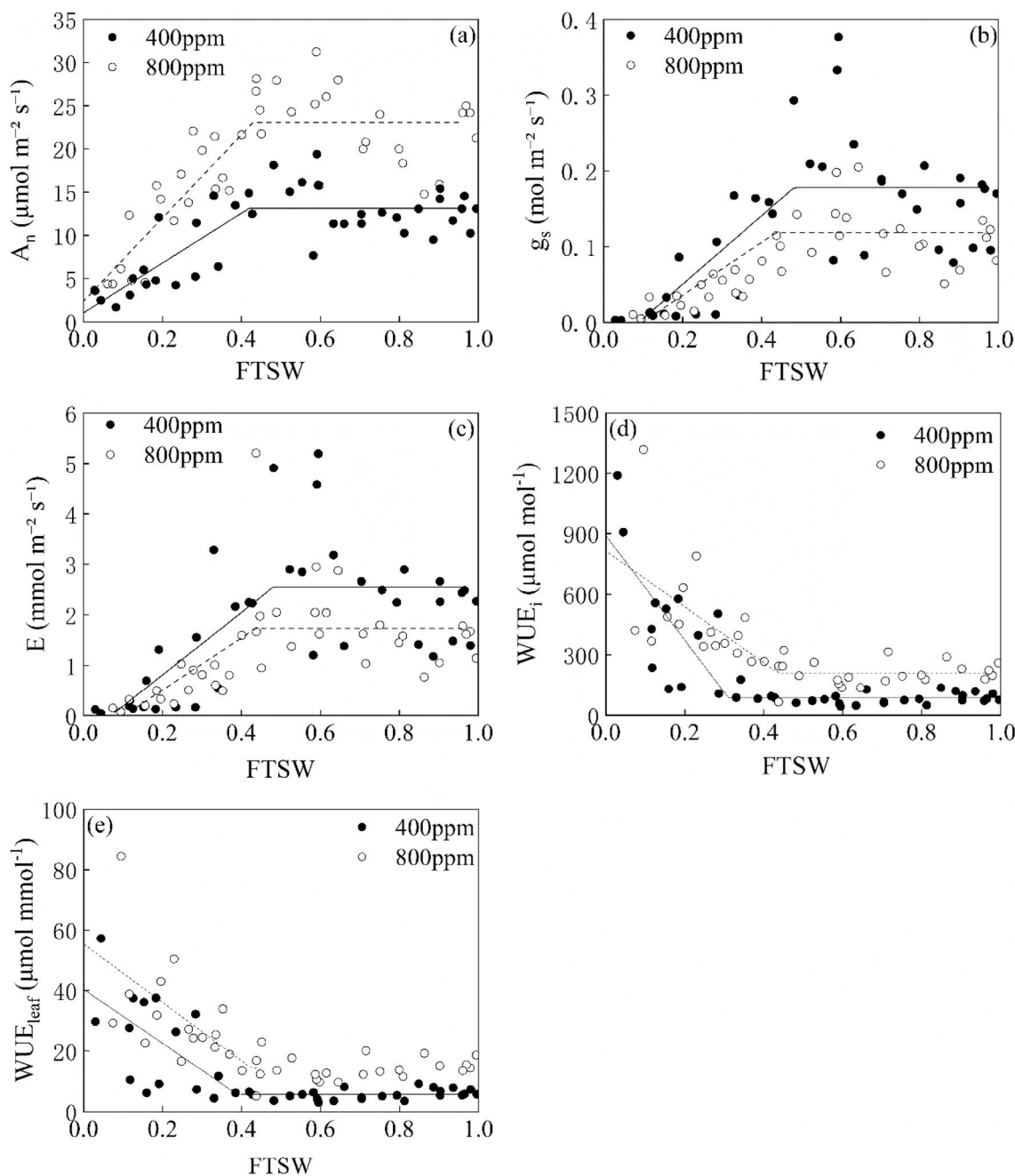


Fig. 1 Changes of net photosynthetic rate (A_n) (a), stomatal conductance (g_s) (b), and transpiration rate (E) (c) intrinsic water use efficiency (WUE_i , A_n/g_s) (d) and leaf water use efficiency (WUE_{leaf} , A_n/T_e) (e) of soybean leaves grown under ambient (400 ppm) and

