

ASSOCIATION OF THE EFFECTS OF ELEVATED TEMPERATURE AND CO₂ ON PLANT GROWTH AND LEAF PHOTOSYNTHESIS WITH CHANGES IN STOMATAL TRAITS, LEAF ANATOMY, AND FOLIAR CARBOHYDRATES IN WINTER WHEAT (*TRITICUM AESTIVUM* L.)

ZHIJIE CHANG¹, LIHUA HAO^{1*}, YI HAN¹, XIAODONG FAN², YUNXIN ZHANG¹, YINSHUAI TIAN³,
LIANG LIU¹, WENNA CHEN³, ZHIPENG WANG¹, WEI SHI¹, JIAWEI YIN³,
YUANYUAN LIU¹ AND YUNPU ZHENG^{1*}

¹School of Water Conservancy and Hydropower, Hebei University of Engineering, Handan, 056038, China;

²School of Water Resources and Architectural Engineering, Northwest Agricultural and Forest University, Yangling 712100, China

³School of Landscape and Ecological Engineering, Hebei University of Engineering, Handan 056038, China.

*Corresponding authors' emails: haolihua_000@sina.com; zhengyunpu_000@sina.com

Abstract

We examined the effects of elevated CO₂ concentration ($e[\text{CO}_2]$), experimental warming (warming) and the two combined environmental factors ($e[\text{CO}_2] \times \text{warming}$) on plant biomass, stomatal characteristics, gas exchange, leaf anatomy, as well as the Rubisco activity and gene expression of winter wheat with environmental growth chambers, where the temperature was set at 21/16°C (day/night) or 26/21°C and the $[\text{CO}_2]$ was controlled at 400 $\mu\text{mol mol}^{-1}$ or 800 $\mu\text{mol mol}^{-1}$, respectively. We found that the plant biomass and leaf photosynthesis were dramatically decreased by $e[\text{CO}_2] \times \text{warming}$ (all $p < 0.05$), although elevated temperature and $[\text{CO}_2]$ were not significantly altered biomass accumulation and leaf photosynthesis (all $p > 0.05$). This down-regulated net photosynthetic rates (P_n) at $e[\text{CO}_2] \times \text{warming}$ partially attributed to the limited effect of CO₂ diffusion processes relating to the declined regularity of stomata distribution, as evidenced by the decrease of stomatal conductance (G_s) at $e[\text{CO}_2] \times \text{warming}$. Additionally, accumulation of nonstructural carbohydrates in leaves of winter wheat can also contribute to the declines of plant biomass and P_n at $e[\text{CO}_2] \times \text{warming}$. These results indicate that the fertilization effect from CO₂ enrichment can be lowered by experimental warming, and thus the potential risks of global change on the net primary productivity of agricultural ecosystems may be underestimated by current process-based ecological models.

Key words: Enhanced $[\text{CO}_2]$; Experimental Warming; Stomata; Photosynthesis; NSC.

Introduction

The carbon dioxide concentration ($[\text{CO}_2]$) in the atmosphere has been drastically increased from about 300 $\mu\text{mol mol}^{-1}$ to 400 $\mu\text{mol mol}^{-1}$ mainly due to fossil fuels burning since the industrial revolution (Sunil *et al.*, 2012), even the global atmospheric $[\text{CO}_2]$ will be higher than 800 $\mu\text{mol mol}^{-1}$ (Anon., 2013). Meanwhile, elevated atmospheric $[\text{CO}_2]$ has already induced climate warming over the past decades, and the earth surface temperature will continually be increased by approximately 5 °C over this century depending on emissions of greenhouse gases (Rasul *et al.*, 2012; Abbas *et al.*, 2017). This elevated atmospheric $[\text{CO}_2]$ and surface temperature may not only dramatically generate global climate change, but also profoundly impact agricultural production all over the world (Cesare *et al.*, 2001; Liu *et al.*, 2020; Xu, 2015; Yu *et al.*, 2012a).

Previous studies have reported that climate warming could affect plant physiological and molecular processes such as photosynthesis (Zong & Shangguan, 2014; Liu *et al.*, 2018), Rubisco content and activity (Hessini *et al.*, 2009) as well as the relationships between soil water and plants (Bencze *et al.*, 2014). It has been estimated that the average global temperature increases every 1°C in the future, the global agriculture yield will be decreased by about 6% (Lipeč *et al.*, 2013). Meanwhile, CO₂ is one of the critical reactants needed by leaf photosynthesis (Ciais *et al.*, 2005; Zheng *et al.*, 2017). Several studies have found that $[\text{CO}_2]$ enhancements may accelerate plant growth (Wu *et al.*, 2017), and increase crop grain yield

(Katny *et al.*, 2005; Wang & Taub, 2010; Yu *et al.*, 2012b) with declining leaf respiration (Arndal *et al.*, 2014;) and meanwhile enhancing the activity and efficiency of Rubisco (Yu *et al.*, 2012a). Therefore, estimating the effects of elevated temperature and $[\text{CO}_2]$ on winter wheat is critical to predicting the impacts of global change on the productivity of agriculture in the near future.

Wheat is one of the most important crops, which plays a pivotal role in supporting food for human beings throughout the world (Tao *et al.*, 2006; Rosegrant & Agcaoili, 2010; Braun *et al.*, 2010). However, previous work has projected that global climate has become warmer in recent years (Fu *et al.*, 2009), and thus climate warming may not only directly affect the growth and development of winter wheat by changing physiological and biochemical processes (Dai *et al.*, 2011), but also indirectly impact grain yield of winter wheat with changing crop phenology and growth stages (Farooq *et al.*, 2011; Bergkamp *et al.*, 2018). In contrast to experimental warming, elevated $[\text{CO}_2]$ can increase leaf photosynthesis and boost plant growth of C₃ species (Long *et al.*, 2006), which can benefit more from CO₂ enrichment and meanwhile suffer less from climate warming under future global change (Kang *et al.*, 2002; Bencze *et al.*, 2014). Therefore, elevated temperature and $[\text{CO}_2]$ may have combined effects on plant growth, water use efficiency, and crop grain yield (Taub *et al.*, 2008; Högy *et al.*, 2009; Nebauer *et al.*, 2011) with modifying leaf photosynthesis (Bencze *et al.*, 2014), dark respiration (Aranjuelo *et al.*, 2011) and transpiration rates (Wei *et al.*, 2018). Nevertheless, most of previous studies investigated

plant growth and leaf photosynthesis in response to single environmental factor such as elevated $[\text{CO}_2]$ or experimental warming (Yu *et al.*, 2012a) and the mechanisms and processes of experimental warming modulating CO_2 fertilization effect on plants/crops are still unclear (Zheng *et al.*, 2020).

This study aims to: (1) investigate the warming effect on stomatal traits, leaf anatomy, and total nonstructural carbohydrates of winter wheat grown at higher $[\text{CO}_2]$; (2) examine the interactive effect of experimental warming and elevated $[\text{CO}_2]$ on plant biomass and net photosynthetic rates; (3) explore the pivotal processes and underline mechanisms governing the physiological response to experimental warming, elevated $[\text{CO}_2]$ and their combined effect associated with leaf anatomy, stomatal traits, and foliar carbohydrates of winter wheat.

