

## Excessive nitrogen application under moderate soil water deficit decreases photosynthesis, respiration, carbon gain and water use efficiency of maize

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### ABSTRACT

The impact of water stress and nitrogen (N) nutrition on leaf respiration (R), carbon balance and water use efficiency (WUE) remains largely elusive. Therefore, the objective of the present study was to investigate the effect of soil water and N stresses on growth, physiological responses, leaf structure, carbon gain and WUE of maize. The plants were subjected to different soil water and N regimes to maturity. The results showed that the photosynthesis ( $A_n$ ) and stomatal conductance ( $G_s$ ) decreased significantly under the water stressed treatments across the N treatments mainly ascribed to the decreased plant water status. The moderate water stress reduced the photosynthetic capacity and activity and also caused damage to the structure of leaves, resulting in the significant reduction of  $A_n$ , and thus decreased  $WUE_i$ . The dark respiration ( $R_d$ ) was significantly decreased due to the damage of mitochondria, however, the  $R_d/A_n$  increased significantly and the carbon gain was seriously compromised, eventually inhibiting biomass growth under the moderately water stressed treatment. Increasing N dose further aggravated the severity of water deficit, decreased  $A_n$ ,  $G_s$  and  $WUE_i$ , damaged the structure and reduced the number of mitochondria of leaves, while increased  $R_d/A_n$  considerably under moderate water stress. Consequently, the biomass accumulation, carbon gain and plant level  $WUE_p$  in the moderately water stressed treatment decreased markedly under the high N supply. Therefore, excessive N application should be avoided when plants suffer soil water stress in maize production.

### 1. Introduction

Water and nitrogen (N) are the two main limiting factors for plant growth and productivity (Sinclair and Rufty, 2012). Maize (*Zea mays* L.) is a major crop used worldwide as a source of food, fuel and animal feed and serves as a key resource for economic and industrial applications. In order to increase yield, N fertilizer in large quantity is usually applied into the soil in crop production. It is reported that N fertilization can increase water stress tolerance in maize (Song et al., 2019), however, the effect of N nutrition on photosynthesis ( $A_n$ ), stomata and water use efficiency (WUE) interacts with soil water regimes (Wang et al., 2010, 2018; Li et al., 2020).

It is well known that plant stress response is initiated and regulated by stress signals. Plant water potential and abscisic acid (ABA) have been reported as important hydraulic and chemical signals in response to water deficit (Comstock, 2002; Wilkinson and Davies, 2002). Earlier studies have shown that root water potential (RWP) and leaf water potential (LWP) decreased under water deficit, while leaf ABA concentration ( $[ABA]_{leaf}$ ) might not be affected significantly, which was, however, influenced by N treatments (Song et al., 2019). Under water deficit, stomatal conductance ( $G_s$ ) regulated by ABA signaling has been widely reported (Comstock, 2002). It was found that  $G_s$  was significantly decreased by high N supply and soil water deficit, contributing to improved WUE at leaf and plant scales, while  $A_n$  was unaffected (Li

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et al., 2020). Wang et al. (2018) reported that reduced soil water regimes under N fertigation resulted in partial closure of stomata via decreased plant water status and intensified root-to-shoot ABA signaling, which increased leaf and plant level WUE, and  $A_n$  was also affected by N fertilization. Leaf respiration (R) plays a major role in carbon cycle at scales from leaf to the globe (Meir et al., 2001). The mitochondrial respiration is necessary to regulate the photosynthesis process in order to protect the photosynthetic organs from the detrimental effects arisen from over-reduction under stresses (Xu et al., 2011; Yoshida et al., 2007). Although some attention has been given to the impact of water deficit on plant R, the results vary with increasing severity of water deficit (Chaves et al., 2003; Flexas et al., 2006). It is reported that leaf R increases proportionally when carbon uptake becomes limited under water stress, which can alter leaf carbon balance (Lawlor and Tezara, 2009).

Both chloroplasts and mitochondria are important organelles where photosynthesis and respiration are the primary pathways of carbon and energy metabolism in plants. Studies have shown that plant responses to water deficit have certain correlation with anatomical structure and changes in ultrastructure of chloroplasts and mitochondria of plants (Wang et al., 2006), and it is important to investigate stress characterization from cell morphology to associate the responses firmly with stress type and intensity (Pinheiro and Chaves, 2011). The function of chloroplasts and mitochondria is closely and tightly coordinated through intracellular metabolite pools (Carrari et al., 2003; Raghavendra and Padmasree, 2003; Nunesnesi et al., 2007; Noguchi and Yoshida, 2008; Araújo et al., 2011, 2014). Besides photosynthesis, mitochondrial metabolism participates in many important cellular processes such as N metabolism and signaling (Nunesnesi et al., 2008).

The impact of soil water and N nutrition and their interactions on leaf R, gas exchange, carbon balance and water use efficiency (WUE) still remain largely elusive. Therefore, the goal of the present study was to investigate soil water and N stresses and their inter-relationships on modification of photosynthesis, respiration, carbon gain and WUE. In this study during the grain filling stage of maize, the photosynthesis, respiration, photosynthetic light and CO<sub>2</sub>-response curves reflecting the photosynthetic capacity, leaf ultrastructure as well as plant water status and WUE were determined to identify the carbon balance between respiration rate and photosynthesis under different water stresses and N deficiency.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The experiment was conducted from April to July 2019 in a glasshouse with temperature-controlling equipment at Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. The seeds of maize (cultivar B73) were pre-germinated and three individual plumules were selected and transplanted into 10.6 L pots (27.5 cm in diameter at the top edge, 20 cm in diameter at the bottom, 24 cm in depth). When three leaves emerged, the plants were thinned to one plant per pot. The soil was sandy loam, having a pH of 7.6, total C of 9.0 g kg<sup>-1</sup> and total N of 1.0 g kg<sup>-1</sup>. The water-holding capacity and permanent wilting point of the soil were 35% and 11%, respectively (Wang et al., 2018). The soil was sieved (5 mm mesh) and then each pot was filled with 12.0 kg of dry soil. The soil bulk density was 1.20 g dry soil cm<sup>-3</sup>. There were holes at the bottom of each pot, though no leaching of water was observed during the treatment period.

### 2.2. Experimental treatments

Maize plants were exposed to three soil water regimes including 90% (W90), 70% (W70) and 50% (W50) of soil water holding capacity, representing well watered, mildly and moderately water stressed treatments, respectively, in interaction with three nitrogen fertilization doses

consisting of 1.0 g N pot<sup>-1</sup> (N1), 2.5 g N pot<sup>-1</sup> (N2.5) and 5.0 g N pot<sup>-1</sup> (N5), representing low, medium and high N supply levels, respectively. The experiment was a completely randomized design comprising nine treatments and each treatment had four replicates, and this totally yielded 36 pots. N fertilizer as NH<sub>4</sub>NO<sub>3</sub> was used, and phosphorus and potassium as KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub> at the rate of 3 g pot<sup>-1</sup> were applied into each pot, which were mixed homogeneously with the soil in the pots. Water treatments were initiated from the jointing growth stages to the end of the experiment when plants were harvested at the maturity stage. Soil water regimes were measured every day and the volumetric soil water content was determined by a TDR (Soil Moisture Equipment Corp., CA, USA) with a pair of TDR probes installed at a depth of 15 cm in the middle of each pot. The plants were irrigated after determination of soil water contents at 16:00 on each day.

### 2.3. Sampling, measurements and analyses

Leaf gas exchange including photosynthetic rate ( $A_n$ ) and stomatal conductance ( $G_s$ ) were measured during the grain filling stage on the upper fully-expanded ear leaves from 9:00 to 11:00 a.m. at the light intensity of 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and CO<sub>2</sub> concentration of 400 ppm with Li-6400 Portable Photosynthesis System (Li-Cor Biosciences, NE, USA). The dark respiration rate ( $R_d$ ) during the grain filling stage was measured after the leaves had been placed in darkness for 30 min, and it was determined without light at the CO<sub>2</sub> concentration of 400 ppm by the above Portable Photosynthesis System. The intrinsic water use efficiency ( $WUE_i$ ) was calculated as  $A_n/G_s$ . The carbon gain was indicated as the ratio between  $R_d$  and  $A_n$  (Pattison et al., 1998).

