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ORIGINAL ARTICLE

DROUGHT STRESS

CO_2 elevation and N fertilizer supply modulate leaf physiology, crop growth and water use efficiency of maize in response to progressive soil drought

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Abstract

Elevated atmospheric CO_2 concentration ($e[CO_2]$) and varied nitrogen (N) fertilization levels may mediate the different responses of C_4 crops to progressive soil drought. In this study, the effects of reduced N (N1, 0.8 g pot^{-1}) and adequate N (N2, 1.6 g pot^{-1}) supply on leaf physiology, plant growth and water use efficiency (WUE) of maize (C_4 crop) exposed to progressive soil drought grown at ambient CO_2 ($a[CO_2]$, 400 ppm) and elevated CO_2 ($e[CO_2]$, 800 ppm) concentration were investigated. The results indicated that compared with $a[CO_2]$, net photosynthetic rate (A_n) and leaf water potential (Ψ_l) at $e[CO_2]$ were maintained in maize leaves, while stomatal conductance (g_s), transpiration rate and leaf hydraulic conductance were decreased, leading to enhanced WUE from stomatal to leaf scale. Despite A_n and Ψ_l of $e[CO_2]$ plants were more sensitive to progressive soil drought under both N fertilization levels, $e[CO_2]$ would increase leaf ABA concentration ($[ABA]_{leaf}$) but decline the g_s response to $[ABA]_{leaf}$ under N1 supply. $e[CO_2]$ coupled with N1 fertilization was conducive to enlarging leaf area, promoting specific leaf area, root and total dry mass, whereas reduced stomatal aperture and plant water use under progressive drought stress, contributing to an improvement in plant WUE, implying a better modulation of maize leaf stomata and water status under reduced N supply combined with $e[CO_2]$ responding to progressive soil drought. These findings in the current study would provide valuable advice for N management on maize (C_4) crop efficient water use in a drier and CO_2-enriched environment.

KEYWORDS
CO_2 elevation, leaf physiology, maize, N fertilization, progressive soil drought, water use efficiency

Key points

- $e[CO_2]$ maintained A_n and Ψ_l, decreased g_s, T_r and K_{leaf}, further enhanced leaf WUE.
- $e[CO_2]$ sensitized maize A_n and Ψ_l decrease during progressive soil drought.
- $e[CO_2]$ increased $[ABA]_{leaf}$ but declined g_s response to $[ABA]_{leaf}$ under reduced N supply.

- e[CO$_2$] combined with reduced N supply decreased stomatal aperture, while enlarged leaf area, root and total dry mass, improved maize WUE under soil drought.

1 | INTRODUCTION

As global warming intensifies, a more CO$_2$-enriched and drier environment will be faced and obviously alter crop growth and production (Hartmann et al., 2013). Moreover, the photosynthetic efficiency and physiological response of the C$_4$ crop to elevated atmospheric CO$_2$ concentration (e[CO$_2$]) could be different from the C$_3$ crop, thereby affecting water use and nutrient absorption. While minerals, especially nitrogen (N) concentration were decreased at e[CO$_2$], leading to the 'hidden hunger' in plants (Li et al., 2016). Therefore, understanding the physiological and growth responses of C$_4$ crops to e[CO$_2$] and soil drought stress, particularly under varied N fertilization levels would be the foundation for achieving sustainable crop production in the future century.

Previous evidence suggested that hormone signalling (mainly xylem sap ABA concentration) from root to shoot was accumulated in dry roots and transported to leaves during soil water deficit (Yan et al., 2017), which profound effects on plant stomata morphology, such as stomatal densityand stomatal size (Zhu et al., 2018), resulting in the decrease of stomatal aperture and stomatal conductance (g_s). In turn, these would decrease the rates of CO$_2$ uptake for carbon assimilation and water loss, and ultimately influence water use efficiency (WUE; Hao et al., 2016). At the same time, drought stress normally could affect crop N nutrition by reducing N bioavailability (i.e. N mineralization) and N uptake (i.e. lowering the diffusion and mass flow from soil solution to root surface) (Marino et al., 2007).

The physiological and morphological responses of different crops to [CO$_2$] were investigated. Numerous pieces of evidence showed that e[CO$_2$] caused partial stomata closure in the short term and reductions of stomatal density in the long term, resulting in higher leaf photosynthetic rate (A_n) and lower g_s in the C$_3$ crop, leading to a significant increase in WUE (Avila et al., 2020; Ullah et al., 2019). In contrast, e[CO$_2$] had little or no effect on A_n and a lower deduction of g_s in the C$_4$ crop (Yang et al., 2022), resulting in higher WUE than the C$_3$ crop. This was attributed to the more efficient CO$_2$ enrichment mechanism and optimized CO$_2$ fixation capacity in the C$_4$ crop (Leakey et al., 2009). However, it did not mean that e[CO$_2$] had no impact on the C$_4$ crop, as other factors changed by e[CO$_2$] may also contribute to the growth stimulation of the C$_4$ crop, particularly when crops were subjected to abiotic stress, such as water shortage and limited N supply (Li et al., 2021; Zhang et al., 2021). It was reported that e[CO$_2$] could alleviate the negative impacts of drought on C$_4$ crops through reducing g_s to limit transpiration rate (T_r) and maintain a higher leaf water potential (Ψ_l; Tausz-Posch et al., 2015). Nevertheless, Ψ_l was not always higher at e[CO$_2$] even if g_s and T_r were found to be lower, and the possible reason was that leaf hydraulic conductance (K_l) might also be decreased at e[CO$_2$] (Fang et al., 2019), counteracting the positive effect of lowered g_s and T_r on Ψ_l.

As the main component of amino acids, proteins, nucleic acids and chlorophyll, N was a key nutrient element in high demand for crops and closely related to A_n (Cai et al., 2012). The lower g_s was observed under low or zero N treatment (Dordas & Sioulas, 2008), while it was also reported that N treatment had the opposite or no effect on g_s (Wang et al., 2018), presenting some contradictory results in the effect of N on g_s. In addition, ABA was involved in the regulation of plant N signal transduction and N uptake (Wang et al., 2010). A previous study showed that N deficiency led to rapid accumulation of ABA (Shamsu et al., 2020). This resulted from the increased stomatal sensitivity to xylem ABA by reducing the transport of cytokinin within the xylem (Rahayu et al., 2005). Generally, a higher leaf N concentration was widely associated with greater photosynthetic capacity, resulting in a more increased A_n than T_r, and a further increase in leaf area, dry mass and WUE (Ashraf et al., 2016). Moreover, the response of photosynthesis in the C$_4$ crop to leaf N content was much stronger than that in the C$_3$ crop (Wang et al., 2020). Accordingly, a slight increase in N content in maize leaves could significantly enhance the rate of carbon assimilation and crop growth.

A previous study revealed that e[CO$_2$] would reduce plant N concentration, especially under drought conditions (Taub & Wang, 2008). One reason for this phenomenon was the increased biomass accumulation leading to diluted N concentration in plants (Gifford et al., 2000). On the other hand, it was observed that e[CO$_2$] would reduce root N uptake to alter the rhizosphere environment (Feng et al., 2015) and decrease the mass flow of nutrients from soil to plant due to the reduction in leaf g_s and T_r (Wang et al., 2018). Hence, Li et al. (2003) found that a high N supply could enhance the positive effect of e[CO$_2$] on wheat WUE. Conversely, an adequate supply of photosynthetic products at e[CO$_2$] promoted the overall uptake of N, thereby modulating plant carbon and nitrogen metabolism (Xu et al., 2022). Nevertheless, recent evidence revealed that compared to the C$_3$ crop, the C$_4$ crop had a different and complex response to combined e[CO$_2$] and N application levels (Geissler et al., 2015). So far, few reports on the interaction between e[CO$_2$] and N fertilizer application focused on the C$_4$ crop. Furthermore, the combined effects of e[CO$_2$] and progressive soil drought on C$_4$ crop physiology and WUE remained largely elusive, particularly coupled with varied N supply.