elevated (800 ppm) atmospheric CO_2 concentrations during progressive soil drying. Fraction of transpirable soil water (FTSW) indicates the degree of dryness of the soil ($n=4$)

dropped in a linear fashion as $[ABA]_{leaf}$ increased, and the output of analysis of co-variance (ANCOVA) showed that the slope of the regression line was less steep at $e[CO_2]$ than at $a[CO_2]$ (Fig. 2b).

At sufficient soil moisture conditions, the initial WUE_i (A_n/g_s) of soybean leaves at $e[CO_2]$ was about 2.54-fold greater than those at $a[CO_2]$ (Fig. 1d), and the initial

WUE_{leaf} (A_n/E) of soybean leaves at $e[CO_2]$ was 2.55 times greater than that under $a[CO_2]$ (Fig. 1e). With the gradual decline of FTSW, WUE_i and WUE_{leaf} remained the initial value at first, and when FTSW decreased to 0.30–0.45, they began to increase linearly. There was no significant difference between the FTSW threshold (the time when WUE began to increase) of plants grown under the two different

Table 1 Significant test for linear-plateau model parameters of stomatal conductance (g_s), net photosynthetic rate (A_n), transpiration rate (E), instict water use efficiency (WUE_i , A_n/g_s) and instantaneous water use efficiency (WUE_{leaf} , A_n/E)

	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)		A_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		E ($\text{mmol m}^{-2} \text{s}^{-1}$)		WUE_i ($\mu\text{mol mol}^{-1}$)		WUE_{leaf} ($\mu\text{mol mmol}^{-1}$)	
	C	$g_{s \text{ max}}$	C	$A_{n \text{ max}}$	C	E_{max}	C	$WUE_{i \text{ min}}$	C	$WUE_{leaf \text{ min}}$
400 ppm	0.48	0.18	0.42	13.10	0.48	2.55	0.31	86.20	0.39	5.70
800 ppm	0.44	0.12	0.43	23.08	0.44	1.74	0.44	209.8	0.43	14.16
P-value	ns	*	ns	**	ns	**	ns	**	ns	**

C, the threshold at which the parameters start to decrease due to drought stress; $g_{s \text{ max}}$, $A_{n \text{ max}}$, E_{max} , $WUE_{i \text{ min}}$ (A_n/g_s) and $WUE_{leaf \text{ min}}$ (A_n/E), the initial values of the variables when the plants were not significantly affected by drought

*Means $p < 0.05$ and **means $p < 0.01$, ns means $P > 0.05$, the same below

Table 2 leaf relative water content (RWC), leaf water potential (Ψ_l), water consumption (WU), Plant water use efficiency (WUE_p), leaf area (LA), leaf dry matter (LDM), specific leaf area (SLA), nitrogen balance index (NBI) and the number of root nodules (NN) of well-watered (WW) and drought-stressed (DS) soybean plants grown under 400 ppm ($a[\text{CO}_2]$) and 800 ppm ($e[\text{CO}_2]$) environments at end of the drought treatment. The data in the table was mean \pm standard error of the means (S.E.) (N=3–4). Letters indicate statistical significance at $p = 0.05$ level