The photosynthetic CO<sub>2</sub> response curve ( $A_n/C_i$ ) and light response curve ( $A_n/Q$ ) were measured during the grain filling stage on the same leaves used for leaf gas exchange measurements. Before the measurement of the responses curves, the leaves were acclimated for 30 min at the light intensity of 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and CO<sub>2</sub> concentration of 400 ppm. The photosynthetic CO<sub>2</sub> response curve was determined from 9:00 to 12:00 at the light intensity of 2000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  by using Li-6400 Portable Photosynthesis System (Li-Cor Biosciences, NE, USA). The measurements were taken at the CO<sub>2</sub> gradient of 400, 350, 250, 150, 100, 50, 400, 600, 800, 1000, 1200 and 1500 ppm. After the measurement, the  $A_n$  values were plotted against the respective intercellular CO<sub>2</sub> concentrations ( $C_i$ ) to produce a response curve. The model of photosynthesis for C<sub>4</sub> plants was used to simulate the photosynthetic CO<sub>2</sub> response curve (Ye and Yu, 2009). The model was expressed as,

$$A_n = a \frac{1 - bC_i}{1 + cC_i} C_i - R_p$$

where  $A_n$  is the net photosynthetic rate;  $C_i$  is the intercellular CO<sub>2</sub> concentration;  $a$  is the initial carboxylation efficiency;  $b$  and  $c$  are coefficients;  $R_p$  is the rate of photorespiration (The dark respiration under light is very small, so the rate of CO<sub>2</sub> release into the air by the leaves under light is approximately regarded as the light respiration rate (Cai and Xu, 2000)). The CO<sub>2</sub> saturated photosynthetic rate ( $A_{\text{sat}}$ ) and saturation intercellular CO<sub>2</sub> concentration ( $C_{\text{isat}}$ ) were calculated as (Ye, 2010),

$$C_{\text{isat}} = \frac{\sqrt{\left(\frac{b+c}{b}\right) - 1}}{c}$$

$$A_{\text{max}} = a \left( \frac{\sqrt{b+c} - \sqrt{b}}{c} \right)^2 - R_p$$

During the same days on which the photosynthetic CO<sub>2</sub> response curves were measured, the photosynthetic light response curves were developed by measuring  $A_n$  at different light intensities by using Li-6400 Portable Photosynthesis System (Li-Cor Biosciences, NE, USA) at the

CO<sub>2</sub> concentration of 400 ppm. The measurements were taken at the light intensity gradient of 2000, 1500, 1000, 700, 500, 300, 200, 100, 50 and 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The photosynthetic light response curve was modeled by a modified rectangular hyperbola. Photosynthetic parameters derived from the light response curve were determined according to the model (Ye, 2007) expressed as,

$$A_n = \alpha \frac{1 - \beta Q}{1 + \gamma Q} Q - R$$

where  $A_n$  is the net photosynthetic rate;  $Q$  is the light intensity;  $\beta$  and  $\gamma$  are coefficients;  $R$  is the dark respiration rate;  $\alpha$  is the initial slope. The maximum net photosynthetic rate ( $A_{\text{max}}$ ) and the saturation irradiance ( $Q_{\text{sat}}$ ) were calculated as (Ye, 2010),

$$Q_{\text{sat}} = \frac{\sqrt{(\beta + \gamma)/\beta} - 1}{\gamma}$$

$$A_{\text{max}} = \alpha \left( \frac{\sqrt{\beta + \gamma} - \sqrt{\beta}}{\gamma} \right)^2 - R$$

During the grain filling stage, leaf samples were collected and frozen immediately in liquid N and stored at  $-80^\circ\text{C}$  for leaf ABA measurement. ABA concentration in the leaves was determined by ELISA method (Yang et al., 2001).

Leaf materials were sampled at the grain filling stage for the observation of leaf ultrastructure. Leaf segments (around  $2 \text{ mm}^2$ ) were cut from the central part of the leaves and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h. They were washed twice in the same buffer for 30 min and post-fixed with 1% OsO<sub>4</sub> for 4 h at  $4^\circ\text{C}$ . After this, the samples were dehydrated through an ethanol series (30-50-70-90%) and dehydrated twice through 100% ethanol and acetone, respectively. Then they were infiltrated in a mixture of acetone and spurr resin (3/7, 5/5, 7/3, v/v), and finally embedded in spurr resin (Grigorova et al., 2012). The samples were made into ultrathin sections and were stained with uranyl acetate and lead citrate, and then examined for chloroplasts, mitochondria, osmiophilic granules and starch grains in bundle sheath cells (BSC) by the HT7700 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) operating at 80 kV. The numbers of chloroplasts, mitochondria, osmiophilic granules and starch grains in bundle sheath cells (BSC) were determined from the images by ImageJ software (version 8.0, National Institute of Health, USA).

At maturity, the leaf area, plant height, root water potential ( $\Psi_r$ ), leaf water potential ( $\Psi_l$ ), total C and total N were measured according to Wang et al. (2018). The water use efficiency ( $\text{WUE}_p$ ) was calculated as dry biomass/plant water consumption.

## 2.4. Statistical analyses

The data were analyzed statistically using SPSS software version 22.0 (IBM Corporation, New York, USA) including one-way and two-way ANOVA. When the analyses of ANOVA were significant among the treatments, the values between the treatments were further evaluated by the Duncan's multiple range test (DMRT) at the significance of  $P < 0.05$ . The figures were made with Origin 8.0 (OriginLab Corporation, MA, USA).

## 3. Results

### 3.1. Plant height and leaf area

Soil water regimes and N treatments as well as their interactions significantly affected plant height (Fig. 1). Across the N treatments, the plant height and leaf area in moderately water stressed treatment were significantly lower than those under the well watered and mildly water stressed treatments. Under the moderately water stressed treatment,

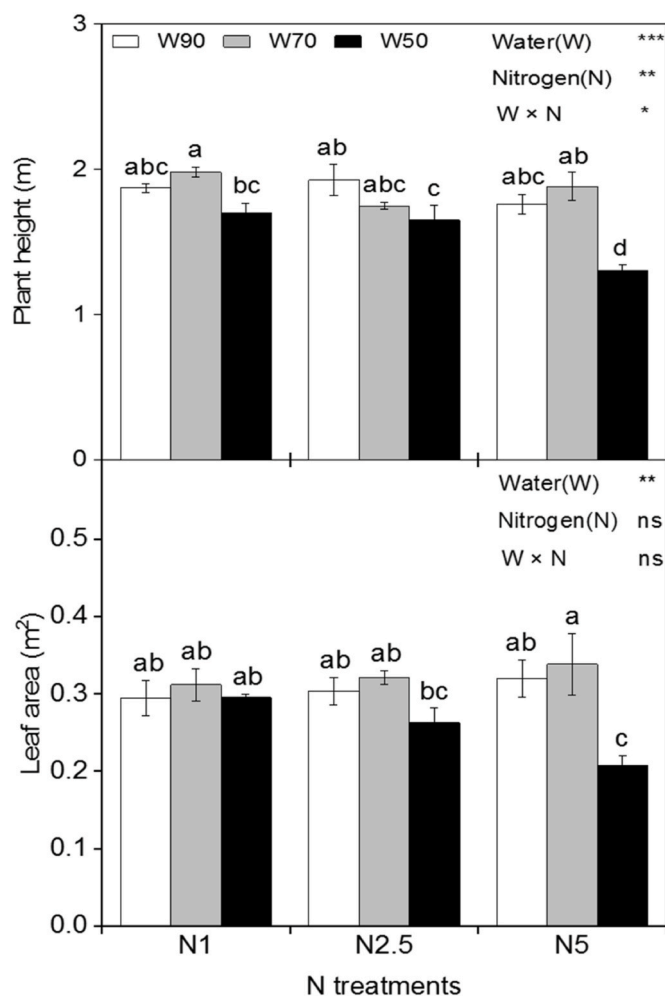


Fig. 1. The plant height and leaf area under different soil water and N treatments at maturity. Different letters above the columns indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*, \*\*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.