Therefore, this study was designed to investigate how both leaf and plant level WUE of C$_4$ crops responded to e[CO$_2$] and reduced N fertilization during progressive soil drought. A progressive soil drought experiment with maize (C$_4$ cereal crop) was conducted at ambient [CO$_2$] (400 ppm, a[CO$_2$]) and e[CO$_2$] (800 ppm) with reduced N (N1, 0.8 g pot^{-1}) and adequate N supply (N2, 1.6 g pot^{-1}) in the climatic controlled greenhouse. Leaf gas exchange, $[ABA]_{leaf}$, plant water relations, leaf area and water use as well as dry mass from root to leaf were determined. It was hypothesized that whether two N fertilization levels would modulate differently the response of maize leaf gas exchange and water relation to progressive soil drought at $e[CO_2]$; $e[CO_2]$ combined with adequate N supply could alleviate the negative effects of maize physiology and WUE in response to soil drought stress.

2 | MATERIALS AND METHODS

2.1 | Crop material and growth conditions

The experiment was conducted in two climate-controlled phytotrons in the South Campus of Northwest A&F University, Yangling, Shaanxi, China, from 20 April to 14 June 2022. The maize (Zhengda 12) seeds were grown in the 4.0 L plastic pot (19.5 cm tall, 19.5 cm in upper diameter and 16 cm in lower diameter) filled with 1 kg peat soil (Pindstrup substrate, Pindstrup Mosebrug A/S, Pindstrup, Denmark), containing N 1.1 $g\,kg^{-1}$, P 0.5 $g\,kg^{-1}$ and K 1.6 $g\,kg^{-1}$ with a pH of 5.9. From sowing, half of the maize plants (48 seedlings) were grown in a phytotron with ambient CO_2 concentration of 400 ppm ($a[CO_2]$), and the other half were grown in a phytotron with elevated CO_2 concentration of 800 ppm ($e[CO_2]$). In both phytotrons, the CO_2 concentration ($[CO_2]$) was sustained by the emission of pure CO_2 from a bottled tank, released 24 h per day from more than one point and distributed evenly in the phytotrons through internal ventilation, and monitored by the automatic control system of the phytotrons. The fluctuated range of $[CO_2]$ concentration was $400/800 \pm 30$ ppm throughout each day. The climatic conditions in two phytotrons were set at $25/18 \pm 2$°C day/night air temperature, 60% relative humidity, 16 h photoperiod, and 500 $mol\,m^{-2}\,s^{-1}$ photosynthetic active radiation (PAR) supplied by high-pressure sodium lamps plus LED lamps.

2.2 | Experimental design

In each phytotron, the plants were divided into two N fertilization levels, i.e., 0.8 g N pot^{-1} denoted as N1 treatment and 1.6 g N pot^{-1} denoted as N2 treatment applied as $CO(NH_2)_2$. In addition, 0.8 g P and 1.0 g K were applied as KH_2PO_4 into each pot. One week after transplanting, the fertilizers were dissolved into water to satisfy the irrigation water to satisfy the maize growth and biomass formation. For each pot, 1.5 cm quartz gravel was covered on the soil surface to minimize evaporation and all pots were constantly watered to 90% of pot water holding capacity (i.e., a pot weight of 3.10 kg).

After 3 weeks, when the plant growth was in the jointing stage, namely water consumption reached 150 mL, four pots of maize plants were harvested for each $[CO_2]$ and N treatments (16 plants) to determine the initial dry mass. Whereafter, the progressive soil drought treatment started on 30th May. In each phytotron, four plants of each N treatment were well-watered (i.e., a pot weight of 3.10 kg) and severed as control, the others (16 plants) were subjected to progressive soil drying by withholding irrigation from the pots for 15 days until the g_s decreased to ca. 10% of the control (i.e., when the pot weight ca. 1.20 kg).

The pot experiment was completely and randomly designed, with a total of eight treatments. Four replicated pots were used for each treatment, and the pots were randomly arranged in each $[CO_2]$ cell. Irrigation water came from tap water and contained negligible concentrations of nutrients.

2.3 | Measurements

2.3.1 | Soil water status

Soil water status was determined by daily weighing the pots using an Analytical Balance (Sartorius Model QA35EDE-S). The pots were weighed on a daily basis at 17:00 h and soil water status was presented as the fraction of transpirable soil water (FTSW), which had been used as an indicator for soil water status of plants experiencing progressive soil drought (Liu et al., 2019). The FTSW value was the ratio between the amount of remaining transpirable soil water in a pot and total transpirable soil water (TTSW)

$$FTSW = \left(WT_n - WT_f\right) / TTSW \tag{1}$$

where WT_n was the pot weight on a given date and WT_f was the pot weight at the last day of drying cycle when the transpiration rate of the progressive soil drought plant reached 10% of the well-watered plants. TTSW was the difference between pot weight at 100% WHC (3.10 kg) and pot weight of progressive soil drought plant on the last day of the drying cycle, namely when the transpiration rate decreased to 10% of well-watered plants (ca. 1.20 kg).

2.3.2 | Leaf gas exchange

During the progressive soil drought, leaf gas exchange rates, including net photosynthetic rate (A_n, $\mu mol\,m^{-2}\,s^{-1}$), stomatal conductance (g_s, $mol\,m^{-2}\,s^{-1}$), and transpiration rate (T_r, $mmol\,m^{-2}\,s^{-1}$) were measured every 3 days on the third upper fully expanded leaf (developed after the onset of progressive soil drought treatment), exactly on the mid portion of the leaf away from the veins with a portable photosynthetic system (LiCor-6800, LI-Cor, NE, USA). The measurements were conducted from 9:00–12:00 h at a PAR of 1500 $mol\,m^{-2}\,s^{-1}$, leaf temperature of 25°C and $[CO_2]$ of 400 ppm for $a[CO_2]$ and 800 ppm for $e[CO_2]$, respectively. For each leaf, the measurement took ca. 6 min until stable readings on A_n and g_s were reached and logged three times. Intrinsic WUE (WUE_i) was calculated as A_n/g_s and instantaneous WUE (WUE_{leaf}) was calculated as A_n/T_r.

2.3.3 | Plant water relation and ABA concentration

Leaf water potential (Ψ_l) was measured on the same leaves for measuring gas exchange rates, with a pressure chamber (SEC3005, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Leaf hydraulic conductance (K_l, $\text{mmol}\,\text{m}^{-2}\,\text{s}^{-1}\,\text{MPa}^{-1}$) was calculated as T_r/Ψ_l. After measuring Ψ_l, the leaf was immediately packed in aluminium foil and frozen in liquid nitrogen. The fresh leaf samples were then frozen in liquid nitrogen and then stored at −80°C. The samples were ground into fine powder and 30 mg of each sample was weighted for ABA assay. 1 mL Milli-Q water was added to the sample and ABA was extracted on a shaken plate overnight at 4°C. The extract was centrifuged at 14,000*g* for 10 min at 4°C. The ABA concentration in the supernatant was determined by ELISA according to Asch (2000).

2.3.4 | Stomatal density and stomatal aperture

The epidermal impression (ca. 1.0 cm^2) was obtained 1 day before the ending of progressive soil drought from surfaces of the upper fully expanded leaf in each plant near noon by applying silicone impression material (Zhermack S.p.A., Badia Polesine, Italy), allowing it to harden and using clear cellophane tape to transfer the imprint to a microscope slide. For each imprint, four images (calibrated size of 320 × 240 μm) were taken using a light microscope (BA210 Digital, MOTIC, China). The number of stomata (i.e., stomatal density; SD) for each image was counted using the Image J2 software (ver. 1.6.0–24; Wayne Rasband, National Institutes of Health, USA). Morphological characteristics of stomata, including stomatal pore aperture length (L_a) and stomatal pore aperture width (W_a) were measured by image-editing software (Motic Images Plus 3.0). Stomatal aperture (SA) was calculated according to Doheny-Adams et al. (2012) using the following equation to estimate SA synthetically with different morphological parameters:

$$SA = \pi \cdot \times L_a \times W_a / 4 \tag{2}$$

where W_a was pore aperture width, L_a was pore aperture length.