Materials and Methods

Experimental design: We employed four environmental growth chambers to sustain plant growth of winter wheat. The environmental growth chambers used in this experiment is independently equipped with a set of automatic $[\text{CO}_2]$ control system, which can accurately control the $[\text{CO}_2]$ in each environmental growth chamber. We randomly selected two growth chambers supplying with the $[\text{CO}_2]$ of $400 \mu\text{mol mol}^{-1}$, and the other two chambers were controlled to $800 \mu\text{mol mol}^{-1}$. Meanwhile, we randomly set up two temperatures of 21/16°C (day/night) and 26/21°C (day/night) for the four chambers, namely this study performed four different treatments in the four environmental growth chambers with Control (Temperature is 21/16°C and CO_2 concentration is $400 \mu\text{mol mol}^{-1}$), experimental warming (Temperature is 26/21°C and CO_2 concentration is $400 \mu\text{mol mol}^{-1}$), elevated $[\text{CO}_2]$ (Temperature is 21/16°C and CO_2 concentration is $800 \mu\text{mol mol}^{-1}$), and $e[\text{CO}_2] \times$ warming (Temperature is 26/21°C and CO_2 concentration is $800 \mu\text{mol mol}^{-1}$). The seeds of winter wheat were grown in pots with a diameter of 20 cm and a height of 60 cm (six plants in each pot). Then, four pots were placed into each of the four chambers, and thus totally 16 pots in the current study ($n=4$). The relative humidity and light intensity in the growth chambers was 60%-70%, and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR respectively, and the photoperiod was 12 hours from 8:00 to 20:00. In addition, we also watered winter wheat plants with 400 mL water three times weekly and fertilized with Hoagland's solution once for each week during the plant growth of winter wheat.

Leaf gas exchange measurement: After the 90-day treatment, new fully expanded winter wheat leaves were randomly selected for measuring gas exchange with a portable photosynthesis measurement system (LI-6400XT; LI-COR, Inc.) equipped with a cuvette chamber, where the environmental factors were strictly controlled as the $[\text{CO}_2]$ of $400 \mu\text{mol mol}^{-1}$, the saturated light of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the temperature of 21°C during the leaf gas exchange measurements. In this study, we performed leaf gas exchange measurements on four pots in each growth chamber ($n=4$).

Stomatal traits measurements: We sampled stomatal imprints on both leaf surfaces with colorless nail varnish. Then, we observed and photographed these stomatal imprints with a microscope. We counted the number of stomata to calculate the stomatal density of winter wheat. To estimate the effects of temperature and $[\text{CO}_2]$ on the stomatal morphology of winter wheat, six digital photographs were randomly selected for measuring stomatal width, stomatal length, stomatal area, and stomatal perimeter with the Auto CAD software. In addition, we selected three photographs for estimating the distribution pattern of stomata. We firstly determined the position of each stoma on winter wheat leaves and then these positions were transferred to spatial points with the ArcGIS software. Then, we employed Ripley's K -function to analyze the spatial pattern of these points distributed on winter wheat leaves (Xu, 2015).

Leaf area and plant biomass measurements: Winter wheat plants were destructively removed for analyzing leaf area and plant biomass after the 90-day treatments. The plant leaves in each pot were collected and determined by a leaf area meter. Then the leaves, stems, and roots of winter wheat from each pot were put into paper bags and dried to constant weight at the temperature of 80°C more than 48 h. The dried plant tissue was weighted by an electronic balance ($n=4$).

Biochemical composition and molecular analysis: According to the methods of Hedrix *et al.*, (1994), we spectrophotometrically analyzed the total nonstructural carbohydrates (glucose, fructose, sucrose, and starch) in winter wheat leaves with a glucose kit (GAHK-20, Sigma, St Louis, MO, USA). Furthermore, total leaf nitrogen (N) and carbon (C) were also analyzed using an elemental analyzer (Elementar Corp., Germany).

We analyzed Rubisco activity of winter wheat according to the method of Jiang *et al.*, (2012). Moreover, we used the actin of winter wheat as the internal standard gene, and then calculated the relative expression level of *RbcL3* and *RbcS2* using real-time quantitative PCR based on the method of Livak *et al.*, (2002). Specifically, the total RNA was extracted with RNA purification kits (Shanghai Shenggong Bioengineering Technology Service Co., Ltd.). According to the sequences of the Gene bank database, we designed the primers of *RbcL3*, *RbcS2* and actin with Primer Premier 5.0 for amplification. *RbcL3* 5'-TAAATCACAGGCCGAAAC-3', 5'-GGCAATAATGAGCCAAAGT-3'; *RbcS2* 5'-AGCAACGGCGGAAGGAT-3', 5'-GCTCACGGAAGACGAAACC-3'; actin 5'-CTCCCTCACAACAACCGC-3', 5'-CATTACCACAGGAAGT-3'. Subsequently, the expression levels of Rubisco genes were determined by a fluorescent quantitative PCR equipment (ABI, Waltham, MA, USA). The 20 μL reaction system contained 10 μL of SybrGreen qPCR Master Mix, 1 μL of forward and reverse primers, 1 μL of template (cDNA sample diluted 8 times), and 7 μL of ddH₂O. Denaturation at 95°C for 3 min; 95°C for 10 s; 60°C for 40 s, 40 cycles.

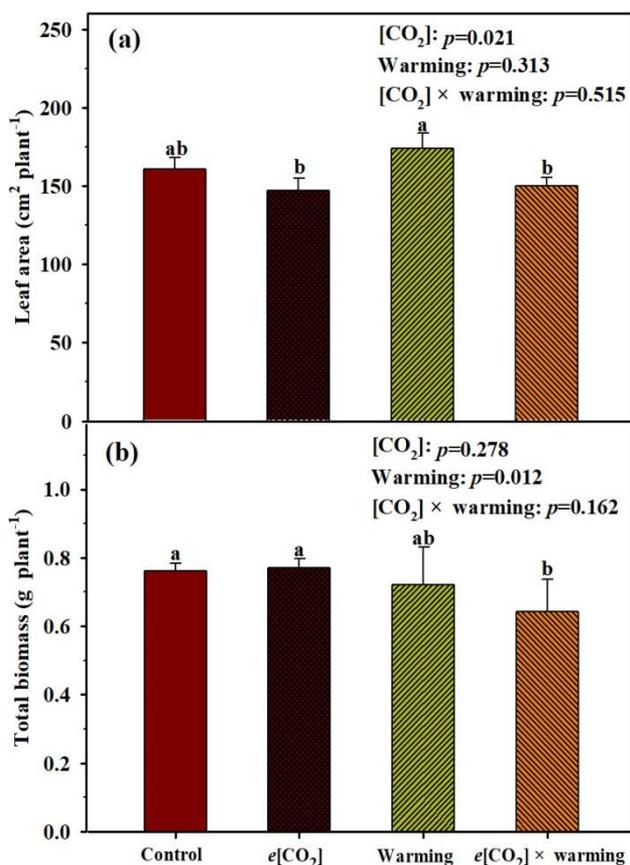


Fig. 1. Effects of elevated temperature and [CO₂] on the leaf area and plant biomass.

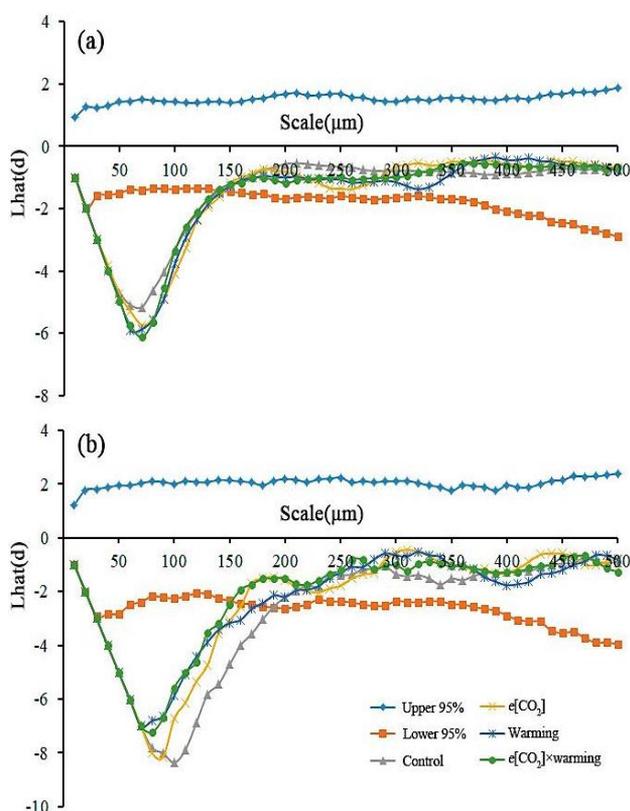


Fig. 2. Effects of elevated temperature and [CO₂] on the spatial distribution pattern of stomata on the adaxial (a) and abaxial (b) surfaces.