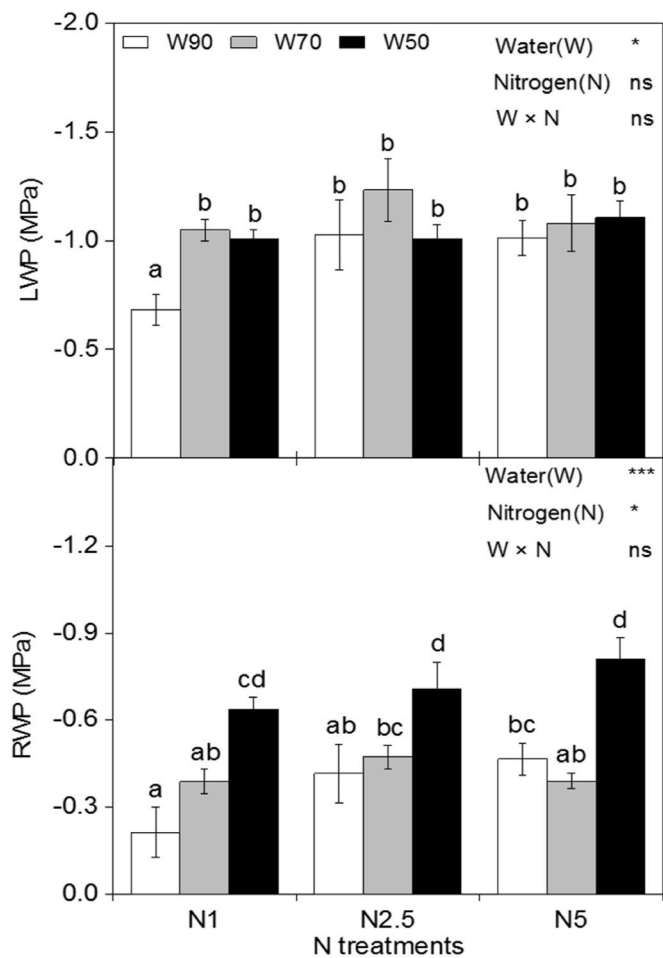
increase of N dose reduced plant height and leaf area by 3% and 11% under the N2.5 treatment, and by 23% and 30% under the N5 treatment, respectively, compared with the N1 treatment.

### 3.2. Plant water status

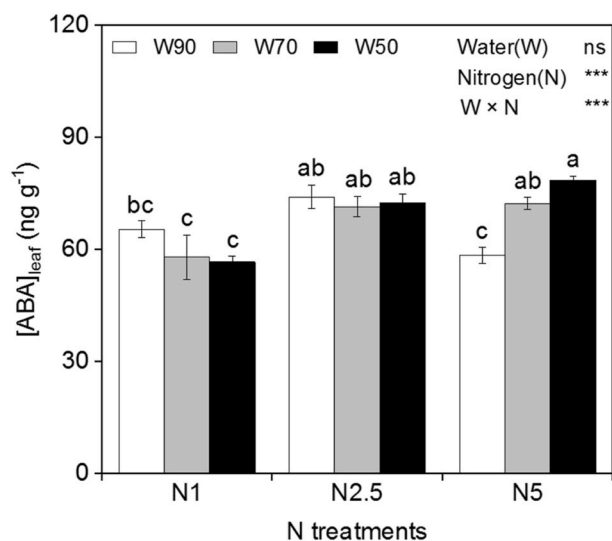
When analyzed across the N treatments, LWP and RWP were significantly affected by soil water regimes (Fig. 2). The moderate water stress decreased RWP significantly. Similarly, the LWP decreased under the mild and moderate water deficit treatments. For RWP, the analysis across the soil water regimes showed that RWP in the medium and high N treatments were significantly lower compared with low N treatment. Nonetheless, across the soil water regimes, LWP was not significantly affected by the increase of N dose.

### 3.3. Leaf ABA concentration

Across the N treatments,  $[\text{ABA}]_{\text{leaf}}$  was not significantly affected by soil water treatments (Fig. 3). The analysis across the soil water regimes showed that  $[\text{ABA}]_{\text{leaf}}$  in the medium and high N treatments were significantly higher than that in the low N treatment. Under the mildly and moderately water stressed treatments, increase of N dose significantly enhanced  $[\text{ABA}]_{\text{leaf}}$  compared with the N1 treatment.



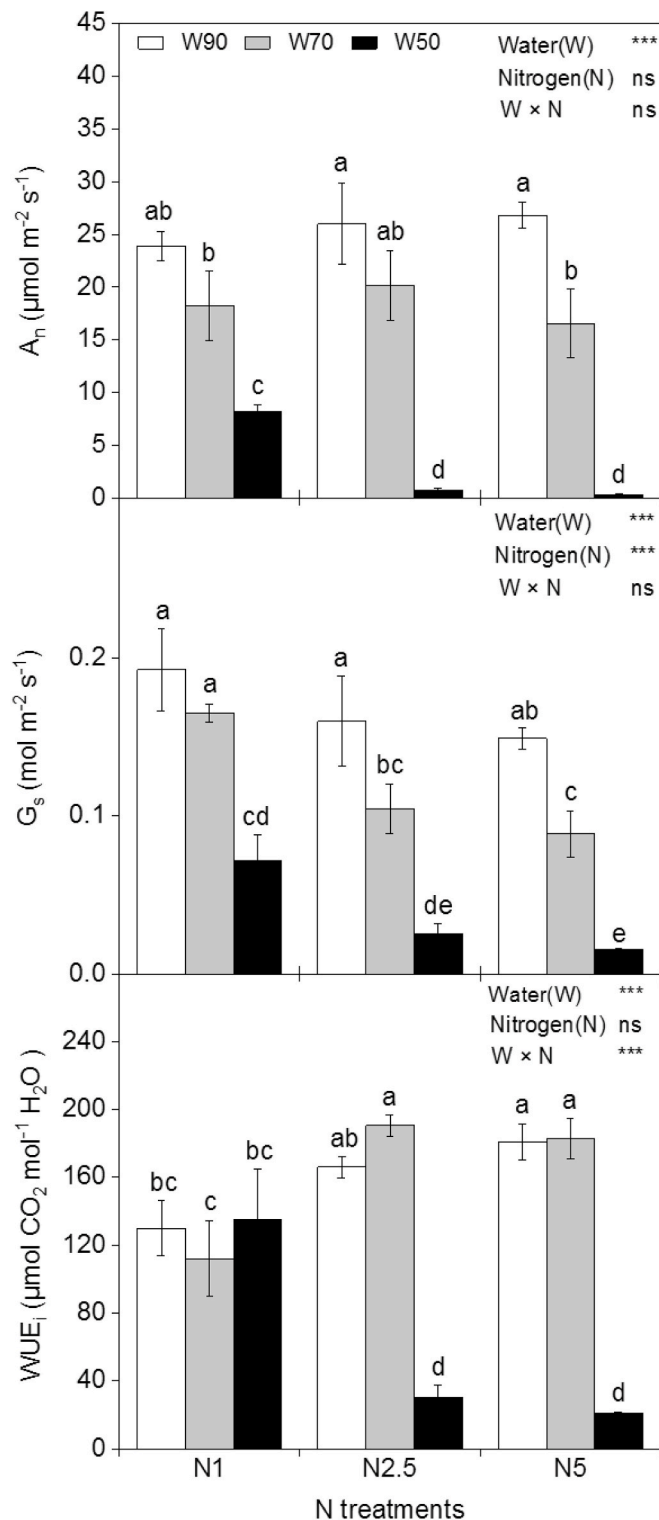
**Fig. 2.** The leaf water potential (LWP) and root water potential (RWP) under different soil water and N treatments. Different letters above the columns indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.