Indicators	Treatments				P value from two-way ANOVA		
	400WW	400DS	800WW	800DS	$[\text{CO}_2]$	[Water]	$[\text{CO}_2] \times [\text{Water}]$
RWC	0.90 \pm 0.01a	0.61 \pm 0.07b	0.89 \pm 0.02a	0.68 \pm 0.04b	0.47	<0.01	0.37
Ψ_l (MPa)	0.46 \pm 0.09c	0.91 \pm 0.08b	0.45 \pm 0.03c	1.23 \pm 0.15a	0.15	<0.01	0.11
WU (cm^3)	2940 \pm 121a	1509 \pm 23c	2369 \pm 191b	1387 \pm 4c	0.02	<0.01	0.09
WUE_p ($\text{kg} \cdot \text{m}^{-3}$)	3.07 \pm 0.10b	3.06 \pm 0.09b	3.68 \pm 0.17ab	4.20 \pm 0.48a	0.01	0.35	0.33
LA (cm^2)	1056 \pm 44.8a	747 \pm 74.5b	1207 \pm 112a	815 \pm 10.0b	0.15	<0.01	0.57
LDM (g)	10.5 \pm 0.43b	6.6 \pm 0.55c	13.8 \pm 0.59a	11.0 \pm 0.81b	<0.01	<0.01	0.39
SLA ($\text{cm}^2 \text{g}^{-1}$)	101.2 \pm 3.7a	112.1 \pm 2.2a	87.3 \pm 4.4b	75.1 \pm 5.0b	<0.01	0.88	0.01
NBI	51.8 \pm 1.01a	46.3 \pm 3.72ab	37.9 \pm 4.19b	40.0 \pm 2.72b	<0.01	0.60	0.26
NN	94 \pm 6.6b	34 \pm 4.0d	113 \pm 1.7a	51 \pm 8.6c	0.02	<0.01	0.87

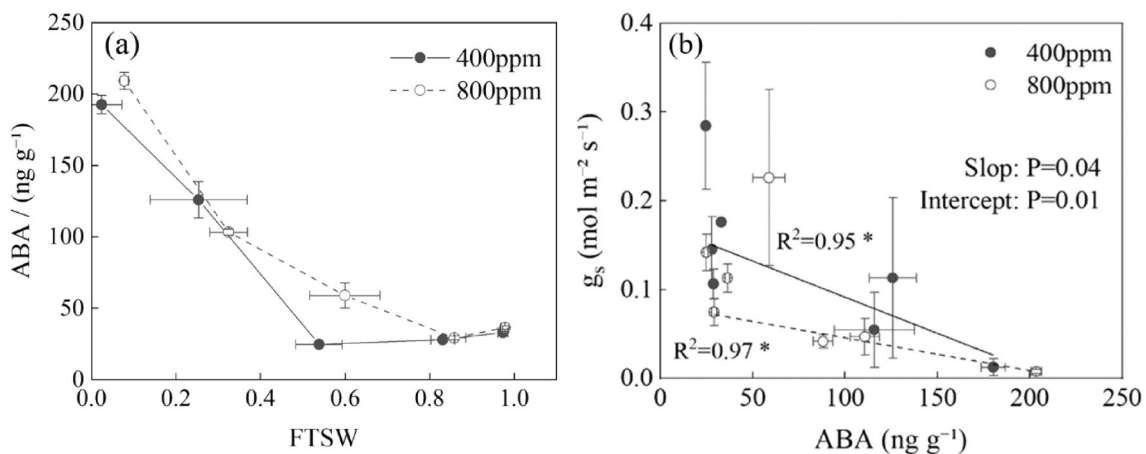


Fig. 2 Changes in ABA concentration (a) and the relationship between leaf ABA concentration and stomatal conductance (g_s) of soybean plants (b) grown under $a[\text{CO}_2]$ and $e[\text{CO}_2]$ during progressive soil drying. Fraction of transpirable soil water (FTSW) indicates

the degree of dryness of the soil. Error bars indicate standard error of the means (SE) (n = 3) *Indicates the regression lines was statistically significantly at $P < 0.05$ level

CO₂ concentrations. Compared with the soybean plants grown at *a*[CO₂] condition, WUE_p of plants at *e*[CO₂] was significantly improved ($P < 0.05$, Table 2).