2.3.5 | Leaf area and specific leaf area

At harvest, leaf area (LA, cm^2) was measured with portable leaf area meter (LI-3100, LI-Cor Inc., Lincoln, NE, USA) and the specific leaf area (SLA, $\text{cm}^2\,\text{g}^{-1}$) was calculated as LA/leaf dry mass (LDM, g).

2.3.6 | Dry mass, plant water use and WUE

Biomass of the plants were harvested at the ending of progressive soil drought. Leaf dry mass (LDM, g), stem dry mass (SDM, g) and root dry mass (RDM, g) of the plants were separately weighed, each dry mass was calculated as the difference of dry mass in leaf, stem and root between the first and last harvest after drying at 70°C in an oven for 48 h to a constant weight. Total dry mass (TDM, g) was the sum of LDM, SDM and RDM. Plant water use (WU, L) was the total water use by weighing the pots since the beginning of progressive soil drought. Plant water use efficiency (WUE_p, $\text{g}\,\text{L}^{-1}$) was calculated as TDM/WU.

2.4 | Statistics analysis

To evaluate the response of A_n, g_s, T_r, Ψ_l and K_l to progressive soil drought, the linear plateau model was modified as

$$\text{If FTSW} > \text{C}, y = y_{\text{initial}}; \tag{3a}$$

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

where y denoted A_n, g_s, T_r, Ψ_l or K_l, y_{initial} denoted the initial values of A_n, g_s, T_r, Ψ_l or K_l (denoted as $A_{n\,\max}$, $g_{s\,\max}$, $T_{r\,\max}$, $\Psi_{l\,\max}$ or $K_{l\,\max}$) when the maize was still not drought stressed, a was the slope of the linear equation, C was the FTSW threshold at which y started to diverge from y_{initial} (denoted as C_A, C_g, C_T, C_Ψ and C_K respectively). The parameters y and C were estimated by PROC NLIN of PC SAS 9.4 (SAS Institute Inc., Cary, NC, USA, 2002–2012) and the coefficient of determination (r^2) was calculated. Statistical comparison of each parameter obtained from the linear-plateau regression between treatments of [CO_2] or [N] was performed by *t*-test using MedCalcstatistical 19.0.7 software.

Three-way analysis of variance (ANOVA) was performed on the data of $[\text{ABA}]_{\text{leaf}}$, SD, SA, LA, SLA, LDM, SDM, RDM, TDM, WU and WUE_p. The effects of the independent factors, CO_2 levels ([CO_2]), N fertilization levels ([N]), and water stress treatment ([W]) as well as their interactions, were detected using the SPSS statistics software (version 22.0, IBM Electronics). Error bars indicated the standard error of the four replications. *, ** and *** indicated significance levels at $p < .05$, $p < .01$ and $p < .001$, respectively. The relationship between increment of g_s and $[\text{ABA}]_{\text{leaf}}$ was evaluated by linear regression. R^2 of the regression lines were calculated and the statistical differences on slopes of the regression lines between g_s and $[\text{ABA}]_{\text{leaf}}$ under *a*[CO_2] and *e*[CO_2] were performed by analysis of covariance (ANCOVA, $[\text{ABA}]_{\text{leaf}}$ as a covariate).

3 | RESULTS

3.1 | Soil water status

Under well-watered condition, the proportion of transpiration soil water (FTSW) under two N fertilization levels at both [CO_2] environment remained above 0.85. After onset the soil drying, the FTSW in drought-stressed plant pot decreased over time until the available water for entire plant was consumed, which took about 14 days (Figure 1a,b).

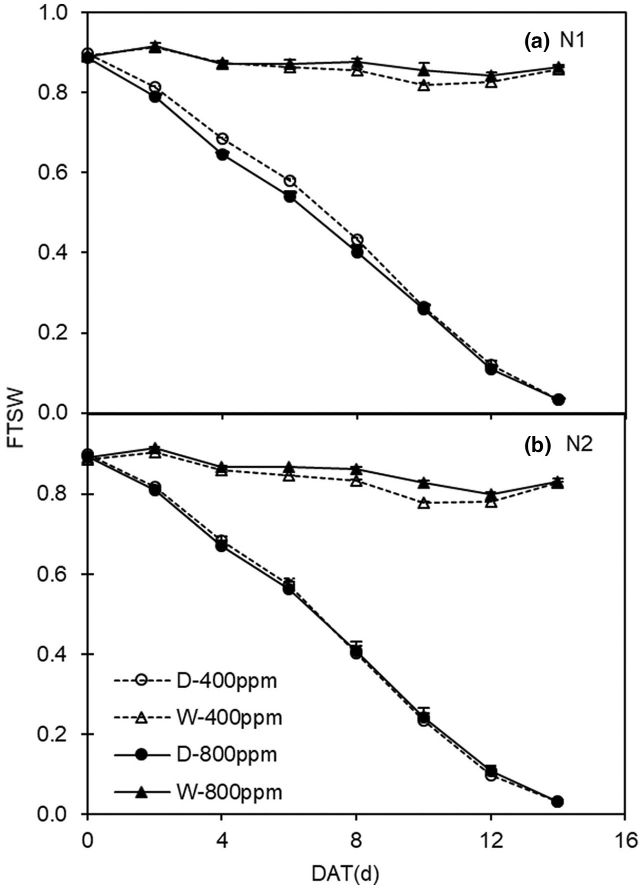


FIGURE 1 The fraction of transpirable soil water (FTSW) of maize as affected by N fertilizers (N1 and N2), respectively, grown at *a*[CO$_2$] (400 ppm) (open symbols) and *e*[CO$_2$] (800 ppm) (closed symbols) under well-watered (triangle shape) and progressive soil drought (circle shape) treatments. Error bars indicated standard error of the four replications.

3.2 | Leaf gas exchange

In the early stage of progressive soil drought, maize was not subjected to obvious drought stress, both [CO$_2$] and N treatments had no significant effect on $A_{\mathrm{n\,max}}$ of maize (Figure 2a,b; Tables 1 and 2). At *a*[CO$_2$], C_{A} of FTSW treated with N1 (0.04) was significantly lower than that of FTSW treated with N2 fertilization (0.13). Moreover, under N1 fertilization, compared with *a*[CO$_2$], *e*[CO$_2$] maize plants had higher C_{A} of FTSW (Figure 2a,b; Tables 1 and 2).

At the early stage of progressive soil drought, the $g_{\mathrm{s\,max}}$ of *e*[CO$_2$] maize plant was significantly decreased by 52.9% and 43.8% under N1 and N2 fertilization, respectively (Figure 2c,d; Tables 1 and 2). Meanwhile, N treatment had no significant effect on $g_{\mathrm{s\,max}}$ of maize plants. With the decrease in FTSW, g_{s} of maize declined under severe soil drying, and there was no significant difference in C_{g} under varied treatments (Figure 2c,d; Tables 1 and 2). As for $T_{\mathrm{r\,max}}$, the response was consistent with g_{s}. *e*[CO$_2$] significantly reduces maize $T_{\mathrm{r\,max}}$ under both N fertilization (Figure 2e,f; Tables 1 and 2).

During progressive soil drought, compared to maize plants grown at *a*[CO$_2$], WUE$_{\mathrm{i}}$ and WUE$_{\mathrm{leaf}}$ at *e*[CO$_2$] were significantly increased by 119.8% and 116.6%, respectively, while both WUE$_{\mathrm{i}}$ and WUE$_{\mathrm{leaf}}$ were not affected by N treatment (Figure 3a,b).

3.3 | Plant water relations

At the early stage of progressive soil drought, $\Psi_{\mathrm{l\,max}}$ had no significant response to both [CO$_2$] and N treatments. [CO$_2$] treatment had a significant impact on maize C_{Ψ} only under N2 fertilization, namely C_{Ψ} at *e*[CO$_2$] was greater than that at *a*[CO$_2$] (Figure 4a,b; Tables 1 and 2). Compared with *a*[CO$_2$], *e*[CO$_2$] reduced 51.6% and 37.1% maize $K_{\mathrm{l\,max}}$ under N1 and N2 fertilization, respectively. Besides, there was no significant difference in C_{K} under varied treatments (Figure 4c,d; Tables 1 and 2).