Measuring leaf anatomical structures: Variations in the internal leaf anatomy of winter wheat plants were examined under light microscopy using leaf cross-sections. Twenty well-defined cells of the palisade layer and 20 cells of the spongy layer were randomly selected from each leaf cross-sectional image. Cell length, cell width, cell area, and cell perimeter were measured using Auto CAD software.

Statistical analysis

We compared the differences in stomatal traits, biomass, gas exchange parameters, and biochemical composition from temperature or [CO₂] with one-way ANOVA followed by Duncan's multiple range tests ($p < 0.05$). Moreover, we also tested the interactions between temperature and [CO₂] on winter wheat using two-way ANOVA followed by Duncan's multiple range tests at $p < 0.05$ level.

Results

Effects of experimental warming and elevated [CO₂] on leaf area and plant biomass: Our results showed that the leaf area of winter wheat under experimental warming is slightly higher than that of plants grown at the Control (*c.* 8%, $p > 0.05$), whereas elevated [CO₂] and e[CO₂] × warming substantially reduced the leaf area of winter wheat by 15.5% ($p = 0.01$) and 13.9% ($p = 0.02$) compared with that of wheat plants exposed to experimental warming (Fig. 1a). Meanwhile, we did not find significant differences in leaf area among the Control and the three treatments (all $p > 0.05$; Fig. 1a). Similarly, we found that e[CO₂] × warming significantly decreased the total biomass by 15.6% ($p = 0.013$), but the total plant biomass was not statistically affected by e[CO₂] or experimental warming (all $p > 0.05$; Fig. 1b). In addition, [CO₂] changed the leaf area of winter wheat ($p = 0.021$), while leaf area was not affected by experimental warming ($p = 0.313$) or [CO₂] × warming ($p = 0.515$; Fig. 1a). By contrast, the total plant biomass of winter wheat was substantially affected by experimental warming ($p = 0.012$), but total biomass of winter wheat was not obviously changed by [CO₂] or [CO₂] × warming (all $p > 0.05$; Fig. 1b).

Effects of experimental warming and elevated [CO₂] on the morphological traits and distribution of stomata: The stomatal density (SD) on wheat leaves was statistically different on both the adaxial surface ($p < 0.01$) and abaxial surface ($p = 0.003$) among the Control and the three treatments (Table 1). The SD of winter wheat was significantly enhanced by *c.* 10% ($p = 0.048$) on adaxial surface and *c.* 35% ($p = 0.013$) on the abaxial leaf surfaces under experimental warming than that of Control (Table 1). The SD under higher [CO₂] was significantly increased by 27.5% ($p < 0.001$) from 51 stomata per mm² to 65 stomata per mm² on adaxial surface and by 37.9% ($p = 0.008$) from 29 stomata per mm² to 40 stomata per mm² on the abaxial leaf surface (Table 1). Similarly, the SD of winter wheat treated with e[CO₂] × warming was also featured much more stomata with 17.6% ($p < 0.001$) on the adaxial surface and 55.2% ($p < 0.001$) than Control (Table 1). Meanwhile, the SD

was significantly affected by experimental warming ($p=0.02$), $[\text{CO}_2]$ ($p<0.001$), and warming \times leaf surface ($p=0.025$) as well as leaf surface ($p<0.001$; Table 2). Moreover, we only found significant differences in stomatal length on abaxial surface ($p=0.047$) and stomatal width on adaxial surface ($p<0.001$) among the Control and the three treatments (Table 1). As a result, the stomatal area on adaxial surface was significantly changed by the three treatments of elevated $[\text{CO}_2]$, experimental warming, and $e[\text{CO}_2] \times$ warming ($p<0.001$), although the stomatal perimeter on both leaf surfaces was not changed by the three treatments (all $p>0.05$; Table 1). Specifically, experimental warming dramatically enhanced the stomatal area on the adaxial surface due mainly to the significantly increased stomatal width (Table 1), while enriched $[\text{CO}_2]$ obviously reduced stomatal openness on adaxial surface, which resulted from the decreased stomatal width ($p=0.021$) and stomatal area ($p=0.012$; Table 1). Interestingly, elevated $[\text{CO}_2]$ barely affected stomatal area on both surfaces under warming conditions (Table 1). Moreover, we also found significant differences in stomatal shape index on the adaxial surface among Control and the three treatments, but the three

treatments not altered the stomatal shape index of abaxial surface (Table 1). Furthermore, $[\text{CO}_2]$ and leaf surface interactively affected the stomatal area ($p=0.039$) and stomatal shape index ($p=0.016$; Table 2). Meanwhile, interactive effects of experimental warming and leaf surface on the stomatal width ($p=0.016$) and stomatal area ($p=0.026$; Table 2) were also detected in this study. Additionally, stomatal length and stomatal perimeter were also significantly affected by $[\text{CO}_2] \times$ warming \times leaf surface.

Our results from spatial pattern analysis also showed that stomatal distribution on winter wheat leaves was highly depending on scales with regular patterns on adaxial surface (spatial scales $<120 \mu\text{m}$) and abaxial surface (spatial scales $<190 \mu\text{m}$). The most regular patterns were appeared at $60 \mu\text{m}$ on adaxial surface and $100 \mu\text{m}$ on abaxial surface, respectively (Fig. 2). Additionally, the three treatments made the distribution of stomata on adaxial surface more regular than that of Control (Fig. 2a), whereas the stomatal distribution regularity on adaxial surface was reduced by experimental warming, elevated $[\text{CO}_2]$, or $e[\text{CO}_2] \times$ warming, due to the higher minimum $L_{\text{hat}}(d)$ values under the three treatments than that of the Control (Fig. 2b).

Table 1. Effect of elevated temperature and $[\text{CO}_2]$ on stomatal morphology.

Stomatal morphology	Leaf surfaces	Control	$e[\text{CO}_2]$	Warming	$e[\text{CO}_2] \times$ warming	<i>p</i> -values
Stomatal density (Number mm^{-2})	adaxial	51 \pm 5c	65 \pm 9a	56 \pm 6b	60 \pm 8a	<0.001
	abaxial	29 \pm 4c	40 \pm 8ab	39 \pm 9b	45 \pm 10a	0.003
Stomatal length (μm)	adaxial	40 \pm 4ab	38 \pm 2b	41 \pm 3a	39 \pm 4ab	0.167
	abaxial	37 \pm 4ab	39 \pm 6a	39 \pm 2a	35 \pm 2b	0.047
Stomatal width (μm)	adaxial	4.1 \pm 0.7b	3.6 \pm 0.5c	5.1 \pm 0.5a	4.3 \pm 0.4b	<0.001
	abaxial	4.0 \pm 1.3a	4.1 \pm 1.0a	4.4 \pm 0.3a	3.9 \pm 0.2a	0.527
Stomatal perimeter (μm)	adaxial	85 \pm 9ab	79 \pm 4b	87 \pm 7a	82 \pm 9ab	0.057
	abaxial	78 \pm 8ab	83 \pm 14a	83 \pm 4a	73 \pm 4b	0.064
Stomatal area (μm^2)	adaxial	167 \pm 54b	130 \pm 21c	211 \pm 29a	163 \pm 22b	<0.001
	abaxial	146 \pm 67a	171 \pm 68a	174 \pm 17a	139 \pm 16a	0.267
Stomatal shape index (%)	adaxial	0.15 \pm 0.01bc	0.14 \pm 0.01c	0.17 \pm 0.01a	0.16 \pm 0.01b	<0.001
	abaxial	0.15 \pm 0.02a	0.15 \pm 0.01a	0.16 \pm 0.01a	0.16 \pm 0.01a	0.170

Notes: Different letters indicate significantly different at $p<0.05$ level among treatments.

Table 2. Interactive effect of elevated temperature and $[\text{CO}_2]$ on stomatal morphology.

