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Original Research

Elevated CO_2 Concentration Alleviates the Adverse Effects of Drought Stress by Modifying Stomatal Traits of Green Pepper (*Capsicum annuum* L.)

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Abstract

We examined the interactive effects of elevated CO_2 concentration and water stress on the stomatal density, stomatal opening, and stomatal distribution pattern of green pepper with environmental growth chambers, whereby the CO_2 concentration was automatically controlled at 400 μmol mol^{-1} ($a[CO_2]$) or 800 μmol mol^{-1} ($e[CO_2]$). Soil water was treated with full irrigation (75-85% field capacity), mild stress (65-75% field capacity), moderate stress (55-65% field capacity), and severe stress (45-55% field capacity). We found that $e[CO_2]$ increased the stomatal density by 65% and 79% on the abaxial and adaxial surfaces, when green pepper plants were treated with mild water stress at the anthesis stage. Water stress obviously changed the stomatal density (SD) at both the early anthesis and maturation stages, whereas had little effect on the SD at the anthesis stage. Moreover, water stress also altered the stomatal aperture size and shape at the early anthesis stage. As a result, $e[CO_2]$ and water stress not only changed the SD at the early anthesis and anthesis stages, but also modified the stomatal opening at the maturation stage. In addition, elevated CO_2 concentration and water stress made the distribution of stomata more regular on green pepper leaves. Our results indicated that green pepper responds $e[CO_2]$

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and water stress not only through modifying the morphology of individual stoma, but also by adjusting stomatal distribution pattern on leaves.

Keywords: elevated CO_2 concentration, water stress, green pepper, stomatal morphology, spatial distribution pattern

Introduction

It is well known that the CO_2 concentration [CO_2] has dramatically increased from 280 μmol mol^{-1} to more than 400 μmol mol^{-1} today since the beginning of the industrial revolution with fossil fuel combustion [1-2]. According to the recently report from Intergovernmental Panel on Climate Change (IPCC), the atmospheric [CO_2] will be continually increased with an average rate of about 2.0 μmol mol^{-1} $year^{-1}$, and the average atmospheric [CO_2] could reach approximately 800 μmol mol^{-1} by the end of the 21th century [2]. Moreover, elevated [CO_2] can result in climate warming through greenhouse effect, and thus lead to temporal and spatial shifts in global precipitation distribution [2]. This unevenly distributed global precipitation may not only generally induce regional drought with declining in the amount of rainfall [3], but also dramatically impact global agricultural productivity through altering the physiological and biochemical processes of crops [4].

Stomata are small pores controlling CO_2 and water exchanges between the plant leaves and atmosphere, and thus have important significance on regulating ecosystem carbon and water cycling [5]. Plant leaves generally optimize their gas exchange by altering stomatal morphology and distribution, which are regulated by both environmental factors [6] and genetic signals [7]. Plants quickly respond to changes of environment by adjusting the pore size of individual stoma, which is controlled by environmental factors such as atmospheric [CO_2] [5] and water deficit [8]. Elevated [CO_2] usually induces stomatal closure, thus further decreases both stomatal conductance and water consumption [9]. Similarly, water stress is another important factor affecting the morphological traits of stomata such as stomatal density and opening [10]. Additionally, elevated [CO_2] and water stress can also modify stomatal distribution on plant leaves [11], which is highly regulated by a number of genes such as *SDDI* and *EPFI* [7].

Green pepper (*Capsicum annuum* L.) is an important vegetable world-widely planted [12]. However, drought stress has recently become a major bottleneck restricting the development of agriculture in China, especially for the vegetable production such as green pepper in the North China Plain, where characterizes arid or semi-arid climates [13]. Several studies have reported that the net photosynthesis rates and growth of C_3 plants are generally stimulated by more than 35% with doubling [CO_2], because these C_3 plants are generally not photosynthetic saturated at current CO_2 concentration [11]. However, few studies investigated the interactive effects of elevated [CO_2] and water deficit on the morphology of individual stoma and the spatial distribution pattern of stomata of green pepper plants. This study aims to: (1) examine the effects of elevated [CO_2] and water deficit on stomatal density and opening as well as stomatal distribution, and (2) explore the potential mechanisms that green pepper plants respond to elevated [CO_2] and water deficit by modifying stomatal traits.