3.4 | Leaf ABA concentration

Irrespective of [CO$_2$] treatment, both N2 fertilization and drought stress significantly increased maize [ABA]$_{\mathrm{leaf}}$ as compared with N1 fertilization and well-watered treatment. Besides, *e*[CO$_2$] had an enhanced tendency in [ABA]$_{\mathrm{leaf}}$ under N1 fertilization across water stress treatment (Figure 5a; Table 3).

Regardless of water stress and N fertilization, g_{s} decreased linearly with [ABA]$_{\mathrm{leaf}}$ of maize plant at both [CO$_2$] environment. Moreover, *e*[CO$_2$] had the notable lower initial value and slope of g_{s} respond to [ABA]$_{\mathrm{leaf}}$ than *a*[CO$_2$] (Figure 6).

3.5 | Stomatal density and stomatal aperture

Each treatment had no effect on SD of maize leaves (Figure 5b; Table 3). SA was significantly affected by N fertilization (Figure 5c; Table 3). Across [CO$_2$] and water stress regimes, SA was 18.4% significantly decreased under N1 as compared with N2 treatment. In addition, under N2 fertilization, drought stress had the decreased trend in SA than well-watered treatment in each [CO$_2$] environment.

3.6 | Leaf area and specific leaf area

e[CO$_2$] and N2 fertilization increased the maize leaf area (LA) by 11.0% and 11.5%, respectively. Drought stress reduced LA by 41.3%. In addition, under drought stress, LA was maximized by *e*[CO$_2$] combined with N1 fertilization (Figure 7a; Table 3). *e*[CO$_2$] increased the maize specific leaf area (SLA). Drought stress had a significant 18.4% reduction in SLA (Figure 7b; Table 3).

3.7 | Dry mass, plant water use and WUE

[CO$_2$] had no significant effect on LDM, SDM, RDM and TDM. Compared with N1 fertilization, N2 fertilization significantly

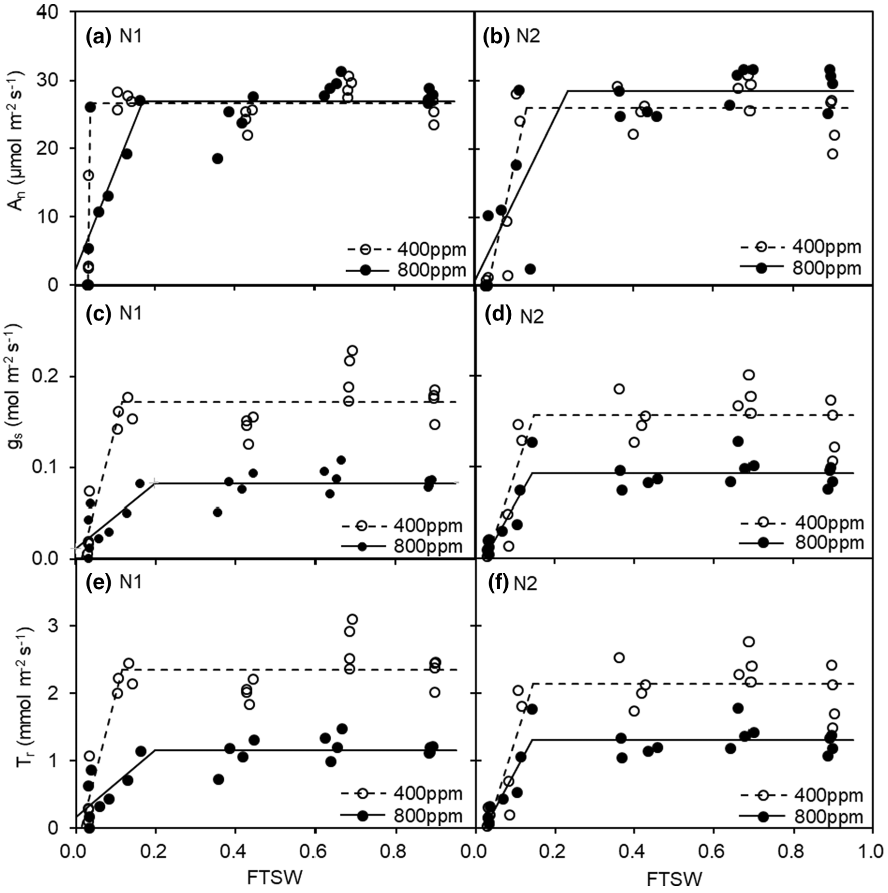


FIGURE 2 Net photosynthesis rate (A_n), stomatal conductance (g_s) and transpiration rate (T_r) of maize as affected by N fertilizers (N1 and N2), respectively, in response to the fraction of transpirable soil water (FTSW) at *a*[CO_2] (400 ppm) (open symbols) and *e*[CO_2] (800 ppm) (closed symbols). Each data point represented measurement on one individual plant.

increased LDM and SDM, resulting in an increased TDM (Figures 8a,b and 9a; Table 3). Drought stress reduced LDM, but had no effect on SDM and RDM, resulting in a reduced TDM (Figures 8a–c and 9a; Table 3). In addition, across N fertilization, *e*[CO_2] significantly increased maize RDM and TDM under drought stress (Figures 8c and 9a; Table 3).

e[CO_2], N1 fertilization and drought stress significantly reduced WU of maize plant, respectively (Figure 9b; Table 3). *e*[CO_2] and drought stress had a greater WUE_p of maize plant than *a*[CO_2] and well-watered regime, respectively. There was no difference in maize WUE_p under each N fertilization (Figure 9c; Table 3).

4 | DISCUSSION

It was widely recognized that the A_n response to *e*[CO_2] in C_4 was weaker than in C_3 crop (Prior et al., 2011) due to the difference in CO_2 metabolism mechanism between C_3 and C_4 crops (Kadam et al., 2014; Liu et al., 2019). Leakey (2006) reported that *e*[CO_2] (550 ppm) had no significant effect on A_n in maize under normal water supply. Besides, it was reported that *e*[CO_2] caused a decrease in g_s in maize and amaranth (Wei et al., 2022). In good agreement with this, here *e*[CO_2] had no effect on A_n in maize, but significantly decreased g_s and T_r at the early stage of progressive soil drought (Figure 2a–f; Tables 1 and 2), leading to significant improved WUE_i and WUE_{leaf} in progressive drought soil (Figure 3a–d). The reason was that maize, as a C_4 crop, possibly required lower CO_2 concentration to reach $A_{n\ max}$ at jointing stage, therefore the photosynthetic gain of *e*[CO_2] was not significant in the current study. Additionally, this might be the reason why *e*[CO_2] did not significantly increase TDM. Meanwhile, *e*[CO_2] concentration could exacerbate the depolarization of protective membrane potential by affecting ion channel activity to close leaf stomata, resulting in the obvious reduced g_s and T_r (Ainsworth & Rogers, 2007), further enhancing leaf WUE under mild and moderate drought stress (Oliveira et al., 2016; Zhang et al., 2018). These indicated that maize, as a C_4 species could better adapt to the future climate changed scenario with increasing CO_2 concentration and worsening drought as WUE_i and WUE_{leaf} were notably improved at *e*[CO_2] in progressive drought soil. More interesting, no significant difference in WUE_i was found between two N treatments, implying the appropriate reduction in N application would be the reasonable and efficient fertilizer and water management for maize production as the no decrease in leaf WUE.