	Stomatal density	Stomatal length	Stomatal width	Stomatal perimeter	Stomatal area	Stomatal shape index
$[\text{CO}_2]$	<0.001	0.054	0.006	0.035	0.013	0.163
Warming	0.020	0.708	0.006	0.816	0.064	<0.001
Leaf surface	<0.001	0.015	0.270	0.030	0.311	0.459
$[\text{CO}_2] \times$ warming	0.025	0.060	0.100	0.057	0.052	0.373
$[\text{CO}_2] \times$ leaf surface	0.870	0.473	0.081	0.346	0.039	0.016
Warming \times leaf surface	0.025	0.255	0.016	0.169	0.026	0.114
$[\text{CO}_2] \times$ warming \times leaf surface	0.471	0.019	0.499	0.028	0.162	0.945

Table 3. Interactive effect of experimental warming and elevated $[\text{CO}_2]$ on gas exchange.

	P_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	T_r ($\text{mmol m}^{-2} \text{s}^{-1}$)	G_s ($\text{mol m}^{-2} \text{s}^{-1}$)	<i>WUE</i> (mmol mol^{-1})	C_i ($\mu\text{mol mol}^{-1}$)	R_d ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
$[\text{CO}_2]$	0.173	<0.001	<0.001	<0.001	0.008	0.054
Warming	0.135	0.091	<0.001	0.231	0.666	0.411
$[\text{CO}_2] \times$ warming	0.020	0.299	<0.001	0.724	0.019	0.037

Table 4. Interactive effect of experimental warming and elevated $[\text{CO}_2]$ on leaf nonstructural carbohydrates.

	Fructose	Sucrose	Glucose	Soluble sugar	Starch	TNC
$[\text{CO}_2]$	<0.001	0.003	0.031	0.025	<0.001	0.676
Warming	0.022	0.004	0.049	<0.001	0.707	0.001
$[\text{CO}_2] \times$ warming	0.066	0.047	0.602	0.102	0.217	0.335

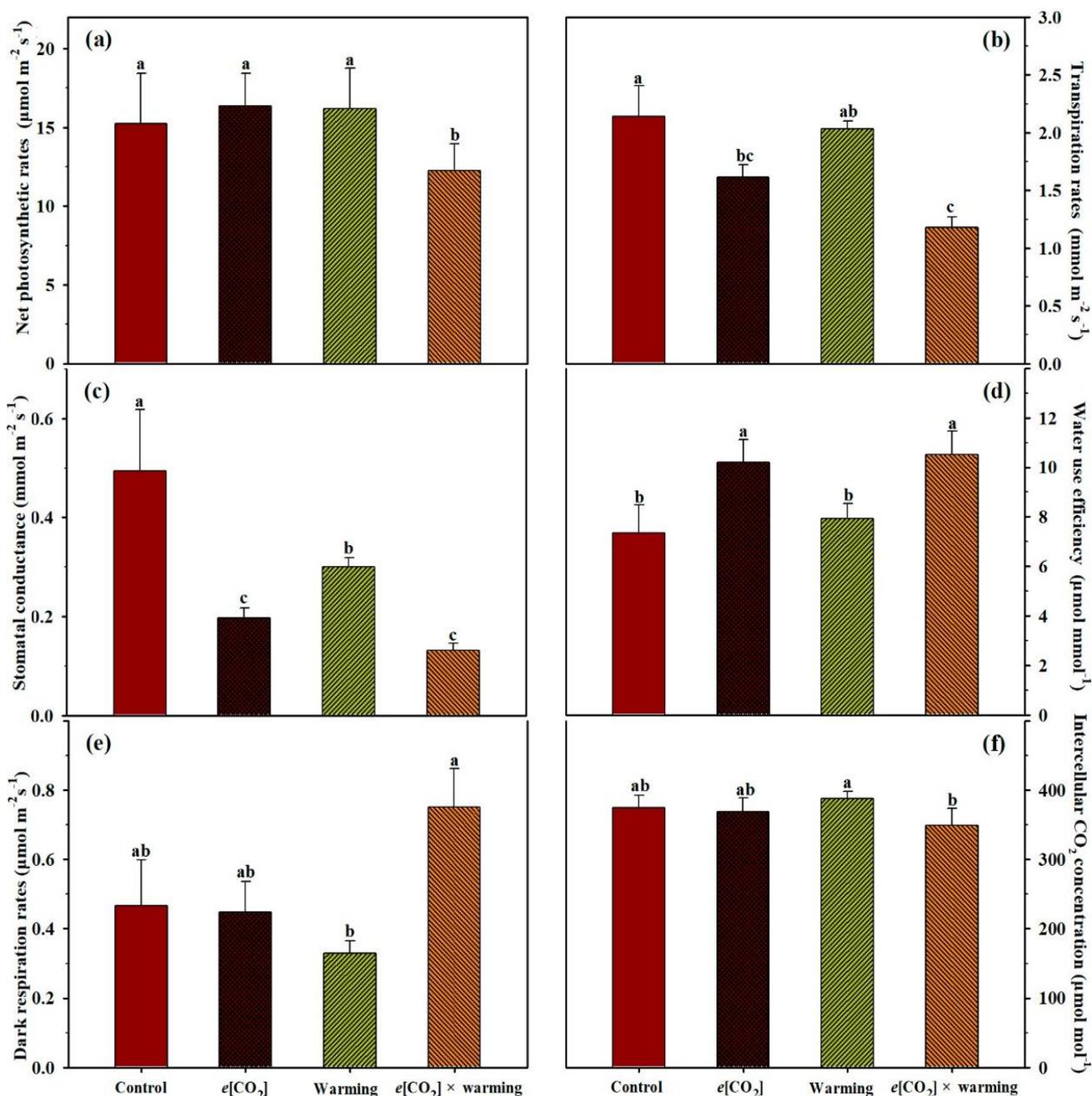


Fig. 3. Effects of elevated temperature and [CO₂] on leaf gas exchange.

Effects of experimental warming and elevated [CO₂] on leaf gas exchange: The net photosynthesis rate (P_n) was significantly decreased by *c.* 20% under the $e[\text{CO}_2]$ × warming conditions compared with the Control ($p=0.049$), although P_n was barely affected by single $e[\text{CO}_2]$ or experimental warming ($p>0.05$; Fig. 3a). Meanwhile, the transpiration rate (T_r) of winter wheat was also substantially reduced by *c.* 25% ($p=0.025$) and 45% ($p<0.001$) under the elevated [CO₂] and $e[\text{CO}_2]$ × warming, respectively (Fig. 3b). Compared with the Control group, the treatments of elevated [CO₂], experimental warming, and $e[\text{CO}_2]$ × warming significantly decreased stomatal conductance (G_s) by *c.* 60%, 40% and 70% (all $p<0.001$; Fig. 3c). When plants exposed to elevated [CO₂] and $e[\text{CO}_2]$ × warming,

water use efficiency (WUE) was substantially increased by *c.* 39% ($p<0.001$) and 40% ($p<0.001$; Fig. 3d). Moreover, both leaf dark respiration (R_d) and intercellular [CO₂] (C_i) of winter wheat were barely changed by elevated [CO₂] and experimental warming (all $p>0.05$), while significant differences on both R_d and C_i were detected between the experimental warming and $e[\text{CO}_2]$ × warming treatments (all $p<0.05$, Fig. 3e and 3f). Additionally, two-way ANOVA indicated elevated [CO₂] dramatically changed T_r , G_s , and WUE (all $p<0.001$), while experimental warming only altered the G_s ($p<0.001$; Table 3). Additionally, elevated [CO₂] and experimental warming interactively affected P_n ($p=0.020$), G_s ($p<0.001$), C_i ($p=0.019$), and R_d ($p=0.037$).