Materials and Methods

Growth Chamber Experiment

The experiment was arranged in a split plot design consisting two factors (CO_2 and water) with [CO_2] as the main plot and water as the subplot. We employed eight environmental growth chambers (Model BDP-2000, Ningbo Prandt Instrument Co., Ltd, China) for sustaining plant growth, where the CO_2 concentration in four growth chambers was supplied with 400 μmol mol^{-1} (a[CO_2]), and the CO_2 concentration in the other four growth chambers was regulated to 800 μmol mol^{-1} (e[CO_2]). In addition to CO_2 concentration, other environmental factors were maintained similarly throughout all of the eight environmental growth chambers with relative humidity (65%), PPFD (1000 μmol m^{-2} s^{-1}), growth temperature (25/20ºC, day/night), and photo period (8:00-20:00).

We grew three bell pepper (*Capsicum annuum* L. cultivar zhongjiao 107) seedlings with seeds in each plastic pot (240 cm^2 at the top and 133 cm^2 at the bottom). These pots were filled with local soil (36% clay, 50 % silt, and 14 % sand with a gravimetric bulk density of 1.58 g cm^{-3}) for sustaining the growth of bell pepper seedlings to enhance the relevance to the field conditions. Bell pepper plants were established in a greenhouse for 30 days after seed sowing with an average temperature of 25/20ºC (day/night) and 700 μmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) in natural sun light, and 65% relative humidity. Then, we put five pots with pepper seedlings in each of the four chambers with ambient or elevated CO_2 concentration, which were treated with four soil water conditions including full irrigation, mild stress, moderate stress, and severe stress. A widely used gravimetric method was adopted to determine the field capacity, and the four water treatments were treated with full irrigation (75-85% FC), mild stress (65-75% FC), moderate stress (55-65% FC), and severe stress

(45-55% FC), respectively. These pepper seedlings were fertilized with half-strength Hoagland's solution weekly (150 mL per pot) during the vegetative growth of green pepper. We defined three growth stages of green pepper plants as the early anthesis (40 days after planting), anthesis (80 days after planting), and maturation stages (120 days after planting).

Stomatal Imprints Sampling

We collected stomatal imprint samples from the middle leaf section with colorless nail polish during 10:00-11:00 am with same canopy light intensity in the growth chamber at the early anthesis, anthesis, and maturation stages. The details of the procedure for sampling stomatal imprints were specifically described by Zheng et al. [14] and Xu [11].

Stomatal Imprint Measurements

We observed and photographed the stomatal imprints with a microscope at the laboratory. We randomly photographed 10 images (10 images * 5 leaves = 50 images) for measuring the length, width, circumference, and area of stomatal aperture. We also calculated the stomatal number per unit leaf area to obtain stomatal density [15]. Moreover, we randomly selected eight stomata from the collected images (50 images * 8 stomata = 400 stomata on each leaf surface) for measuring stomatal length, width, circumference, area, and shape index [14].

Spatial Distribution Pattern Analysis

We took five images from each stomatal imprint to estimate stomatal distribution patterns on each leaf surface (5 images * 5 leaves = 25 images). The details for analyzing stomatal distribution pattern can be found at Ripley [16] and Xu [11].

Statistical Analysis

We tested the effects of CO_2 concentration and water stress on stomatal morphology with one-way and two-way ANOVA followed by Duncan's multiple range test ($p<0.05$) using the *SPSS* 13.0 software (Chicago, IL).

Results and Discussion

Effects of [CO_2] and Water Stress on the Stomatal Density of Green Pepper

Stomatal density (SD) on the abaxial surface were substantially greater than that on the adaxial surface of green pepper leaves at the early anthesis stage (all $p<0.05$; Table 1). Elevated [CO_2] barely affected the SD on the abaxial leaf surface of the green pepper except for these plants subjected to mild water stress ($p>0.05$), while the SD on the adaxial leaf surface substantially increased under water stress at the early anthesis stage (all $p<0.05$; Table 1). However, elevated CO_2 concentration dramatically increased the SD by 65% and 79% on both leaf surfaces treated with mild water stress at the anthesis stage (all $p<0.05$; Table 2). In contrast to the early anthesis stage and the anthesis stage, elevated [CO_2] significantly increased the SD of the adaxial surface by 75% and 46% under full irrigation and moderate water stress at the maturation stage ($p<0.05$; Table 3), whereas the SD of the abaxial surface was not changed by elevated CO_2 concentration regardless of the soil water status (all $p>0.05$; Table 3). Although the SD of the green pepper was obviously different between leaf surfaces regardless of treatments during the three development stages, soil water status significantly affected SD at the early anthesis and maturation stages (Table 4 and Table 6) and elevated [CO_2] significantly changed the SD at the anthesis and maturation stages (Table 5 and Table 6). Moreover, we also found that SD was substantially affected by [CO_2] × water at the anthesis stage ($p<0.05$; Table 5). However, our results showed interactive effects of [CO_2] × water, [CO_2] × leaf surface, water × leaf surface, and [CO_2] × water × leaf surface on the SD of green pepper plants at the early anthesis stage (all $p<0.05$; Table 4).