When FTSW decreased to a certain value, A_n, g_s, T_r, Ψ_l and K_l began to decline. Several authors reported a delayed response of A_n and g_s to drought stress in C_3 crop tomato grown under *e*[CO_2] (Liu et al., 2019; Yan et al., 2017). However, the recent study showed that the FTSW threshold of A_n and g_s in C_4 crop maize was higher at *e*[CO_2] (Wei et al., 2022). In this study, under N1 treatment, a higher FTSW threshold of A_n was observed at *e*[CO_2]. While *e*[CO_2] did not significantly change the sensitivity of g_s and T_r to drought, but showed an increasing trend of FTSW under N2 fertilization at *e*[CO_2]

TABLE 1 Results of the linear-plateau regression analyses of the responses of net photosynthetic rate (A_n), stomatal conductance (g_s), transpiration rate (T_r), leaf water potential (Ψ_l) and leaf hydraulic conductance (K_l) of maize to the reduction in fraction of transpirable soil water (FTSW) under ambient CO_2 concentration ($a[CO_2]$, 400 ppm) and elevated CO_2 concentration ($e[CO_2]$, 800 ppm) with reduced N (N1, 0.8 g N pot^{-1}) and adequate N supply (N2, 1.6 g N pot^{-1}).

	A_n		g_s		T_r		Ψ_l		K_l	
Factor	$A_{n\,max}$	C_A	$g_{s\,max}$	C_g	$T_{r\,max}$	C_T	$\Psi_{l\,max}$	C_Ψ	$K_{l\,max}$	C_K
N1										
400 ppm	26.66 ± 1.27	0.04 ± 0.00	0.17 ± 0.02	0.12 ± 0.03	2.35 ± 0.20	0.12 ± 0.03	−3.73 ± 0.43	0.15 ± 0.01	0.64 ± 0.08	0.19 ± 0.06
800 ppm	26.96 ± 3.50	0.17 ± 0.07	0.08 ± 0.01	0.20 ± 0.11	1.16 ± 0.15	0.20 ± 0.11	−4.02 ± 1.47	0.16 ± 0.09	0.31 ± 0.07	0.18 ± 0.12
N2										
400 ppm	26.06 ± 2.86	0.13 ± 0.03	0.16 ± 0.02	0.15 ± 0.05	2.14 ± 0.25	0.15 ± 0.04	−3.83 ± 1.03	0.13 ± 0.02	0.62 ± 0.13	0.19 ± 0.15
800 ppm	28.40 ± 3.75	0.24 ± 0.16	0.09 ± 0.01	0.14 ± 0.05	1.30 ± 0.14	0.14 ± 0.03	−3.48 ± 0.67	0.16 ± 0.02	0.39 ± 0.06	0.20 ± 0.11

Note: $A_{n\,max}$, $g_{s\,max}$, $T_{r\,max}$, $\Psi_{l\,max}$ and $K_{l\,max}$ indicated the initial values (mean ± standard error) of the parameters when the plants were not significantly affected by drought; C (C_A, C_g, C_T, C_Ψ or C_K) indicated the threshold at which the parameter (A_n, g_s, T_r, Ψ_l or K_l, respectively) start to decrease due to progressive soil drought.

(Figure 2a–f; Tables 1 and 2). These results implied that $e[CO_2]$ could moderate the acclimation of maize A_n, being more sensitive to progressive soil drought under reduced N supply due to the weaker leaf photosynthetic capacity and no longer to be sustained in N1 treatment. The earlier stomatal closure at $e[CO_2]$ could reduce water use, maintain soil water status, and lengthen the plant survival time under long-term drought (Yan et al., 2017).

At the early stage of progressive soil drought, Ψ_l was not affected at $e[CO_2]$, while K_l was decreased in this study (Figure 4a–d; Tables 1 and 2). These implied that maize plant did not suffer much water stress in the period of better water status, leading to no significant response of Ψ_l to $e[CO_2]$. The decrease of K_l was corresponding to the decline in T_r at $e[CO_2]$, reducing the water transport capacity and affecting nutrients uptake in maize crop (Yao et al., 2020). During progressive drought stress, Ψ_l at $e[CO_2]$ decreased earlier under N2 treatment, while the plant water status was maintained longer at $a[CO_2]$ (Figure 4a,b; Tables 1 and 2). This was mainly due to the similar sensitivity of g_s and T_r between $a[CO_2]$ and $e[CO_2]$, resulting in no decrease in water use under drought and a decrease in plant water status under both N treatments.

As a key component of protein, nucleic acid and chlorophyll, etc., N was an essential nutrient for crop growth (Xu et al., 2012). Moderate N application could promote the synthesis of chlorophyll and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), being conducive to enhance Rubisco, carbonic anhydrase activity and chlorophyll content, and finally improving the photosynthetic rate and accumulation of plant assimilates (Bassi et al., 2018). In this experiment, two N supply levels had no significant effect on leaf gas exchange parameters and water relations at the beginning of drought stress (Figure 2a–f; Tables 1 and 2). The reason for this discrepancy might be considered about the relatively better and steady soil water status under N1 treatment due to less plant water use, thus facilitating to maintain the leaf physiological response, and the difference among growing stages, maize species needed further investigation. In earlier experiment, high N treatment accelerated the decline of A_n, g_s and T_r at varied drought degrees in tomato plant (Yang et al., 2022). However, following the intensified drought stress, a higher FTSW threshold of $a[CO_2]$ maize A_n decrease was observed under N2 treatment. This physiological response could be attributed to the increased proportion of bound water and enhanced resistance of CO_2 entering stomata resulted from the possible leaf photosynthetic acclimation of adequate N supply, thus reducing the supply of CO_2 and ultimately being detrimental to A_n under moderate drought. Adversely, N1 supply retarded the maize A_n decline and maintained growth rate during progressive drought stress, while this phenomenon would also merit further investigation.

Earlier studies suggested that $[ABA]_{leaf}$ played a crucial role in stomatal regulation during progressive soil drought at $e[CO_2]$, gradually increasing with the decrease in FTSW (Tazoe & Santrucek, 2015; Yang et al., 2022). $[ABA]_{leaf}$ of maize growing at $e[CO_2]$ was reported to be greater than that of $a[CO_2]$, whereas the opposite was true for amaranth (Wei et al., 2022). Nevertheless, this study found that although change of $[ABA]_{leaf}$ was no difference in two CO_2

TABLE 2 Results of statistical analysis of the linear-plateau regression of net photosynthetic rate (A_n), stomatal conductance (g_s), transpiration rate (T_r), leaf water potential (Ψ_l) and leaf hydraulic conductance (K_l) of maize response to the reduction in fraction of transpirable soil water (FTSW).

	A_n		g_s		T_r		Ψ_l		K_l	
Factor	$A_{n\,max}$	C_A	$g_{s\,max}$	C_g	$T_{r\,max}$	C_T	$\Psi_{l\,max}$	C_Ψ	$K_{l\,max}$	C_K
N1										
400 ppm	0.8693	0.0004	<0.0001	0.1312	<0.0001	0.1265	0.6952	0.8066	<0.0001	0.9674
800 ppm	ns	***	***	ns	***	ns	ns	ns	***	ns
N2										
400 ppm	0.3007	0.1915	<0.0001	0.865	<0.0001	0.9069	0.5513	0.0397	0.0021	0.8945
800 ppm	ns	Ns	***	ns	***	ns	ns	*	**	ns
400 ppm										
N1	0.6840	<0.0001	0.1897	0.2535	0.1646	0.2434	0.8516	0.0745	0.7619	0.9928
N2	ns	***	Ns	ns	ns	ns	ns	ns	ns	ns
800 ppm										
N1	0.5556	0.4389	0.1225	0.3174	0.1385	0.3012	0.4867	0.9293	0.0874	0.8593
N2	ns	Ns	Ns	ns	ns	ns	ns	ns	ns	ns

Note: *, **, and *** indicated the differences of the parameters between ambient CO_2 concentration ($a[CO_2]$, 400 ppm) and elevated CO_2 concentration ($e[CO_2]$, 800 ppm) with reduced N (N1, 0.8 g N pot^{-1}) and adequate N supply (N2, 1.6 g N pot^{-1}) at $p < .05$, $p < .01$ and $p < .001$ levels, respectively; ns indicated no significant difference. $A_{n\,max}$, $g_{s\,max}$, $T_{r\,max}$, $\Psi_{l\,max}$ and $K_{l\,max}$ indicated the initial values (mean ± standard error) of the parameters when the plants were not significantly affected by drought; C (C_A, C_g, C_T, C_Ψ or C_K) indicated the threshold at which the parameter (A_n, g_s, T_r, Ψ_l or K_l, respectively) start to decrease due to progressive soil drought. *, **, and *** indicated the significant difference at $p < .05$, $p < .01$ and $p < .001$ levels, respectively; ns indicated no significant difference.