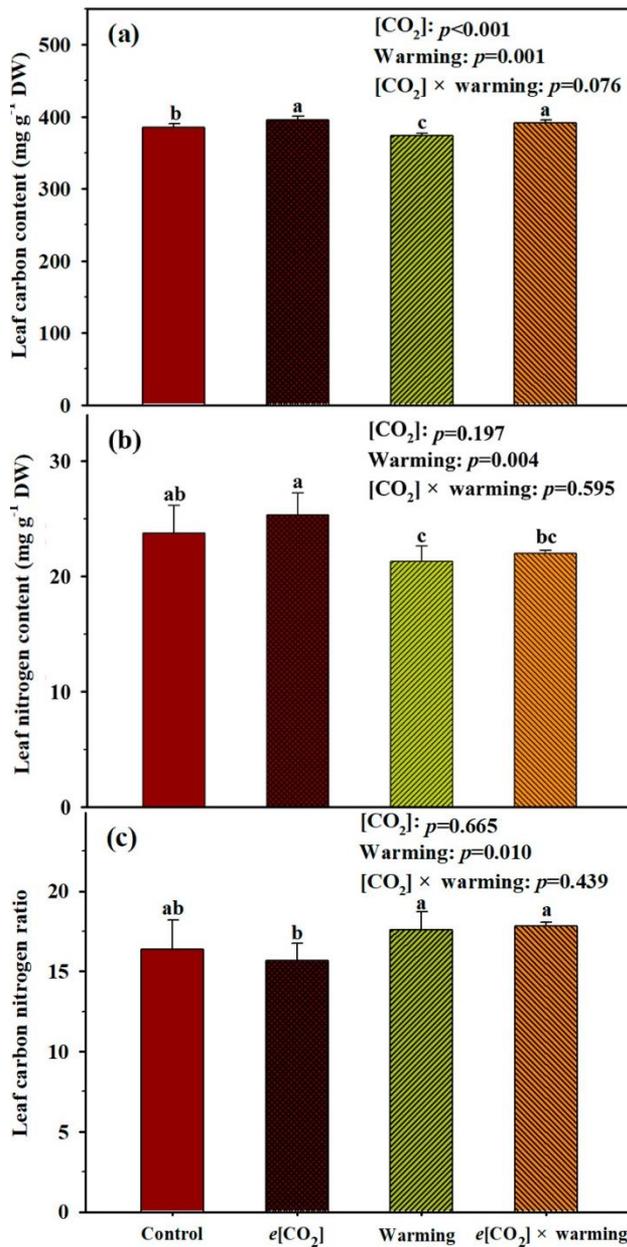


Fig. 4. Effect of elevated temperature and [CO₂] on leaf carbon (C) and nitrogen (N) as well as C/N ratio.

Effects of experimental warming and elevated [CO₂] on leaf carbon and nitrogen as well as nonstructural carbohydrates: Leaf C and N were significantly decreased 2.9% ($p = 0.001$) and 10.3% ($p = 0.049$) by experimental warming, whereas elevated [CO₂] and e[CO₂] × warming substantially increased the C by 2.7% ($p = 0.003$) and 1.6% ($p = 0.044$; Fig. 4a). In contrast to leaf C and N, leaf carbon nitrogen ratio (C/N) were barely changed by experimental warming, elevated [CO₂], and e[CO₂] × warming ($p > 0.05$; Fig. 4b and 4c), although we found significant differences in leaf N and C/N of winter wheat among the three treatments (all $p < 0.05$; Figs. 4b and 4c).

In addition to leaf C and N, the leaf nonstructural carbohydrates were also affected by experimental warming, elevated [CO₂], and e[CO₂] × warming (Fig. 5). Elevated [CO₂] obviously decreased the fructose content by *c.* 63% ($p < 0.001$; Fig. 5a), and thus reduced the soluble

sugar content by *c.* 37% ($p < 0.001$; Fig. 5e). However, elevated [CO₂] significantly increased the starch content by *c.* 39% ($p = 0.010$; Fig. 5d). Moreover, experimental warming dramatically decreased the total nonstructural carbohydrates (TNC) by *c.* 19% ($p = 0.033$; Fig. 5f), mainly due to the *c.* 32% decline in starch content ($p = 0.029$; Fig. 5d), because the soluble sugar content was not changed under experimental warming conditions (Fig. 5e). Similarly, the TNC was also substantially decreased by *c.* 27% ($p = 0.005$; Fig. 5f) under the e[CO₂] × warming conditions, which resulted from the declines in soluble sugar of *c.* 29% ($p = 0.001$; Fig. 5e). This declined soluble sugar was mainly attributed to the significantly decreased sucrose content of *c.* 54% ($p < 0.001$) at the treatment of e[CO₂] × warming (Fig. 5b). Additionally, enhanced [CO₂] significantly changed fructose, sucrose, glucose, soluble sugar (all $p < 0.05$), and starch ($p < 0.001$; Table 4). Meanwhile, experimental warming marginally affected the contents of fructose, glucose, sucrose, soluble sugar and TNC (Table 4).

Effects of experimental warming and elevated [CO₂] on anatomy of mesophyll cells: Experimental warming significantly increased mesophyll cell length by *c.* 10% ($p = 0.020$; Table 5), but marginally decreased the cell width, cell area, cell perimeter, and leaf thickness of winter wheat by 8.8% ($p = 0.007$), 13.6% ($p = 0.030$), 6.8% ($p = 0.041$), and 8.3% ($p < 0.001$), respectively (Fig. 6). However, the mesophyll cell width and leaf thickness of winter wheat was significantly reduced by 14.5% ($p = 0.001$) and 22.7% ($p < 0.001$) under elevated [CO₂]. Meanwhile, mesophyll cell width and leaf thickness were also marginally reduced by 11.1% ($p = 0.001$) and 4.8% ($p = 0.003$) under e[CO₂] × warming conditions (Table 5; Fig. 6). Furthermore, elevated [CO₂] substantially affected the mesophyll cell length ($p = 0.007$) and leaf thickness ($p < 0.001$; Table 6). Experimental warming dramatically affected the mesophyll cell length ($p = 0.002$), mesophyll cell width ($p = 0.002$), and leaf thickness ($p < 0.001$; Table 6; Fig. 6). Moreover, [CO₂] and experimental warming interactively affected the mesophyll cell width ($p = 0.021$) and leaf thickness ($p < 0.001$).

Effects of experimental warming and elevated [CO₂] on the activity and gene expression of Rubisco: The Rubisco activation state was different among Control and the three treatments (Fig. 7), where the Rubisco activation state was markedly increased by 38%, 157%, and 63% under elevated [CO₂], experimental warming, and e[CO₂] × warming (Fig. 7). These enhanced Rubisco activation states of winter wheat mainly attributed to the decreases of total Rubisco activity of winter wheat plants grown at the three treatments, because the initial Rubisco activity was slightly changed by elevated temperature and [CO₂] (Fig. 7). In addition, we also found that the amount of gene expression in *RbcL3* and *RbcS2* under elevated [CO₂] was substantially higher than Control (all $p < 0.001$; Fig. 8). Nevertheless, experimental warming obviously declined the amount of gene expression in *RbcL3* and *RbcS2* by 80% ($p = 0.015$) and 91% ($p = 0.0059$; Fig. 8), while e[CO₂] × warming barely affected the amount of gene expression in *RbcL3* and *RbcS2* (all $p > 0.05$; Fig. 8).