Effects of CO₂ and drought on plant biomass and nutrition

Soybean leaf dry matter (LDM) was mainly influenced by both [CO₂] and water treatments. In arid condition, the LDM of the *e*[CO₂] plants was 66.0% higher than that of the *a*[CO₂] plants, while under well-watered condition, the LDM of the *e*[CO₂] plants was 31.6% higher than that of the *a*[CO₂] (Table 2). Compared with plants grown under drought stress, the leaf area (LA) of plants under well-watered condition was 41.4% and 48.1% greater at *a*[CO₂] and *e*[CO₂], respectively (Table 2). The specific leaf area (SLA) was notably influenced by CO₂ growth environment (Fig. 3b). In contrast to the *a*[CO₂] treatment, *e*[CO₂] treatment significantly decreased the SLA of plants by 13.7% and 33.0% at well-watered and drought-stressed conditions, correspondingly. Water and [CO₂] both had significantly effect on the number of root nodules (NN). In contrast to the *a*[CO₂] treatment, *e*[CO₂] significantly improved the number of root nodules by 20.2% and 50.0% under well-watered and drought condition, respectively. Drought stress significantly decreased NN under either CO₂ growth environment (Table 2).

Elevated [CO₂] notably reduced the leaf nitrogen concentration ($[N]_{\text{leaf}}$) ($P < 0.05$). In contrast to the *a*[CO₂] treatment, *e*[CO₂] plants decreased 21.4% and 23.4% $[N]_{\text{leaf}}$, respectively, under well-watered and drought conditions (Fig. 3a). There was a significant interaction between [CO₂] and water treatment on specific leaf nitrogen content (SLN) (Fig. 3b). The SLN was similar between well-watered and drought-stressed treatments at *a*[CO₂], but at *e*[CO₂] SLN was notably higher under drought. Elevated [CO₂] significantly decreased the leaf nitrogen balance index (NBI). The NBI is the ratio of chlorophyll to flavonoids. NBI values decrease when nitrogen deficiency occurs, it can be used to evaluate plant nitrogen status. In contrast to *a*[CO₂] treatment, *e*[CO₂] treatment significantly decreased the NBI of by 26.8% and 13.6% under well-watered and drought-stressed environments, respectively. [CO₂] and water treatments significantly affected plant N content. Compared to *a*[CO₂] treatment, *e*[CO₂] decreased plant N content by 26.6% and 12.3% at well-watered and drought-stressed environments, respectively. In contrast to well-watered treatment, drought stress reduced plant N content by 25.0% and 10.3% under *a*[CO₂] and *e*[CO₂], respectively. Moreover, elevated CO₂ significantly decreased the effect of drought on N content in soybean plants due to the interaction of [CO₂] and water treatments (Fig. 3c).