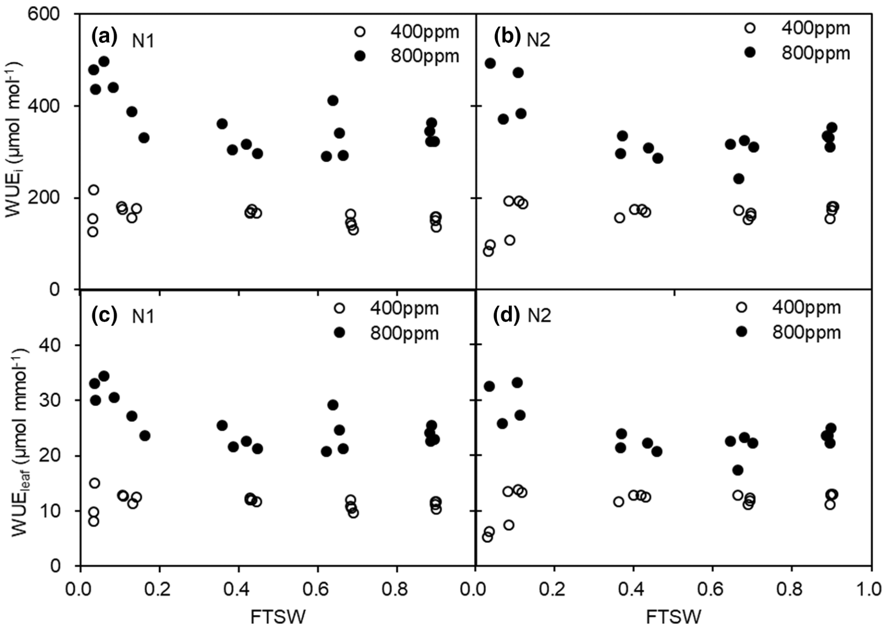


FIGURE 3 Intrinsic WUE (WUE$_i$, A_n/g_s) and instantaneous WUE (WUE$_{leaf}$, A_n/T_r) of maize as affected by N fertilizers (N1 and N2), respectively, in response to the fraction of transpirable soil water (FTSW) at $a[CO_2]$ (400 ppm) (open symbols) and $e[CO_2]$ (800 ppm) (closed symbols). Each data point represented measurement on one individual plant.

concentrations (Figure 5a; Table 3), the g_s was negatively correlated with $[ABA]_{leaf}$ and the slope at $e[CO_2]$ was notably lower than that at $a[CO_2]$ (Figure 6), indicating that the decrease of g_s at $e[CO_2]$ might be induced by ABA-mediated stomatal closure. Moreover, N2 treatment resulted in higher $[ABA]_{leaf}$ as compared with N1 treatment, especially under drought stress condition in this study (Figure 5a; Table 3). This was primarily ascribed to the drought stress combined with N2 treatment causing the root to produce a large amount of ABA to decline g_s, thus further reducing water loss (Xu et al., 2018). However, in fact, N2 treatment had no higher FTSW threshold of g_s, but there was a decrease tendency at $e[CO_2]$, and an opposite tendency at $a[CO_2]$. The possible explanation was that the response of g_s to $[ABA]_{leaf}$ was influenced by two $[CO_2]$ concentrations during progressive soil drought, and the response of g_s was less sensitive and decreased later at $e[CO_2]$.

Earlier study indicated that $e[CO_2]$ significantly increased the SD of maize (Wei et al., 2022), and moderate drought increased SD (Fraser et al., 2009), while excessive drought decreased SD (Xu

FIGURE 4 Leaf water potential (Ψ_l) and leaf hydraulic conductance (K_l) of maize as affected by N fertilizers (N1 and N2), respectively, in response to the fraction of transpirable soil water (FTSW) at $a[CO_2]$ (400 ppm) (open symbols) and $e[CO_2]$ (800 ppm) (closed symbols). Each data point represented measurement on one individual plant.

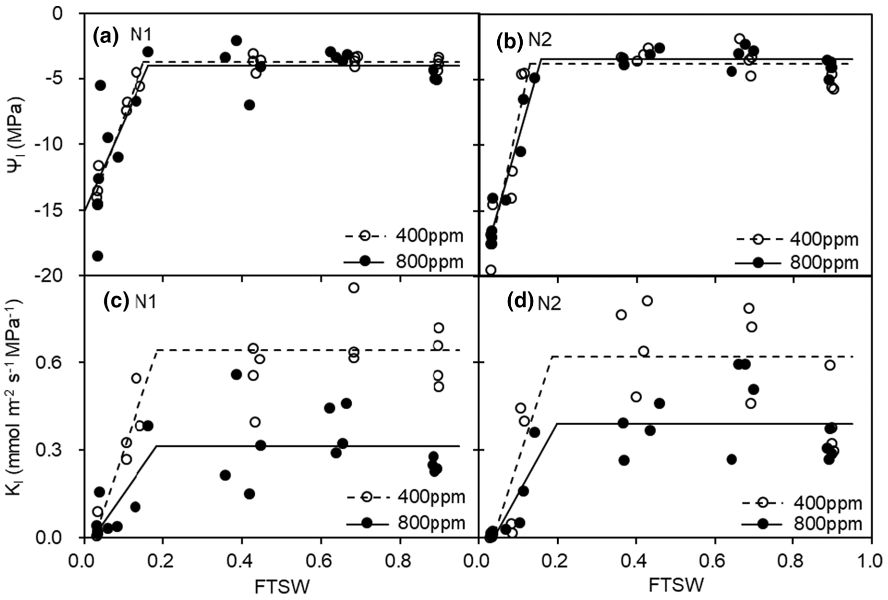
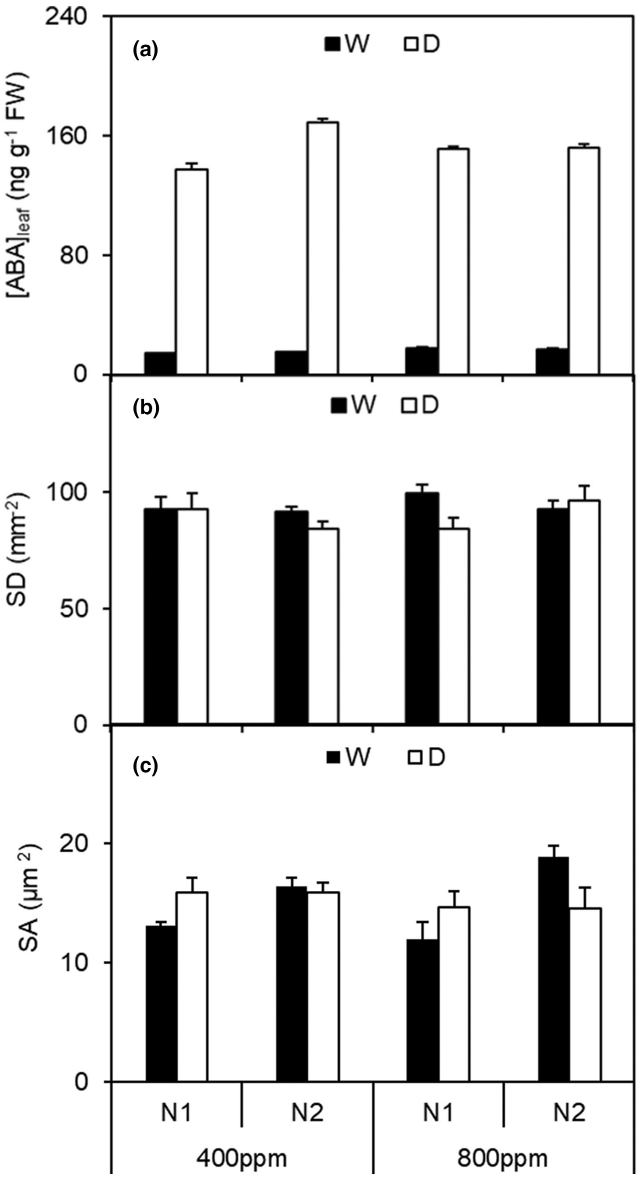


FIGURE 5 Leaf ABA concentration ($[ABA]_{leaf}$), stomatal density (SD) and stomatal aperture (SA) of maize respectively as affected by different CO_2 levels, N fertilizations and water stress treatments. W denoted well-watered treatment and D denoted progressive soil drought treatment. Error bars indicated the SE ($n = 4$).