**Fig. 3.** The  $[ABA]_{leaf}$  under different soil water and N treatments. Different letters above the columns indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*\*\* and ns indicate the significances at  $P < 0.001$  and no significance, respectively, for the experimental factors.

### 3.4. $A_n$ , $G_s$ and $WUE_i$

Across the N treatments, moderately and mildly water stressed treatments significantly decreased  $A_n$  and  $G_s$  during the grain filling stage (Fig. 4). When analyzed across the water treatments,  $G_s$  decreased



**Fig. 4.** The  $A_n$ ,  $G_s$  and  $WUE_i$  of maize plants under different soil water and N treatments during the grain filling stage. Different letters above the columns indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.



with soil N levels, while  $A_n$  was not significantly affected by N treatments. Increase of N dose considerably decreased  $A_n$  and  $G_s$  compared with the N1 treatment in the moderately water stressed treatment.

Across the N treatments,  $WUE_i$  was higher in the well watered and mildly water stressed treatments compared with the moderate water deficit treatment. High and medium N treatment significantly reduced  $WUE_i$  in the moderately water stressed treatment compared with the low N treatment. In contrast, increase of N dose improved  $WUE_i$  under the well watered and mildly water stressed treatments.

### 3.5. Photosynthetic $CO_2$ and light response curve

Either soil water regimes or N treatments affected the photosynthetic  $CO_2$  and light response curves of maize plants (Fig. 5). Fig. 5a-c and Table 1 showed that both  $\alpha$  and R were significantly affected by water treatments. Across the N treatments, the  $\alpha$  and R were highest in the well watered treatment compared to the other water treatments. Across the N treatments, the  $A_{max}$  and  $Q_{sat}$  were highest in the well watered or mildly water stressed treatments compared with the moderately water stressed treatment, though the difference was not statistically significant under the water and N treatments.

Both  $a$  and  $R_p$  were significantly affected by water treatments and the interaction between water and N treatments (Table 2). However, the  $R_p$  and  $C_{isat}$  were significantly affected by N treatments and the interaction between water and N treatments. Across the N treatments, the  $a$ ,  $R_p$  and  $A_{max}$  were lowest in the moderately water stressed treatment compared with other water treatments. The analysis across the water treatments showed that the  $R_p$  and  $C_{isat}$  were significantly higher under the low N treatment compared with other N treatments. Under the low and medium N treatment, the  $a$ ,  $R_p$  and  $A_{sat}$  were highest in the mildly water stressed treatment compared with well watered and moderately water stressed treatments. Nevertheless, under the high N treatment, the  $a$ ,  $R_p$  and  $A_{sat}$  increased with the increase of water levels. Across the water treatments, the  $C_{isat}$  decreased with the increasing N dose significantly.

**Table 1**

Effect of soil water and N treatments on the initial slope ( $\alpha$ ), the maximum net photosynthetic rate ( $A_{max}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), the dark respiration rate (R,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and the saturation irradiance ( $Q_{sat}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) derived from the photosynthetic light–response curves (Fig. 5a–c) of maize plants.

N treatments	Water treatments	$\alpha$	R	$A_{max}$	$Q_{sat}$
N1	W50	0.049 ab	2.34abc	21.2a	1865.9a
	W70	0.050 ab	2.09bc	24.3a	2325.3a
	W90	0.051 ab	3.30a	21.3a	2478.9a
N2.5	W50	0.055a	2.42abc	21.0a	1891.8a
	W70	0.042b	1.84c	23.7a	1925.2a
	W90	0.055a	3.04 ab	25.9a	1999.6a
N5	W50	0.053a	2.60abc	22.3a	2106.1a
	W70	0.045 ab	2.05bc	28.2a	1943.9a
	W90	0.047 ab	2.49abc	27.1a	2362.5a
Water (W)		*	**	ns	ns
Nitrogen (N)		ns	ns	ns	ns
W × N		ns	ns	ns	ns

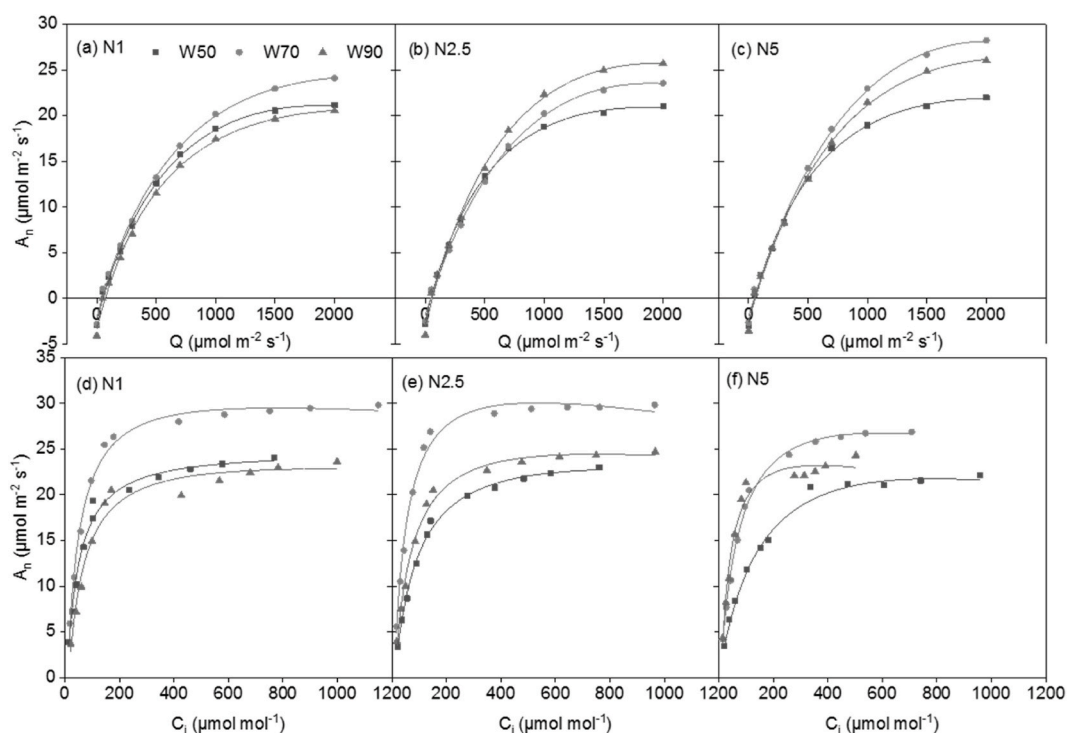
Different letters in the column of each experimental factor indicate significant differences at  $P < 0.05$ . \*, \*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.01$  and no significance, respectively, for the experimental factors.

### 3.6. $R_d$ and $R_d/A_n$

Across the N treatments, the moderately water stressed treatment significantly decreased  $R_d$ , while significantly increased  $R_d/A_n$  compared with the well watered and mildly water stressed treatments (Fig. 6). When analyzed across the soil water regimes, the  $R_d$  was similar among the N treatments, however, the  $R_d/A_n$  was highest in the high N treatment compared with the low and medium N treatments. In the moderately water stressed treatment, increase of N dose significantly increased  $R_d/A_n$ , which was not affected by N doses under the well watered and mildly water stressed treatments.