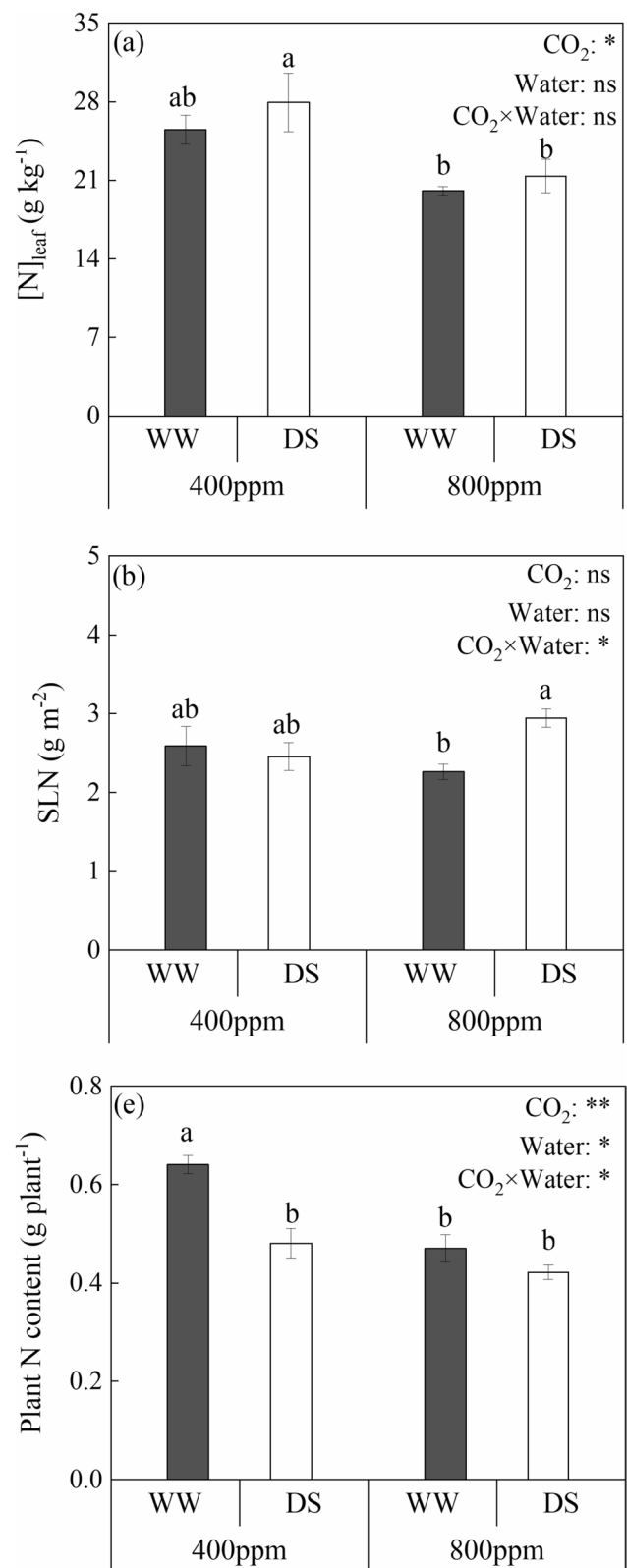


Fig. 3 Effects of water and [CO₂] on nitrogen nutrition in soybean (n = 3). *means $p < 0.05$ and **means $p < 0.01$, ns means $P > 0.05$

PCA and correlation analysis among physiological parameters

Principal component analysis (PCA) shows the association among the physiological parameters of soybean plants exposed to the four treatments (Fig. 4). The results showed that PC1 and PC2 explained 47.9% and 24.0% of the total variance, separately. Among them, parameters ABA, Ψ_1 , g_s and LA, WU contribute the most to PC1, while SLA and $[N]_{\text{leaf}}$ contribute the most to PC2. The irrigation treatment was mainly influenced by PC1, and the $[\text{CO}_2]$ treatment was mainly influenced by PC2 (Fig. 4). Therefore, the water treatment affected mainly on parameters including WU, Ψ_1 and ABA, while the CO_2 treatment mainly affected parameters of SLA, $[N]_{\text{leaf}}$ and NBI. In addition, PCA analysis showed that the irrigation treatment separated the parameters into different clusters, while the clusters of the $[\text{CO}_2]$ treatment were not as clearly separated. And under drought conditions, the $e[\text{CO}_2]$ treatment was more clearly distinguished from the $a[\text{CO}_2]$ treatment. The PCA plots also reveal that the number of root nodules was directly related to RWC, A_n , g_s , LA and WU, while it was adversely related to ABA and Ψ_1 . The NBI was directly related to plant N content and SLA, while negatively correlated with WUE_i and WUE_p . The $[N]_{\text{leaf}}$ was directly related to SLA and adversely related to LDM.