& Zhou, 2008). Disagreement with this, maize SD under $e[CO_2]$ and progressive drought did not change significantly in the present study (Figure 5b; Table 3), this mainly was resulted from the inhibited cell elongation under drought stress, resulting in simultaneous reduction of leaf area and stomatal number, that was, no significant change in SD (Xie & Zhang, 2018). However, SA under progressive soil drought was lower at $e[CO_2]$ (Figure 5c; Table 3). Furthermore, compared to N2, N1 treatment significantly decreased SA, suggesting that drought combined with reduced N supply would synergistically decline stomatal aperture and reduce maize g_s at $e[CO_2]$.

It was reported that the increase in CO_2 concentration of C_4 crop facilitated the number of tanner cells in leaf cells to increase (Ferris et al., 2001), which contributed to the significant enhanced LA of $e[CO_2]$ maize plant in this study, while the SLA of $e[CO_2]$ maize was also increased here (Figure 7b; Table 3), this was not consistent with the observed decreasing effect on maize SLA in Wei et al. (2022) and merited further exploration. As for biomass, it might have no significant effect at $e[CO_2]$ (Fang et al., 2021). Similarly, we found that $e[CO_2]$ had no increased dry mass of maize (Figures 8a–c and 9a; Table 3). Besides, N2 had larger LA as compared with N1 supply (Figure 7a; Table 3) as the proper application of N fertilizer could enlarge LA and chlorophyll content, and further has a positive effect on promoting the photosynthetic process (Badr et al., 2016), implying that $e[CO_2]$ could mitigate the negative effect of drought stress on LA under both N fertilization, especially under N2 supply. Leaf expansion growth of maize under soil drought was more sensitive to abiotic stress than carbon assimilation, leading to lower SLA (thicker leaves) (Wei et al., 2021). In good

TABLE 3 Output of three-way ANOVA of leaf ABA concentration ($[ABA]_{leaf}$), stomatal density (SD), stomatal aperture (SA), leaf area (LA), special leaf area (SLA), leaf dry mass (LDM), stem dry mass (SDM), root dry mass (RDM), total dry mass (TDM), plant water use (WU) and water use efficiency (WUE_p) of well-watered (W), progressive soil drought (D) maize plants under atmospheric $[CO_2]$ (400 and 800 ppm) with reduced N (N1, 0.8 g N pot^{-1}) and adequate N supply (N2, 1.6 g N pot^{-1}).

Factor	$[ABA]_{leaf}$ (ng g^{-1}FW)	SD (mm^{-2})	SA (μm^2)	LA (cm^2)	SLA (cm^2 g^{-1})	LDM (g)	SDM (g)	RDM (g)	TDM (g)	WU (L)	WUE_p (g L^{-1})
$[CO_2]$	ns	ns	Ns	***	*	ns	ns	ns	ns	***	***
[N]	***	ns	**	***	ns	***	***	ns	***	***	ns
[W]	***	ns	Ns	***	***	***	ns	ns	***	***	***
$[CO_2]\times[N]$	***	ns	Ns	*	ns	ns	ns	ns	ns	ns	ns
$[CO_2]\times[W]$	ns	ns	Ns	*	ns	**	*	*	*	***	ns
$[N]\times[W]$	***	ns	**	***	ns	***	***	**	***	***	*
$[CO_2]\times[N]\times[W]$	***	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns

Note: *, **, and *** indicated the differences of the parameters between 400 and 800 ppm with N1 and N2 under well-watered treatment and progressive soil drought treatment at $p < .05$, $p < .01$, $p < .001$ level, respectively; ns indicated no significant difference.

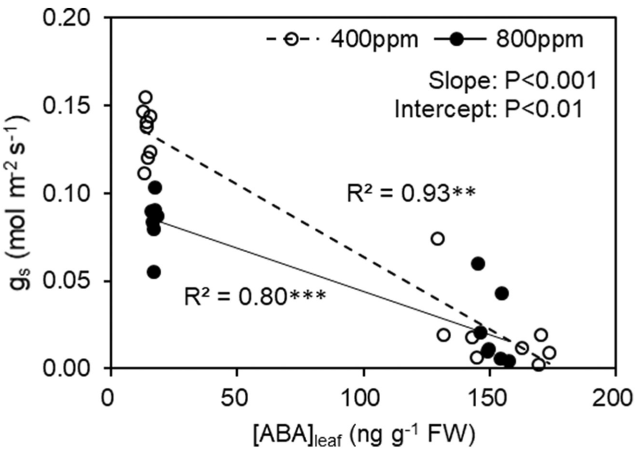


FIGURE 6 Relations between leaf ABA concentration ($[ABA]_{leaf}$) with stomatal conductance (g_s) of maize grown under atmospheric $[CO_2]$ (400 and 800 ppm). ** and *** indicated the regression line was statistically significance at $p < .01$ and $p < .001$ level, respectively.

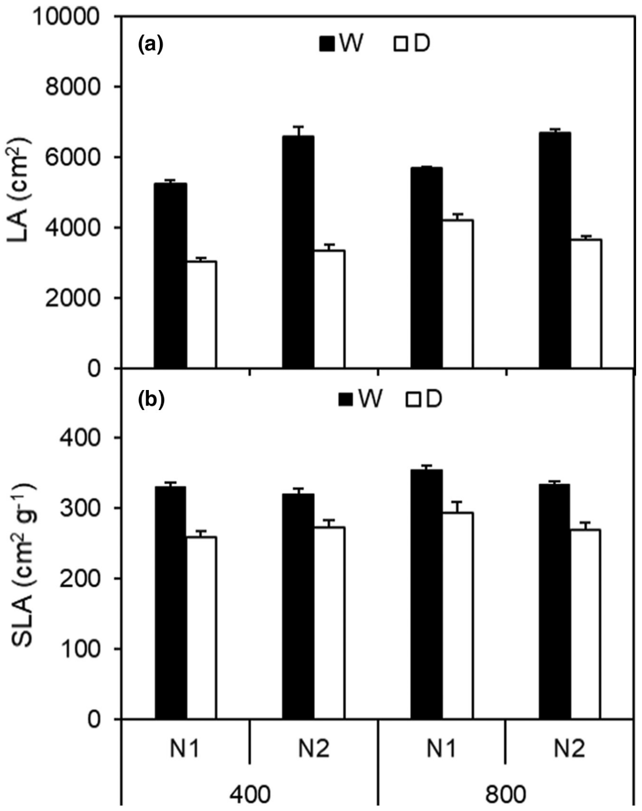


FIGURE 7 Leaf area (LA) and specific leaf area (SLA) of maize respectively as affected by different CO_2 levels, N fertilizations and water stress treatments. W denoted well-watered treatment and D denoted progressive soil drought treatment. Error bars indicated the SE ($n = 4$).

accordance with this, here the SLA under soil drought was lower than that under well-watered condition (Figure 7b; Table 3), being resulted from the increased crop dry mass. Moreover, considering plants grown under drought stress, $e[CO_2]$ maize possessed no decreased LDM and SDM, increased RDM and TDM at two N supply (Figures 8a–c and 9a; Table 3). This was consistent with the former study reported that exposure to $e[CO_2]$ significantly enhanced the biomass accumulation of C_4 crop under soil drought (van der Kooi et al., 2016) and indicated that in drought soil, $e[CO_2]$ was beneficial to the root development for maintaining water absorbing capacity of maize crop grown under both N supply, particularly for N1 fertilization.