### 3.7. The ultrastructure of chloroplasts and mitochondria

With the increase of water stress, the chloroplasts clung on the cell wall became swollen (Fig. 7A–C, D–F and G–I). Meanwhile, the



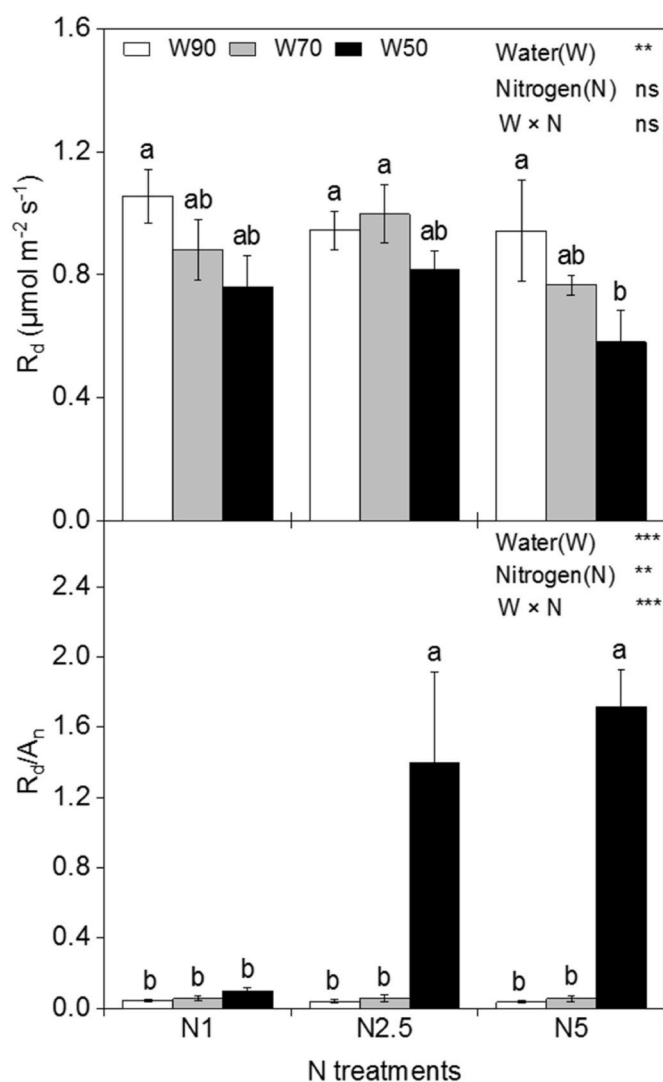
**Fig. 5.** The photosynthetic  $CO_2$  and light response curves of maize plants exposed to different soil water and N treatments.

**Table 2**

Effect of soil water and N treatments on the initial carboxylation efficiency ( $a$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), the rate of the photorespiration ( $R_p$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), the photosynthetic capacity ( $A_{\text{sat}}$ ,  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), and saturation intercellular  $\text{CO}_2$  concentration ( $C_{\text{isat}}$ ,  $\mu\text{mol m}^{-1}$ ) derived from the photosynthetic  $\text{CO}_2$ -response curves (Fig. 5d–f) of maize plants.

N treatments	Water treatments	$a$	$R_p$	$A_{\text{sat}}$	$C_{\text{isat}}$
N1	W50	0.885bcd	5.63bc	24.1a	857.6 ab
	W70	1.172 ab	8.43 ab	29.7a	807.1abc
	W90	0.750bcde	7.95 ab	22.8a	907.9a
N2.5	W50	0.526cde	3.27cd	26.3a	593.3cde
	W70	1.088abc	7.49 ab	30.2a	552.7de
	W90	0.779bcde	5.57bc	25.9a	705.9abcd
N5	W50	0.181e	0.15d	21.5a	768.6abcd
	W70	0.414de	2.33cd	25.6a	630.2bcd
	W90	1.621a	10.35a	23.3a	374.6e
Water (W)		*	***	ns	ns
Nitrogen (N)		ns	*	ns	***
W × N		**	**	ns	*

Different letters in the column of each experimental factor indicate significant differences at  $P < 0.05$ . \*, \*\*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.



**Fig. 6.** Effect of soil water and N treatments on the dark respiration rate ( $R_d$ ) and  $R_d/A_n$  of maize leaves. Different letters above the columns indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*\*, \*\*\* and ns indicate the significances at  $P < 0.01$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.

chloroplast envelope was partially ruptured and the thylakoid membranes were loose, and the thylakoids were overly disorganized. In addition, the number of chloroplasts and starch grains decreased, while the number of osmiophilic granules increased in the moderately water stressed treatment in comparison to the well watered and mildly water stressed treatments (Table 3, Fig. 7A–C). High N supply decreased the number of starch grains and osmiophilic granules while the size of starch grains increased (Fig. 7G–I).

Compared with the well watered and mildly water stressed treatments, the number of mitochondria in the moderately water stressed treatment decreased, and the mitochondria swelled irregularly and the membrane was partially ruptured, meanwhile, fractured arrangement of cristae were observed or disappeared in the moderately water stressed treatment (Table 3, Fig. 8A–C, D–F and G–I). Under water stress especially the moderately water stressed treatment, compared with low N treatment, the mitochondria were more severely damaged under medium and high N supply, and the number of mitochondria was also reduced (Table 3, Fig. 8A, D and G). Additionally, the cristae was broken or even disappeared under the high N treatment (Fig. 8G–I).

### 3.8. Above-ground dry biomass, plant water use and $WUE_p$

Across the N treatments, the above-ground dry biomass and plant water use were significantly higher in the well watered and mildly water stressed treatments compared with the moderately water stressed treatment (Table 4). However, they decreased with the increase of N dose when analyzed across the soil water treatments. Across the N treatments, the mildly water stressed treatment had the highest  $WUE_p$ , followed by the well watered and the moderately water stressed treatments. The analysis across the soil water treatments showed that the  $WUE_p$  was not affected by the N levels. Under the mildly water stressed treatment, increase of N dose improved  $WUE_p$ , however, under the moderately water stressed treatment, the high N treatment significantly reduced the above-ground dry biomass, plant water use and  $WUE_p$  compared with the low and medium N treatments.