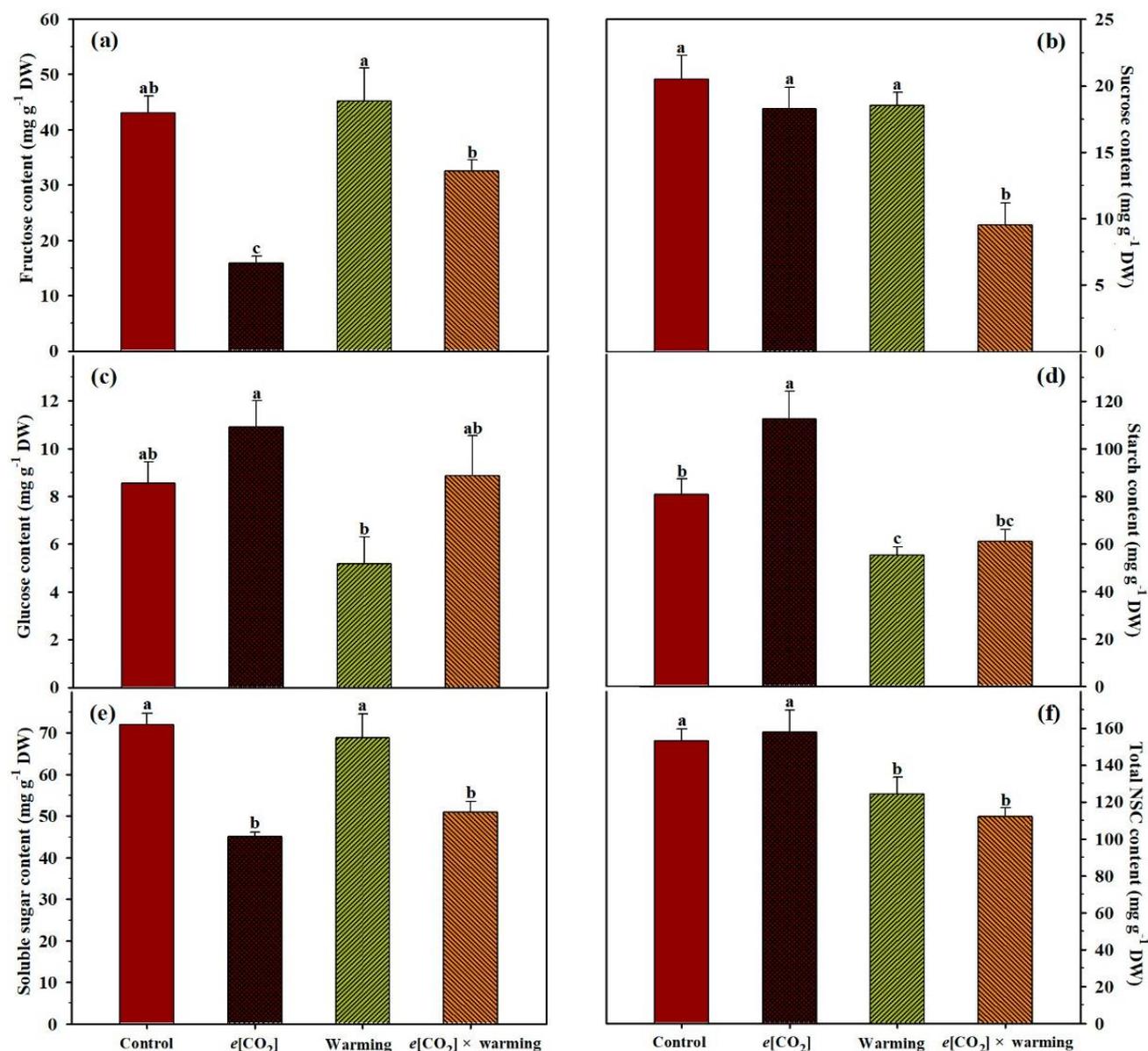


Fig. 5. Effect of elevated temperature and [CO₂] on leaf nonstructural carbohydrates.

Discussion

Responses of plant biomass to elevated temperature and [CO₂]: Several studies have demonstrated that experimental warming may boost plant growth through increasing leaf photosynthesis mainly due to the enhanced Rubisco activity when the growth temperature below their optimum (Liu *et al.*, 2020; Hatfield *et al.*, 2011). However, the plant biomass of winter wheat was barely affected by experimental warming with elevating growth temperature from 21°C to 26°C in this study, which is directly supported by the slightly changed leaf photosynthesis. These results suggested that winter wheat plants may still grow at their optimal temperature range under experimental warming. Previous study also reported that the optimal temperature of winter wheat for plant growth and leaf photosynthesis ranges from 20°C to 30°C (Hatfield *et al.*, 2011). However, the optimal growth temperature may also be different among species, ecotypes and functional groups (Hatfield *et al.*, 2011).

Both photosynthesis and biomass of winter wheat (*C₃* species) were not significantly changed by experimental warming in the current study. Nevertheless, Zheng *et al.*, (2018) reported that experimental warming not changed plant biomass and grain yield of maize (*C₄* species), although leaf photosynthesis was obviously increased under warming conditions. Meanwhile, it should be noted that the extension of experimental warming may result in the changes of plant biomass and leaf photosynthesis, because the growth temperature was elevated by 5°C in this study, which was higher than the elevated temperature of 2°C in other studies (Hatfield *et al.*, 2011; Zheng *et al.*, 2018). In addition to species and warming extension, the facility of experimental warming may also impact the responses of plant biomass. Zheng *et al.*, (2018) found that the plant biomass and grain yield of maize was slightly changed by 2°C warming with infrared heaters, but Wang *et al.*, (2021) have claimed that the plant biomass and grain yield of maize was increased by *c.* 20% and *c.* 30% by elevating temperature 2°C with Open Top

Chambers (OTC). Therefore, these conflicted results of previous studies about plant biomass in response to experimental warming may attribute to different warming facilities, which is indirectly confirmed by the unaffected plant biomass of winter wheat grown at growth chambers as found in this study.

It is well documented that elevated $[\text{CO}_2]$ enhances leaf photosynthesis and thus promote plant biomass (Xu, 2015; Bencze *et al.*, 2014; Yu *et al.*, 2012a). While several studies reported that $[\text{CO}_2]$ enrichment not changed plant growth (Wang *et al.*, 2016) or even had an adverse effect on leaf photosynthesis and biomass accumulation of crops (Högy *et al.*, 2013; Xu, 2015). In this study, plant biomass was marginally affected by elevated $[\text{CO}_2]$, which can probably be explained by the different duration of plants exposed to high $[\text{CO}_2]$. Wang *et al.*, (2016) have reported that the total plant biomass of winter wheat was not changed under CO_2 enrichment in the first two years, while an averaged increase of 15.5% in total plant biomass of winter wheat was observed after two more years later. Furthermore, we also found a significant increase in leaf starch content of winter wheat under high $[\text{CO}_2]$, indicating that plant growth might be constrained by the accumulation of nonstructural carbohydrates, namely the imbalance between source and sink (Liu *et al.*, 2020).

Interestingly, the plant biomass of winter wheat was substantially decreased by $e[\text{CO}_2] \times$ warming, although plant biomass was barely changed by elevated $[\text{CO}_2]$ or experimental warming, indicating that elevated $[\text{CO}_2]$ and experimental warming may have combined effects on wheat grain yield, which may suffer much more under future climate change coming with elevated temperature

and higher $[\text{CO}_2]$ (Wang *et al.*, 2021; Wang *et al.*, 2016). Previous studies have already found that the plant biomass and grain yield of maize was obviously declined at experimental warming of 3°C and the $[\text{CO}_2]$ of $550 \mu\text{mol mol}^{-1}$ (Abebe *et al.*, 2016).

Responses of leaf photosynthesis to elevated temperature and $[\text{CO}_2]$ associated with the stomatal diffusion processes as well as the activation state and gene express of Rubisco:

It is well known that stomata are crucial transmission channels for plants to exchange H_2O and CO_2 between leaves and atmosphere (Xu, 2015), and thus plants usually regulate stomatal traits for adapting current environments and resisting external stresses (Zheng *et al.*, 2013). In current study, the stomatal density of winter wheat was enhanced under experimental warming, elevated $[\text{CO}_2]$, or $e[\text{CO}_2] \times$ warming, indicating winter wheat plants may consistently develop much more stomata at elevated temperature and $[\text{CO}_2]$. However, the response of stomatal openness to experimental warming and elevated $[\text{CO}_2]$ are inconsistent, where the stomatal openness of winter wheat is declined at elevated $[\text{CO}_2]$ and increased at experimental warming, even the stomatal openness is not significantly changed by elevated temperature and $[\text{CO}_2]$. These results demonstrated that modifying stomatal number should be more efficiency than modulating stomatal openness of winter wheat in response to elevated temperature and $[\text{CO}_2]$. Zhu *et al.*, (2016) reported that blueberries mitigated high temperature stress and improved leaf gas exchange efficiency through increasing stomatal frequency rather than stomatal openness.