Here, plant water use was decreased at $e[CO_2]$, N1 supply and drought stress (Figure 9b; Table 3). It was well known that $e[CO_2]$ had lowered T_r and WU, thus could retain the soil water condition to prolong the time of subsequent soil water use (Li et al., 2020). This would be helpful for the N fertilizer uptake and utilization, and further partially explain the slight and no difference in maize plant water use and WUE_p between two N fertilizer levels under progressive soil drought in this study. Therefore, irrespective of N fertilization supply, $e[CO_2]$ and drought stress simultaneously increased maize WUE_p (Figure 9c; Table 3). This was mostly ascribed to the decrease in plant water use and in good line with the previous report on tomato plant WUE respond to drought coupled with $e[CO_2]$ environment (Wei et al., 2021).

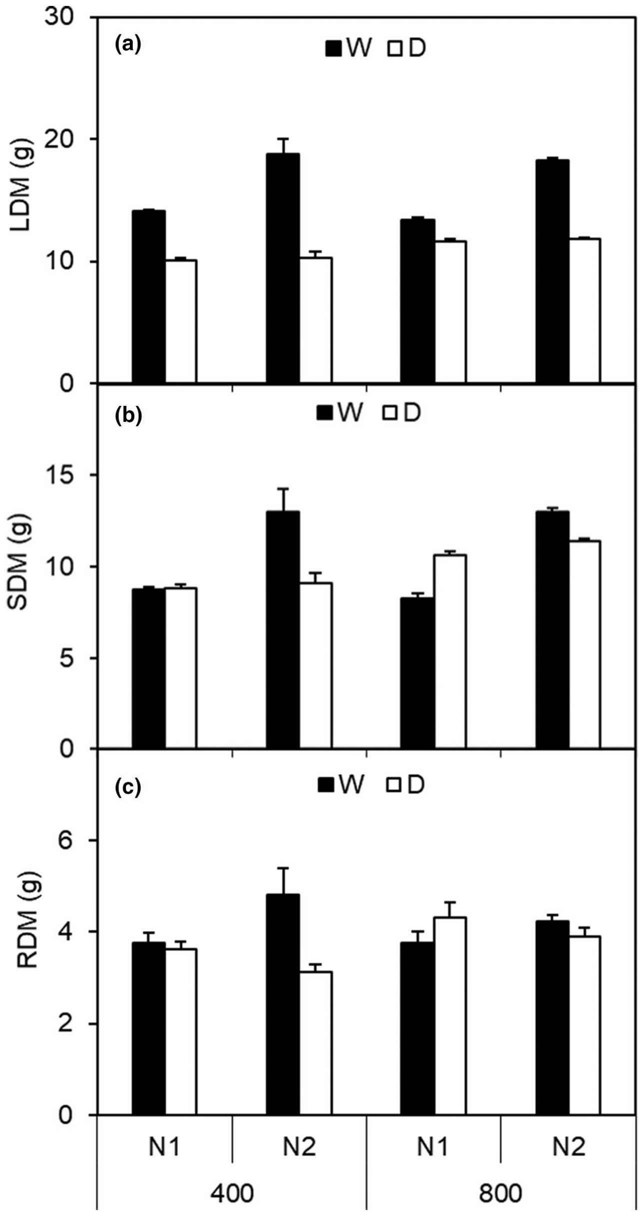


FIGURE 8 Leaf dry mass (LDM), stem dry mass (SDM) and root dry mass (RDM) of maize respectively as affected by different CO_2 levels, N fertilizations and water stress treatments. W denoted well-watered treatment and D denoted progressive soil drought treatment. Error bars indicated the SE ($n=4$).

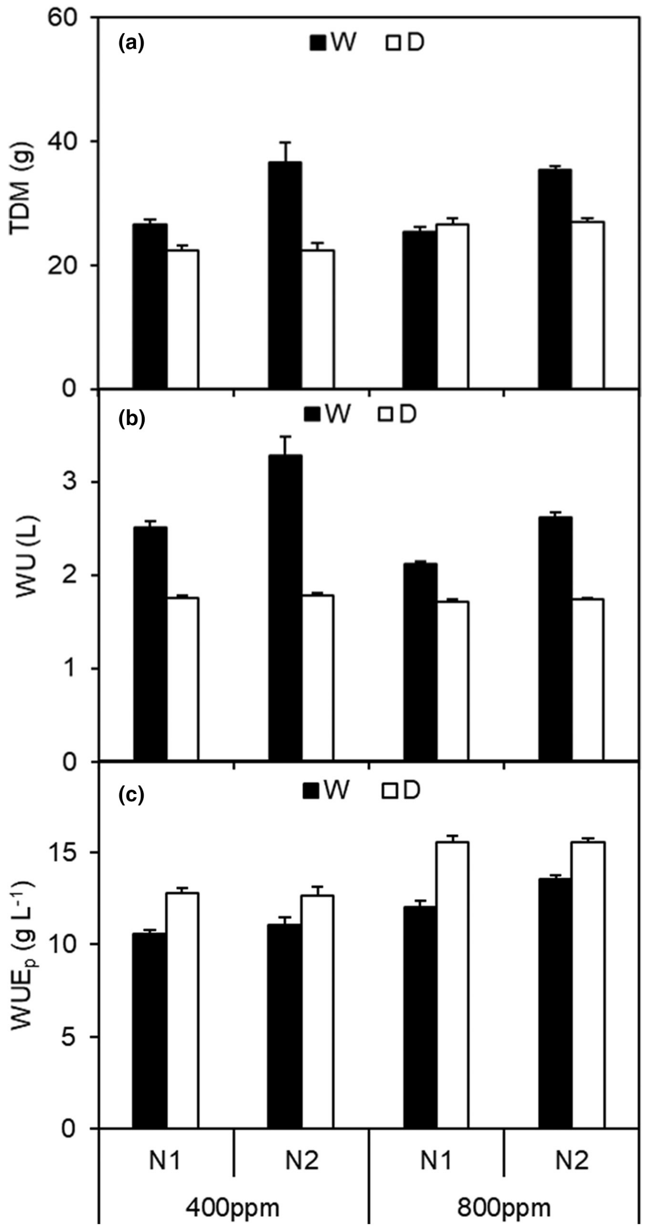


FIGURE 9 Total dry mass (TDM), plant water use (WU) and plant WUE (WUE$_p$) of maize respectively as affected by different CO_2 levels, N fertilizations and water stress treatments. W denoted well-watered treatment and D denoted progressive soil drought treatment. Error bars indicated the SE ($n=4$).

5 | CONCLUSION

Collectively, compared to $a[CO_2]$ environment, $e[CO_2]$ could maintain maize leaf A_n and Ψ_l, but notably decrease g_s, T_r and K_l, leading to increased WUE from stomatal to leaf scale under two N supply. Although A_n and Ψ_l of $e[CO_2]$ maize decreased at a higher FTSW threshold, $e[CO_2]$ intended to increase $[ABA]_{leaf}$ under N1 fertilization and reduced the g_s respond to $[ABA]_{leaf}$. Moreover, N1 fertilization coupled with $e[CO_2]$ decreased SA and WU, sustained LDM and SDM, increased LA, SLA, RDM and TDM under drought stress, resulting in an enhanced WUE$_p$ of maize crop, indicating a more reasonable stomata and water control at $e[CO_2]$ combined with N1 fertilization supply in response to progressive soil drought. These findings in this study provide one useful suggestion for improving WUE of C_4 crop maize under N application respond to the drier and CO_2-rich climates.

AUTHOR CONTRIBUTIONS

Manyi Zhang: Conceptualization; investigation; methodology; formal analysis; writing – original draft; visualization. **Guiyu Wei:** Investigation. **Bingjing Cui:** Investigation. **Chunshuo Liu:** Investigation. **Heng Wan:** Investigation. **Jingxiang Hou:** Investigation. **Yiting Chen:** Investigation. **Jiarui Zhang:** Investigation. **Jie Liu:** Investigation.

Zhenhua Wei: Conceptualization; methodology; writing – review and editing; funding acquisition; supervision; validation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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