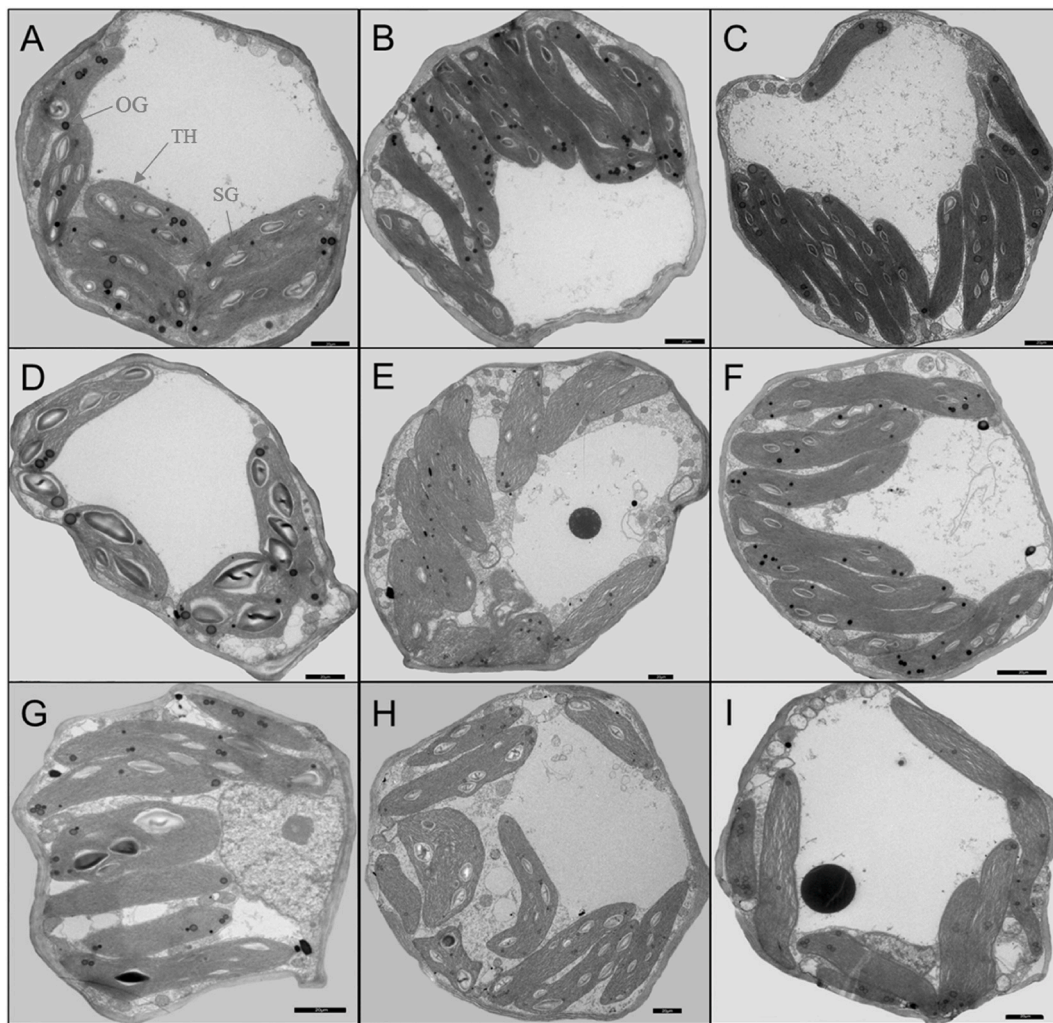
### 3.9. Plant C and N accumulation

When analyzed across the N treatments, the C and N accumulation was significantly higher in the well watered and mildly water stressed treatments compared with the moderately water stressed treatment (Fig. 9). Across the water treatments, the N accumulation was highest under the N2.5 treatment compared to other N treatments. Under the moderately water stressed treatment, increase of N dose significantly decreased the C accumulation under the N5 treatment compared with the N1 and N2.5 treatments.

Increase of N dose under the moderately water stressed treatment significantly decreased N accumulation, while the N accumulation under the well watered and mildly water stressed treatments were not affected under different N doses. Increase of N dose increased N accumulation in the well watered treatment, while the N accumulation was similar under different N doses in the mildly and moderately water stressed treatments. There was a significant negative linear correlation between plant C accumulation and  $R_d/A_n$  (Fig. 10).

## 4. Discussion

Water stress and N deficiency are the two most common abiotic stresses plants encounter during growth. There are still controversial conclusions on the interaction of water deficit and N deficiency on leaf gas exchange, respiration and WUE. Many previous studies reported that increasing N supply alleviated water stress and improved drought resistance (Song et al., 2019; Pinheiro et al., 2004). Nonetheless, some other studies found that N fertilization can weaken crops under drought conditions (Araus et al., 2020), and decrease  $G_s$  with high nitrate supply after three days of drought stress compared with the plants supplied



**Fig. 7.** The ultrastructure of chloroplast in the bundle sheath cells (BSC) under different soil water and N treatments. Scale bars = 20 μm. OG, TH and SG represent osmiophilic granule, thylakoid and starch grain. Figures from A to I are ultrastructures of chloroplast under N1W50, N1W70, N1W90, N2.5W50, N2.5W70, N2.5W90, N5W50, N5W70 and N5W90, respectively.

**Table 3**

Effect of soil water and N treatments on the number of osmiophilic granules, starch grains, chloroplasts and mitochondria in the bundle sheath cells (BSC).

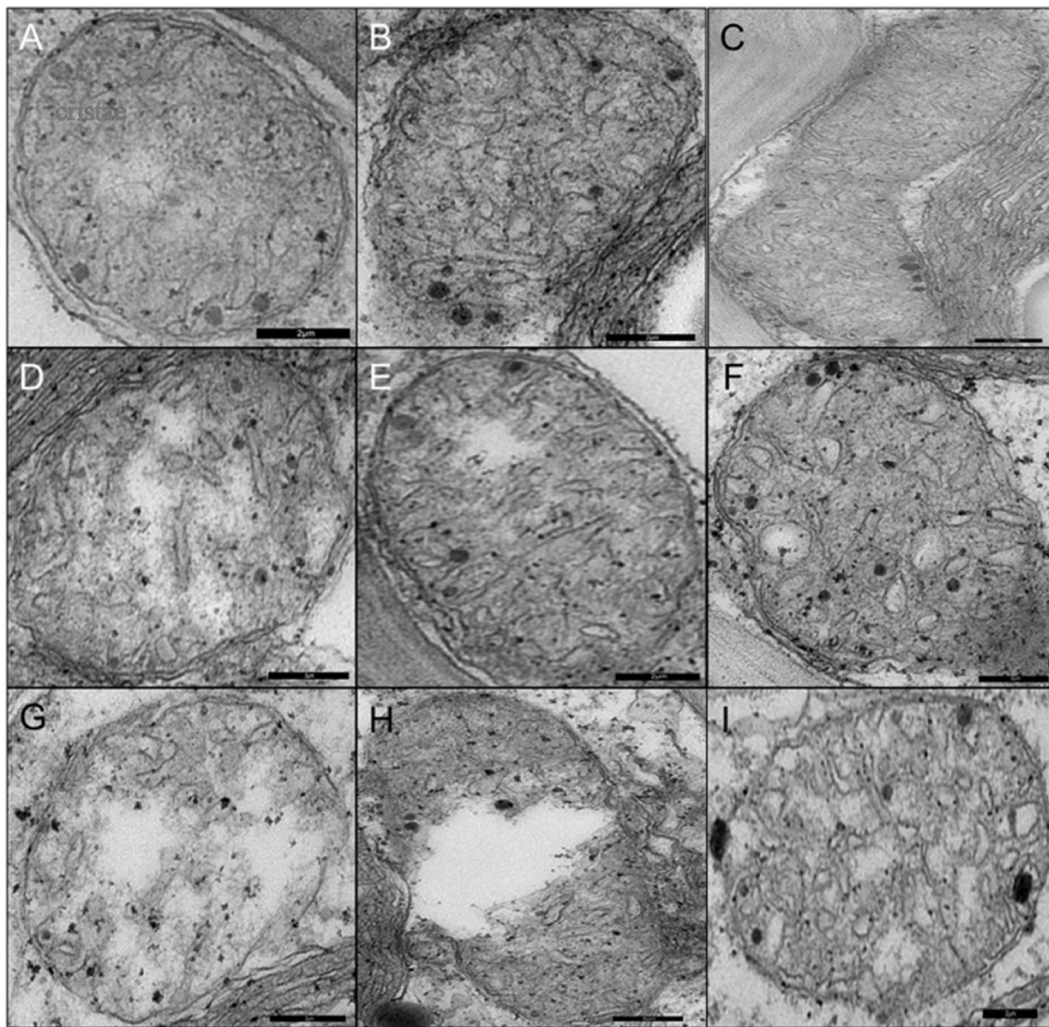
N treatments	Water treatments	Number			
		Osmiophilic granules	Starch grains	Chloroplasts	Mitochondria
N1	W50	42a	29 ab	8 ab	13a
	W70	34 ab	32 ab	9 ab	14a
	W90	30 ab	35a	9 ab	10a
N2.5	W50	44a	27 ab	9 ab	8a
	W70	39 ab	20b	8 ab	12a
	W90	29 ab	36a	9 ab	11a
N5	W50	31 ab	22 ab	7b	8a
	W70	35 ab	24 ab	11a	9a
	W90	24b	31 ab	10a	10a
Water (W)		*	*	ns	ns
Nitrogen (N)		ns	ns	ns	ns
W × N		ns	ns	ns	ns

Different letters in the column of each experimental factor indicate significant differences among the treatments at  $P < 0.05$ . \* and ns indicate the significances at  $P < 0.05$  and no significance, respectively, for the experimental factors.

with water (Ding et al., 2018). However, this should depend upon soil N fertility levels, as in the present study, it was found that increase of N dose did not alleviate water stress for plants. On the contrary, it aggravated water stress especially under the moderately water stressed treatment, and decreased photosynthesis and carbon gain as well as

damaged the leaf structure, resulting in reduction in plant growth and WUE.





**Fig. 8.** The ultrastructure of mitochondria in the bundle sheath cells (BSC) under different soil water and N treatments. Scale bars = 2 µm. Figures from A to I are ultrastructures of mitochondria under N1W50, N1W70, N1W90, N2.5W50, N2.5W70, N2.5W90, N5W50, N5W70 and N5W90, respectively.