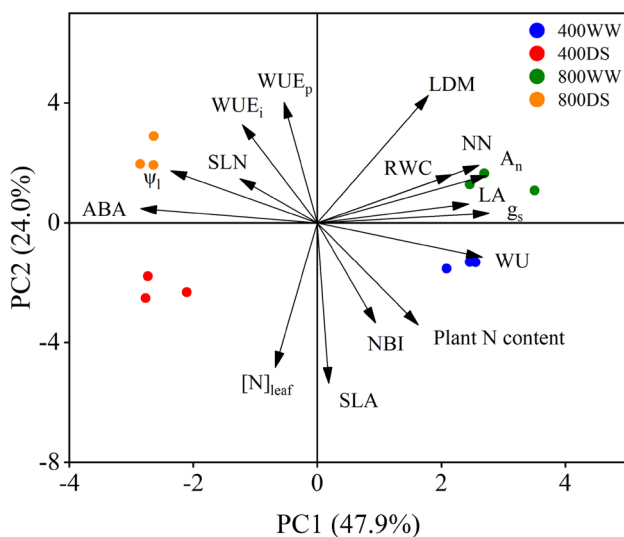


Fig. 4 Principal component analysis (PCA) of soybean physiological parameters for soybeans grown under two water conditions and two $[\text{CO}_2]$. “400WW, 400DS, 800WW and 800DS” refer to $a[\text{CO}_2]$ well-watered, $a[\text{CO}_2]$ drought-stressed, $e[\text{CO}_2]$ well-watered and $e[\text{CO}_2]$ drought-stressed conditions, respectively

Discussion

Physiological response of soybean plants to $e[\text{CO}_2]$

Improvement of A_n in soybean by elevated $[\text{CO}_2]$ treatment was stronger than in other crops, which may be an additional gain due to nitrogen fixation in soybean. In addition, the photosynthetic capacity of soybeans under $e[\text{CO}_2]$ was improved by increased leaf thickness. In this research, $e[\text{CO}_2]$ improved the WUE of soybean leaves due to an increased A_n and decreases in g_s and E, consistent with earlier finding in soybean plants (Wang et al. 2018). Many research indicates that $e[\text{CO}_2]$ often results in an increase in A_n (Fan et al. 2020; Yang et al. 2020), which is due to that elevated $[\text{CO}_2]$ facilitates the carboxylation of Rubisco while depressing the photorespiration (Bowes 1991). The increase of A_n in soybean leaves (Ye et al. 2019) upon growing at $e[\text{CO}_2]$ is consistent with the findings in other species, but unlike non-nitrogen fixing crops, the stimulating effect of $e[\text{CO}_2]$ on A_n to a much larger extent than that in other crops (85.7% in soybean vs. 35–60% in other crops). This could have been related to the N-fixation role played by soybean root nodules as the number of root nodules (NN) was significant increased by $e[\text{CO}_2]$ (Table 2). Coincided with this, the PCA revealed that the A_n was most strongly correlated with the NN (Fig. 4). The increased A_n at $e[\text{CO}_2]$ could provide more carbon to root supporting nodulation and nodule growth, contributing to enhanced N fixation thus better plant N nutrition. In this study, $e[\text{CO}_2]$ did not affect leaf area (LA), while significantly increased leaf dry mass (LDM) (Table 2), resulting in a significant reduction in SLA, which indicates an increase in the thickness of the leaf. Similar increases in leaf thickness were also observed in experiments with oilseed rape and soybean under $e[\text{CO}_2]$ environment by Uprety and Mahalaxmi (2000) and Ainsworth et al. (2002). Increased leaf thickness could improve leaf light absorption and better light distribution inside the leaf, improving the photosynthetic capacity (Brodrick et al. 2013). This would have contribute to the significant increase in photosynthetic capacity of soybean when grown at $e[\text{CO}_2]$ environment. The increase in leaf photosynthetic rate and leaf thickness was generally coupled with an improvement in leaf N concentration (Marengo et al. 2017), but N_{leaf} was significantly lower at $e[\text{CO}_2]$ environment. Zhu et al. (2009) suggest that this may be a result of increased protease activity in the leaves caused by elevated CO_2 , which enables the remobilization. The reduction in N_{leaf} caused a reduction in Rubisco content, which in turn led to a down-regulation of photosynthetic rate, resulting in photo acclimation, but this was not found in the present research (Aranjuelo et al. 2005).

Response of soybean leaf stomata to Progressive drought under $e[\text{CO}_2]$

Soybeans grown at $e[\text{CO}_2]$ had a delayed g_s response to progressive soil drying compared to the $a[\text{CO}_2]$ treatment, coinciding with a reduced sensitivity of g_s to the ABA signaling, though they tended to maintain a better leaf water status under drought than the $a[\text{CO}_2]$ -grown plants.