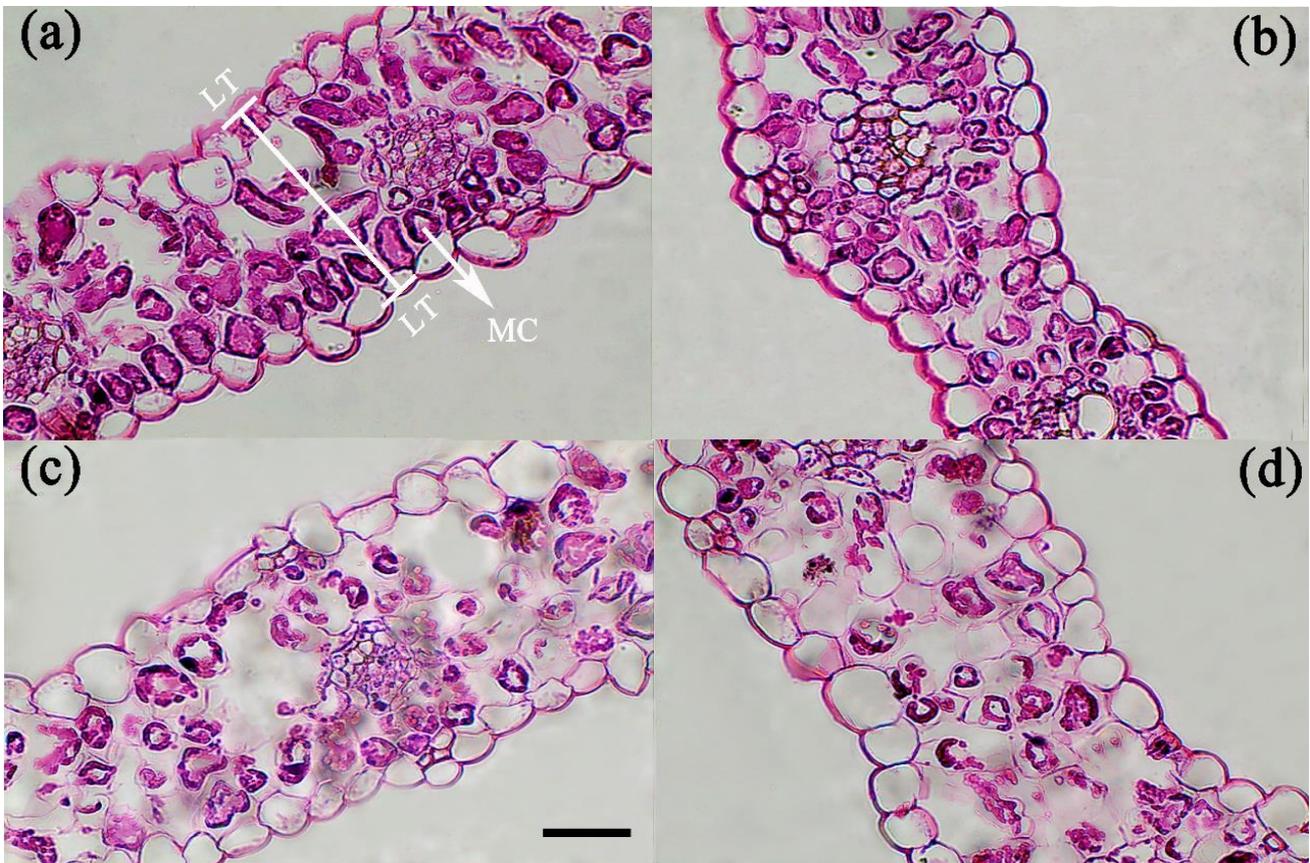


Fig. 6. Effects of elevated temperature and $[\text{CO}_2]$ on leaf anatomy of winter wheat.

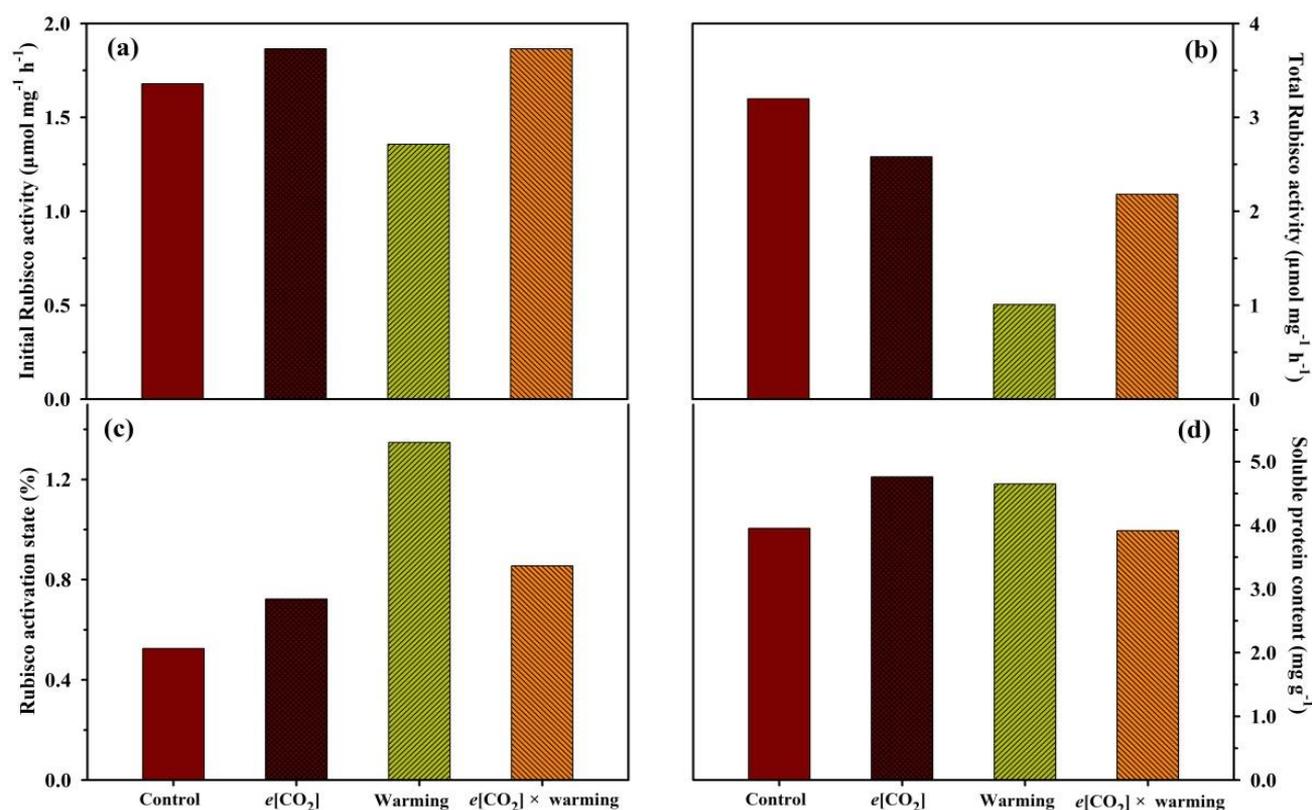


Fig. 7. Effects of elevated temperature and [CO₂] on Rubisco activity and soluble protein of winter wheat.

Table 5. Effect of elevated temperature and [CO₂] on the anatomic traits of leaf mesophyll cells.

Mesophyll cell traits	Control	e[CO ₂]	Warming	e[CO ₂] × warming	<i>p</i> -values
Mesophyll cell length (μm)	22.5 ± 2.1b	20.8 ± 1.0b	24.1 ± 2.5a	22.2 ± 2.2b	0.001
Mesophyll cell width (μm)	17.5 ± 1.7a	14.9 ± 1.3b	15.9 ± 2.4b	15.5 ± 1.2b	0.002
Mesophyll cell area (μm ²)	332 ± 65a	294 ± 37.7ab	287 ± 80b	290 ± 62ab	0.139
Mesophyll cell perimeter (μm)	67.0 ± 6.7a	64.1 ± 4.5ab	62.4 ± 9.0b	63.0 ± 6.1ab	0.199
Leaf thickness (μm)	132 ± 9a	102 ± 5d	121 ± 6c	126 ± 8b	<0.001

Table 6. Interactive effect of elevated temperature and [CO₂] on leaf anatomy of winter wheat.