**Table 4**  
Effect of soil water and N treatments on the above-ground dry biomass, plant water use and water use efficiency (WUE<sub>p</sub>).

N treatments	Water treatments	Above-ground dry biomass (g plant <sup>-1</sup> )	Plant water use (L plant <sup>-1</sup> )	WUE <sub>p</sub> (g L <sup>-1</sup> )
N1	W50	74.4b	22.1d	3.4b
	W70	96.3a	29.6bc	3.3b
	W90	103.1a	34.3a	3.0bc
N2.5	W50	69.7b	20.4d	3.4b
	W70	97.7a	28.9bc	3.4b
	W90	100.7a	31.6b	3.2b
N5	W50	46.2c	16.5e	2.8c
	W70	106.9a	27.5c	3.9a
	W90	93.1a	28.9bc	3.2b
Water (W)		***	***	**
Nitrogen (N)		*	***	ns
W × N		**	ns	**

Different letters in the column indicate significant differences among the treatments according to Duncan's multiple range test at  $P < 0.05$ . \*, \*\*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.

**4.1. Moderate soil water stress inhibited photosynthetic rate by reduced photosynthetic capacity and activity and decreased carbon gain, resulting in reduced plant biomass**

The increase of water deficit severity decreased both  $A_n$  and  $G_s$  (Fig. 4), which was mainly due to the decreased root water status (Fig. 2). The  $WUE_i$  was improved under the well watered and mildly water stressed treatments compared with the moderately water stressed treatment across the N treatments ascribed to the more pronouncedly increased  $A_n$  than  $G_s$  (Fig. 4). Accordingly, for the moderately water stressed treatment, the  $WUE_i$  was significantly reduced due to the severely inhibited  $A_n$  compared with the reduction in  $G_s$ . It is known that  $A_n$  is not only affected by  $G_s$ , but also by  $CO_2$  and light use efficiency of leaves. The  $\alpha$  from the light response curve was not depressed by the moderately water stressed treatment, indicating that the increase of  $A_n$  to  $A_{max}$  with increasing light intensity was not affected. Nonetheless, the  $A_{max}$  was decreased by the moderately water stressed treatment compared with the well watered or mildly water stressed treatment, reflecting the declined maximum photosynthetic capacity. The decreased  $A_n$  from the  $CO_2$  response curve under the moderately water stressed treatment indicated that the amount of Rubisco and its activity reduced in the low intercellular  $CO_2$  concentration range under moderate water stress (Ye, 2010). In consequence, the  $WUE_i$  was reduced in the moderately water stressed treatment compared with well watered and mildly water stressed treatments (Fig. 4), resulting in the reduced

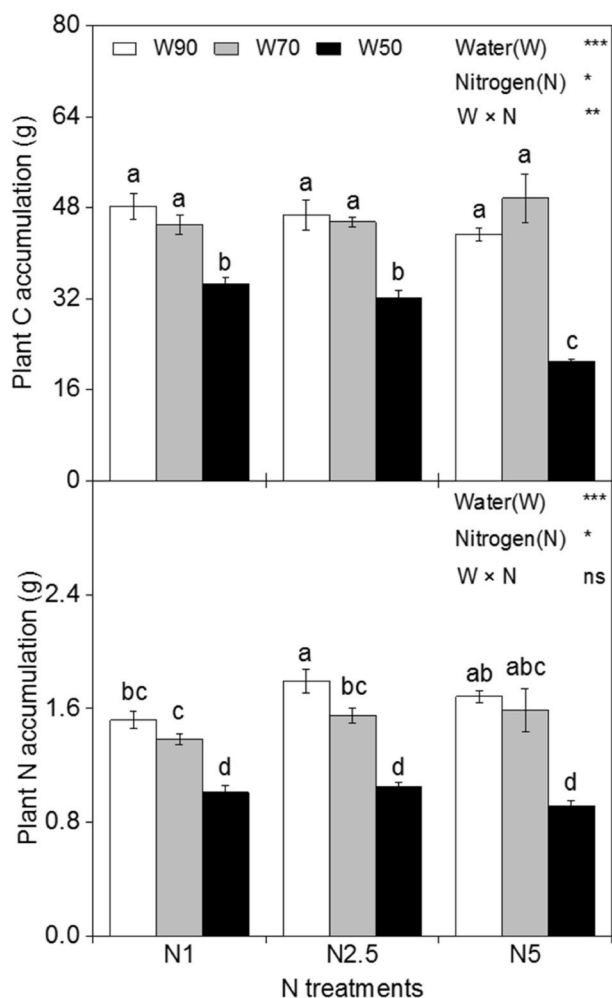


Fig. 9. Effect of soil water and N treatments on plant C and N accumulation of maize plants. \*, \*\*, \*\*\* and ns indicate the significances at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  and no significance, respectively, for the experimental factors.

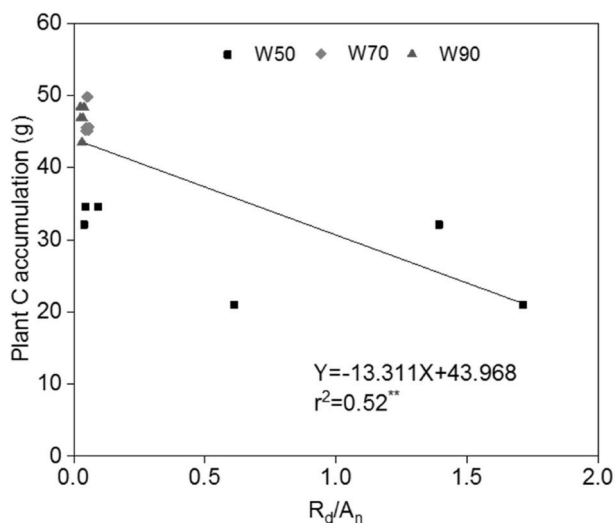


Fig. 10. Relationship between plant C accumulation and  $R_d/A_n$  of maize plants during the growth stage under different soil water and N treatments. \*\* indicates the significance of the regression line at  $P < 0.01$ .

accumulation of biomass at the suboptimal soil water growing condition.

Moreover, the damaged structure of leaves induced under water stress caused the significant reduction of  $A_n$  and photosynthetic efficiency in the moderately water stressed treatment (Lawlor, 2002). It is found that the chloroplasts swelled in order to increase light absorption area alleviating stress damage (Fig. 7A, D and G). In contrast, the chloroplasts became elongated ellipses and clung on the wall of cells that contained well-arranged thylakoid along with numerous starch grains and less osmiophilic granules under well watered and mildly water stressed treatments (Fig. 7B–C, E–F and H–I). These results indicated that the plants under the moderately water stressed treatment had developed certain drought resistance. In addition, the number of chloroplasts and starch grains decreased, while the number of osmiophilic granules increased in BSC under the moderately water stressed treatment. The increase in the number and volume of osmiophilic granules under water stress was caused by the degradation of chloroplast thylakoids and the accumulation of lipids in the degraded products, implying that the chloroplasts were seriously damaged. Simultaneously, the thylakoid membranes became loose, and the thylakoids were disorganized. The increase in the number of osmiophilic granules further increased cytoplasmic concentration and decreased osmotic potential (Zhang et al., 2015). It is known that chloroplasts in BSC of  $C_4$  plants often contain a lot of starch grains. The number of starch grains decreased under the moderately water stressed treatment, as the water stress promoted the degradation of starch grains to synthesize more organic solutes to regulate osmotic pressure, and thus reducing water loss.