In this study, g_s of soybean leaf was remarkably reduced by $e[\text{CO}_2]$ (Fig. 1b; Table 1). The $e[\text{CO}_2]$ -induced stomatal closure is thought to be a response by guard cells membrane channels to the increase in intercellular CO_2 concentration (Maurel et al. 2016), on the other hand, it has been considered as the decrease in stomatal size and density (Xu et al. 2016). The decrease in g_s reduces evapotranspiration and allows the plant to maintain a better water status. As can be seen in S2 (in the supplementary figures), the FTSW at $e[\text{CO}_2]$ treatment were consistently greater than that at $a[\text{CO}_2]$, indicating that the reduced g_s and E had lowered plant water consumption thus soil water depletion during the progressive drought. In addition, along with the decrease of FTSW, g_s of the soybean leaves at $e[\text{CO}_2]$ began to decrease gradually when FTSW below 0.44, this FTSW threshold was slightly lower than that under $a[\text{CO}_2]$. Such delayed stomatal closure during soil drying under $e[\text{CO}_2]$ has also been identified in tomato plants (Liu et al. 2019, Li et al. 2019). This suggests that the reduction in g_s under $e[\text{CO}_2]$ leads to a reduction in plant water consumption, conserving soil water and improving plant growth at drought conditions (Aranjuelo et al. 2014, Bowes 1991). In contrast, Gray et al. (2016) reported that did not protect soybean plants from drought stress. The discrepancy among different studies could be due to the diverse cultivars tested or the varied environmental conditions applied in the experiments.

Research has indicated that ABA, as an early chemical signal, can induce stomatal closure at moderate drought stress (Zhang et al. 2018). Besides, it has also been shown that ABA is involved in the stomatal CO_2 response (Raschke 1975). In the adequate water supply environment, the ABA concentration of soybean leaf was slightly higher at $e[\text{CO}_2]$ than that at $a[\text{CO}_2]$ (Table 2). With the intensification of drought, and the N_{leaf} grew exponentially as the FTSW decreased. Similar trends were found for plants at both $[\text{CO}_2]$ conditions, though at a given FTSW level, a slightly higher ABA concentration was shown in the $e[\text{CO}_2]$ plants (Fig. 2a). Moreover, regression analysis revealed a negative correlation between ABA concentration and g_s , and the ANCOVA showed that the slope of the regression line was less steep at $e[\text{CO}_2]$ than at $a[\text{CO}_2]$ (Fig. 2b). In line with this, Yan et al. (2017) found similar results in tomato plants. This suggests that the g_s of soybean leaf become less sensitive to ABA signaling at $e[\text{CO}_2]$, in agreement with results obtained for non-nitrogen-fixing crops such as tomato

and wheat (Li et al. 2019; Wei et al. 2020). Numerous field trials had also been carried out in Illinois investigating the interactive effects of droughts and $e[\text{CO}_2]$ on soybean growth and physiology, and in particular, the study on the stomatal response of ABA to $e[\text{CO}_2]$ pointed out that $e[\text{CO}_2]$ enhanced the stomata sensibility to ABA (Gray et al. 2016). Obviously, this disagrees with our findings in the present study, which probably attributed to the different environmental conditions and soybean varieties used among different studies. Nonetheless, such uncertainties merit further studies.