	Mesophyll cell length	Mesophyll cell width	Mesophyll cell area	Mesophyll cell perimeter	Leaf thickness
[CO ₂]	0.007	0.307	0.145	0.117	<0.001
Warming	0.002	0.002	0.307	0.534	<0.001
[CO ₂] × warming	0.882	0.021	0.221	0.341	<0.001

Elevated temperature and [CO₂] can also change the division and differentiation of epidermal cells (Zheng *et al.*, 2013), which largely determines the distribution pattern of stomata on plant leaves (Shpak *et al.*, 2005; Nadeau *et al.*, 2002). We found divergently response to elevated temperature and [CO₂] between adaxial and abaxial surfaces in stomatal distribution, as demonstrated by the higher regularity of stomatal distribution on the abaxial surface than that on the adaxial surface, indicating the leaf gas exchange efficiency of abaxial surface might be higher than that of the adaxial surface. Meanwhile, our results that e[CO₂] × warming reduced both the regularity of stomata distribution on abaxial surface and stomatal conductance also directly supported the above conclusion that the stomata distributed on abaxial surface might contribute to the efficiency of leaf gas exchange much

more at elevated temperature and [CO₂]. However, it should be noted that leaf photosynthesis was also markedly declined under e[CO₂] × warming treatment, although the Rubisco activation state and soluble protein content were substantially enhanced, suggesting that leaf photosynthesis might be limited by CO₂ diffusion processes through stomata, which were closely related to stomatal frequency, stomatal openness, and stomatal distribution. The obviously decreased stomatal conductance under e[CO₂] × warming conditions in this study also strongly confirmed these conclusions that the declines of leaf photosynthesis could partially attribute to the decreased stomatal conductance, which associated with the stomatal morphological traits and stomatal distribution pattern of winter wheat plants treated with e[CO₂] × warming. Additionally, it is noted that leaf

photosynthesis of winter wheat was unaffected by experimental warming or elevated $[\text{CO}_2]$, although stomatal conductance was significantly reduced under both the elevated $[\text{CO}_2]$ and experimental warming treatments. These results could partially be explained by the boosted Rubisco activation state at experimental warming and the enhanced amount of gene expression in *Rbc S2* and *Rbc L3* at elevated $[\text{CO}_2]$. Overall, these results demonstrated that the effect of CO_2 enrichments could be lowered by experimental warming, and thus the risks of climate change on regional and/or global wheat yield might be underestimated when the elevated atmospheric $[\text{CO}_2]$ combined to experimental warming in the near future.

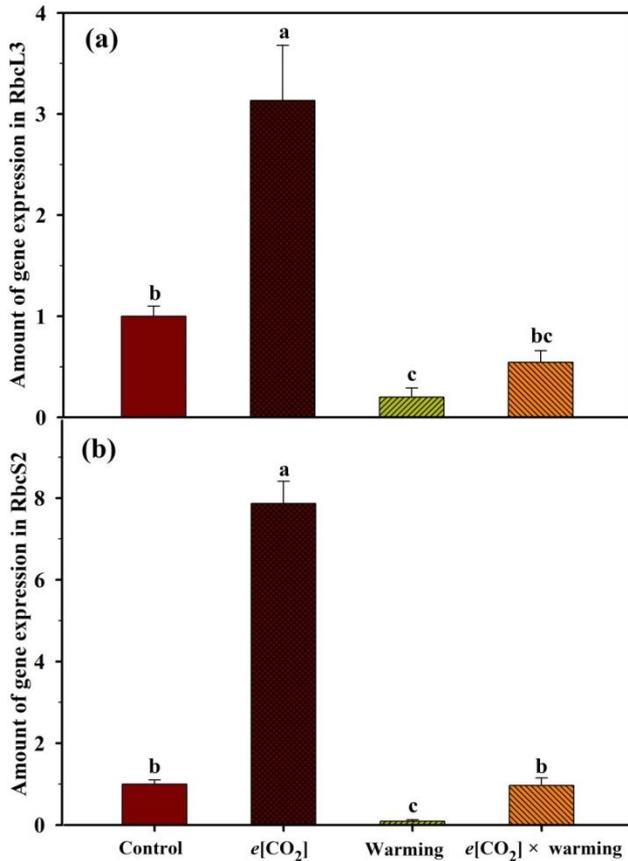


Fig. 8. Effects of elevated temperature and $[\text{CO}_2]$ on the amount of gene expression of Rubisco in winter wheat leaves.

Response of water use efficiency to elevated temperature and $[\text{CO}_2]$ related to the balance between leaf photosynthesis and transpiration of winter wheat:

Water use efficiency (*WUE*) of plants/crops usually reveals the balance between carbon gain/crop yield and water consumption, and the leaf-level *WUE* characterizes the ratio of leaf photosynthesis and transpiration, which are simultaneously affected by elevated temperature and $[\text{CO}_2]$ (Yuan *et al.*, 2018). It has been demonstrated that elevated $[\text{CO}_2]$ generally increases the net photosynthetic rate (P_n) and meanwhile decreases the transpiration rate (T_r) of plants/crops, thereby enhances the leaf-level *WUE* of plants/crops (Chun *et al.*, 2010; Robredo *et al.*, 2007). While the responses of leaf-level *WUE* to elevated temperature and $[\text{CO}_2]$ are still unclear, because leaf photosynthesis and transpiration in response to

experimental warming are different with elevated $[\text{CO}_2]$, thus understanding the potential mechanisms of leaf-level *WUE* in response to the simultaneously elevated temperature and $[\text{CO}_2]$ are critical to understanding the changes in carbon/water balance of agricultural ecosystems (Liu *et al.*, 2020). Our results suggested that the markedly enhanced leaf-level *WUE* under elevated $[\text{CO}_2]$ mainly resulted from the decreased leaf transpiration, because leaf photosynthesis was not changed by elevated $[\text{CO}_2]$. However, Kimball *et al.* (2002) reported that elevated $[\text{CO}_2]$ enhanced the leaf-level *WUE* with increasing leaf photosynthesis, but the leaf transpiration was not obviously changed by elevated $[\text{CO}_2]$. In addition to elevated $[\text{CO}_2]$, the leaf-level *WUE* of winter wheat was increased by $e[\text{CO}_2] \times$ warming, although leaf photosynthesis and transpiration was simultaneously decreased under elevated temperature and $[\text{CO}_2]$. These results indicated that leaf photosynthesis might contribute much more than leaf transpiration on the enhanced leaf-level *WUE* of winter wheat under $e[\text{CO}_2] \times$ warming treatments.

It should be noted that the combined effects of elevated temperature and $[\text{CO}_2]$ on leaf-level *WUE* of winter wheat may also confound with other environmental factors such as water deficits, nitrogen deposition, and ozone concentration, which is already happening and is most likely to be worse in most temperate regions where winter wheat grows under future climate change. Given the lacking of vernalization for winter wheat in environmental growth chambers, we did not further proceed this experiment to investigate the combined effects of elevated temperature and $[\text{CO}_2]$ on *WUE* and crop grain yield. Therefore, the *WUE* of agriculture ecosystem, namely the balance between crop yield and water consumption, should be exactly estimated through long-term field experiments with multiple factors to improve the projection accuracy of process-based ecological models.

Conclusions

Plant biomass and leaf photosynthesis were substantially constrained by $e[\text{CO}_2] \times$ warming, although plant biomass and leaf photosynthesis were barely affected by elevated $[\text{CO}_2]$ or experimental warming. The limiting effect of $e[\text{CO}_2] \times$ warming on plant biomass and leaf photosynthesis might attribute to the impacts on stomatal diffusion processes as well as the activity and gene express of Rubisco. Overall, our results suggest that the simultaneously increases in global surface temperature and $[\text{CO}_2]$ may lower plant biomass and leaf photosynthesis, and thus reduced the regional grain yield of winter wheat.

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