Respiration, as the second most important physiological basis, is central to survival and productivity of plants. The previous study indicated that the limitation of plant growth imposed by water stress is mainly due to reductions in plant carbon gain, which is dependent on the balance between photosynthesis and respiration defined as  $R_d/A_n$  (Flexas et al., 2006). The current study found that  $R_d$  was significantly decreased under the moderately water stressed treatment across the N treatments, which is contrary to some of the previous results where  $R_d$  was found to be insensitive to water deficit (Gimeno et al., 2010), as water stress could cause a significant shift of electrons from the cytochrome to the alternative pathway, while the total respiratory oxygen uptake was not significantly different at any water stress levels (Ribascarbo et al., 2005). Inhibition of respiration under water stress has been observed previously and interpreted as decreased availability of substrates to mitochondria because of low photosynthesis (Ribascarbo et al., 2005). In addition, respiration is also affected by the damage of mitochondria under the moderately water stressed treatment. With increasing severity of water deficit especially under the moderately water stressed treatment, the number of mitochondria in BSC decreased, and furthermore, the mitochondrial membrane became ruptured, and cristae was broken, disintegrated and disappeared, resulting in loss of normal cell function. Consequently, the mitochondrial respiratory metabolism was severely impaired.

Contrary to the decreased  $A_n$  and  $R_d$ , the  $R_d/A_n$  increased significantly under the moderately water stressed treatment, indicating that the carbon balance of maize was significantly modified by water deficit. Under this circumstance, the carbon gain was seriously compromised, eventually leading to carbon starvation, and thus inhibiting C accumulation as well as the growth of plants (Flexas et al., 2006; Valladares and Sánchez-Gómez, 2006). One of the reasons for this is that the most sensitive organelles affected by stress were chloroplasts, followed by mitochondria, i.e. mitochondria were more tolerant to water stress than chloroplasts and the change of mitochondrial structure was slightly slower than that of chloroplasts. Therefore, to a certain extent, the asynchronous response of organelles to abiotic stresses ensures the adaptability of cells, i.e., the damage of chloroplasts increases the cytoplasmic concentration and reduces the osmotic potential by disintegration of thylakoids and degradation of starch grains, so that the cells retain water, and then other organelles including mitochondria work



normally under water stress. However, this process relied on the severity of water stress. There was a significant negative linear correlation between  $R_d/A_n$  and plant C accumulation, implying that during the grain filling stage, even moderately soil water regime significantly decreased plant C accumulation, whereas the plant carbon gain was more than carbon consumption under sufficient or mildly water stressed treatments compared to moderately water stressed treatment. This resulted in the significantly higher above-ground biomass accumulation in these treatments (Table 4).

#### 4.2. Increasing N dose aggravated water stress and reduced stomatal conductance and carbon gain, leading to decreased biomass accumulation and $WUE_p$ under moderate water deficit

Multiple studies have indicated that N fertilization can improve WUE and increase drought tolerance (Song et al., 2019). However, in the present study, it was found that increasing N dose aggravated severity of water deficit especially under the moderately water stressed treatment. It is noteworthy that under the mildly and moderately water stressed treatment, compared with low N supply, the  $[ABA]_{leaf}$  was further reinforced with increasing N dose. The  $A_n$ ,  $G_s$  and  $WUE_i$  decreased significantly under high N supply, while the  $R_d/A_n$  increased considerably under the high N compared with the low and medium N treatments due to the significant reduction in  $A_n$  under the moderate water stress (Figs. 4 and 6). Consequently, plant growth and  $WUE_p$  in the moderately water stressed treatment also decreased markedly under the high N supply (Table 4, Fig. 1). The possible mechanisms for the interaction of water stress and N application levels are discussed below.

The changes of  $G_s$  in low N-grown plants in previous studies were variable, including decreased (Broadley et al., 2000), increased or no effect (Grassi et al., 2002). ABA as a key phytohormone in stress signal transduction plays an important role in regulating plant water balance and inducing stress tolerance (Lu et al., 2013). Previous studies have shown that ABA may correlate with plant N nutrition (Wang et al., 2010). In the present study, the analysis across the water treatments showed that the increase of N dose significantly increased  $[ABA]_{leaf}$  (Fig. 3). Increasing N dose can alter N partitioning and metabolism within the leaves and increase the amount of active ABA, resulting in elevated ABA concentration in the leaves (Song et al., 2019). Furthermore, studies have shown that stress-induced ABA accumulation and stomatal sensitivity to ABA were independently affected (John et al., 1982). The stomatal sensitivity to ABA can be modulated by N nutrition (Pinheiro and Chaves, 2011). Both the accumulation of ABA and apparent stomatal sensitivity to ABA could increase with the increasing N dose particularly under water stress. In addition, increasing N supply was favorable to maintain the activities of nitrate reductase ( $NR_{ase}$ ) and NR-mediated NO synthesis, which was required for ABA-induced stomatal closure (Desikan et al., 2002). Therefore, under water stress treatments, increasing N dose not only further increased  $[ABA]_{leaf}$  concentration, but also could have increased stomatal sensitivity to ABA by increasing NO synthesis. The N signaling genes regulating ABA and drought-responsive gene expression could also be involved in the crosstalk between N supply and water stress (Araus et al., 2020). In addition, the application of N fertilizer across the water treatments significantly decreased the root water potential (Fig. 2), as N can serve as an osmolyte, and thus the nitrogen applied into the soil decreased soil osmotic potential, and the root water potential had to decrease in order to absorb water from the soil normally, which aggravated the plant water status. Consequently, the stomata became partially closed and the  $G_s$  decreased significantly to reduce transpirational water loss (Fig. 4). The leaf area was also decreased with the increase of N application rate (Fig. 1). Both the root-to-shoot ABA and hydraulic signals could contribute to the significantly decreased  $G_s$  with the increase of N application rate particularly under water stress (Fig. 4). In consequence, the  $A_n$  decreased significantly in the high N treatment under moderate water stress (Fig. 4).

The  $R_d/A_n$  is indicative of leaf carbon balance. In the present study,  $R_d$  was not affected by increasing N levels under each soil water treatment. Nevertheless, the  $R_d/A_n$  increased significantly when N supply increased under the moderately water stressed treatment due to the significantly decreased  $A_n$  with increasing N supply (Fig. 4), indicating that plants assimilated lower  $CO_2$  at a more respiratory cost, thus the C consumption was higher than C uptake with increasing N supply under the moderately water stressed treatment. Therefore, plant C accumulation was significantly reduced in the moderately water stressed treatment, which were not observed in the well watered and mildly water stressed treatments (Fig. 9).

In addition, increasing N dose under the moderately water stressed treatment severely damaged the structure of mitochondria (Fig. 8A, D and G). High N supply also showed decreased number of mitochondria and starch grains (Table 3). It is known that chloroplasts in BSC of  $C_4$  plants contain a lot of starch grains for leaf growth stored as energy for plant growth at normal growth conditions, and the decrease of starch grains is related to the decrease of chloroplast photosynthetic capacity and other metabolisms reflecting the plant growth may be degraded. In consequence, the plant growth and biomass accumulation were significantly decreased with increasing N dose particularly under the moderately water stressed treatment.

## 5. Conclusions

The moderate water stress resulted in decreased photosynthetic rate by depressed photosynthetic capacity and activity, and the moderate water stress also induced damaged structure of leaves, resulting in lower  $WUE_i$  and biomass compared with well watered and mildly water stressed treatments. The  $R_d/A_n$  increased significantly under the moderately water stressed treatment, and the carbon gain was seriously compromised, eventually inhibiting the C accumulation as well as the growth of plants.

Increasing N dose aggravated severity of water deficit, decreased  $A_n$ ,  $G_s$  and  $WUE_i$  under the moderately water stressed treatment due to the intensified ABA and hydraulic signals, while increased  $R_d/A_n$  considerably. Increasing N dose severely damaged the structure of mitochondria and reduced the number of mitochondria. In consequence, plant growth and  $WUE_p$  in the moderately water stressed treatment also decreased markedly under the high N supply. Therefore, in maize production decline in N fertilization is suggested during the period when plants suffer moderate soil water deficit.

### Author contributions

Y.W. and W.Z. conceived and designed the study. H.X., C.W. and L.L. performed the experiment, the sample measurements and analyses. H.X. wrote the draft of the manuscript. Y.W., W.Z., X.L., N.C., W.H. and F.L. contributed to analyzing the data and improving the manuscript.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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