Response of soybean growth to drought and $e[\text{CO}_2]$ environment

Dilution of plant nitrogen concentration at $e[\text{CO}_2]$ was not altered by the increase in nitrogen fixation capacity, i.e. increase in NN. The CO_2 fertilization effect in previous studies was that increased photosynthesis generally promotes increased plant growth when $[\text{CO}_2]$ is elevated (Zheng et al. 2020). However, sufficient N is required for plants to fully utilize the increased carbon supply at $e[\text{CO}_2]$, plants that are not capable of biological N fixation are often limited by N availability (Ainsworth et al. 2007). The reduction in NBI at $e[\text{CO}_2]$ in this study suggests that soybean leaves remain N-limited under elevated CO_2 as other non-N-fixing crops, so the extra N fixed by enhanced N fixation must be available to plant growth. Many studies about the influence of $e[\text{CO}_2]$ on the N trophic have shown that N concentration decreases in the presence of atmospheric CO_2 enrichment (Liu et al. 2019; Wang et al. 2021). This is likely to be because the “dilution effect” caused the large accumulation of non-structural carbohydrates and biomass under $e[\text{CO}_2]$. Similar results were obtained in this study, with elevated CO_2 significantly reduced $[N]_{\text{leaf}}$. However, unlike other non-nitrogen fixing crops, SLN is not affected by $e[\text{CO}_2]$ (Liu et al. 2019; Zheng et al. 2020). This could be attributed to the presence that biological N fixation provides additional N to the plants, alleviating N limitation under $e[\text{CO}_2]$ condition. Soybean plants thus have both a greater carbon sink capacity and the ability to match their N and carbon supplies at $e[\text{CO}_2]$ (Ainsworth et al. 2007). Analysis of the N content of the whole soybean plants revealed that both $e[\text{CO}_2]$ and DS reduced plant N content, but the drought had a smaller effect on plant N content in elevated CO_2 environment (Fig. 3c). Jin et al. (2019) also showed that legumes are influenced by dilution effects to a lesser extent and can satisfy the N requirements for $e[\text{CO}_2]$ conditions by enhancing N fixation. In this research, the results showed that N density in the leaves remained dilutive at $e[\text{CO}_2]$, suggesting that N fixation in soybean is inadequate to alleviate the dilutive impact of $e[\text{CO}_2]$ on leaf N concentrations; but the drought

impact on $[N]_{\text{leaf}}$ was mitigated at $e[\text{CO}_2]$, with differences between the various water treatments reduced.

Through the previous analysis we found that compared to the $a[\text{CO}_2]$ treatment, the greater number of nodules of the $e[\text{CO}_2]$ plants would lead to an enhanced N-fixation, which, however, did not improve N nutrition of the plants. Nevertheless, by sustaining the SLN, the soybean plants were able to enhance A_n when growing at $e[\text{CO}_2]$, particularly under dry conditions. PCA plots illustrate that there were more root nodules, better leaf water status and more dry matter in the WW treated plants. While under drought, soybean plants grown under $e[\text{CO}_2]$ had greater WUE_p and SLN compared to the $a[\text{CO}_2]$, indicating that the drought stress were alleviated by elevated CO_2 . In addition, PCA plots reveal that the effect of drought was greater than that of $e[\text{CO}_2]$ (Fig. 4). This may be due to the fact that under severe drought stress, leaves were mainly subject to hydraulic regulation and were more affected by water deficits (Fan et al. 2020).

Conclusions

Elevated $[\text{CO}_2]$ -treated plants had significantly greater A_n compared to $a[\text{CO}_2]$ -treated plants, but lower g_s and E , resulting in enhanced WUE_i and WUE_{leaf} . In addition, soybeans grown at $e[\text{CO}_2]$ had a delayed g_s response to progressive soil drying compared to the $a[\text{CO}_2]$ treatment, coinciding with a reduced sensitivity of g_s to the ABA signaling, though they tended to maintain a better leaf water status under drought than the $a[\text{CO}_2]$ -grown plants. Although the leaf nitrogen concentration and the total plant N content were markedly lower in plants grown at $e[\text{CO}_2]$, there was no remarkable difference in SLN between the two $[\text{CO}_2]$ treatments. Compared to the $e[\text{CO}_2]$ treatment, the greater number of nodules of the $e[\text{CO}_2]$ plants would lead to an enhanced N-fixation, which, however, did not improve N nutrition of the plants. Nevertheless, by sustaining the SLN, the soybean plants were able to enhance A_n when growing at $e[\text{CO}_2]$, particularly under dry conditions. This provides a theoretical basis for how to better utilize the nitrogen fixation of soybeans in future climates, and also provides a scientific basis for future exploration of water-saving irrigation of soybeans in drought and CO_2 rich climate conditions.

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Declarations

Competing interest The authors have not disclosed any competing interests.

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