It has been well demonstrated that stomatal morphology of individual stoma as well as stomatal distribution patterns can affect the net photosynthetic rate, transpiration rate, and thus the leaf level water use efficiency [17]. The stomatal density on the adaxial leaf surface of green pepper was obviously decreased by water stress combined with ambient CO_2 concentration at the early anthesis stage and with elevated CO_2 concentration at the maturation stage. Previous studies have reported that plants subjected to water stress usually decreased the transpiration rates and thus enhanced the water use efficiency of plants [18-19]. Also, Li et al. [20] has found a positive correlation between stomatal density and transpiration rate in a kind of drought-resistant winter wheat.

Effects of [CO_2] and Water Stress on the Stomatal Opening of Green Pepper

Our results showed that stomatal opening was also different between the two leaf surfaces with larger stomata on the abaxial leaf surface at the early anthesis stage of green pepper plants. The stomatal opening on the abaxial leaf surface at the early anthesis stage was obviously greater under water stress conditions (Fig. 1), mainly due to the larger stomatal aperture width (SAW, all $p<0.05$) on the abaxial surface regardless of [CO_2] (Table 1 and Fig. 1). Mild water stress increased the stomatal opening on the adaxial surface under elevated [CO_2] ($p<0.05$; Fig. 1), mainly due to the larger stomatal aperture length (SAL) at the early anthesis stage ($p<0.05$; Table 1 and Fig. 1). Additionally, interactive

Table 1. Effects of $e[CO_2]$ on the morphological traits of stomata on green pepper leaves under water stresses at the early anthesis stage.

Stomatal traits	$a[CO_2]$				$e[CO_2]$			
	Full irrigation	Mild stress	Moderate stress	Severe stress	Full irrigation	Mild stress	Moderate stress	Severe stress
Adaxial surface								
SD (No. mm^{-2})	99.5±2.6a	25.5±4.6c	57.8±5.3b	24.0±0.6c	27.0±0.8c	64.0±11.4b	92.5±6.6a	55.3±4.6b
SAL (μm)	18.1±2.2ab	18.6±1.6ab	19.2±2.1ab	19.5±1.8a	16.6±0.4b	20.5±0.8a	19.7±0.9a	19.1±1.5ab
SAW (μm)	6.1±0.5ab	5.5±0.4b	6.6±0.5ab	6.4±0.8ab	6.6±0.2a	7.1±0.4a	6.3±0.3ab	6.2±0.5ab
SAC (μm)	40.9±4.7a	41.4±3.3ab	43.2±4.9ab	44.4±3.9ab	38.3±0.9b	46.3±1.5a	43.9±1.4ab	43.2±3.2ab
SAA (μm^2)	87.5±18.0ab	82.2±11.5ab	100.2±18.6ab	100.5±18.3ab	80.0±3.1b	110.8±6.3a	95.2±6.9ab	94.2±11.9ab
SASI (%)	0.22±0.003b	0.22±0.003b	0.23±0.009ab	0.22±0.006b	0.24±0.003a	0.23±0.006ab	0.22±0.003b	0.22±0.007ab
Abaxial surface								
SD (No. mm^{-2})	67.5±4.9ab	76.5±7.1a	90.8±15.6a	79.5±11.2a	42.0±3.6b	45.8±3.2b	66.0±5.9ab	87.3±15.0a
SAL (μm)	17.8±1.4a	20.3±0.6a	19.1±1.6a	20.7±1.8a	17.7±2.7a	20.2±1.0a	20.0±1.3a	19.5±0.5a
SAW (μm)	6.5±0.4ab	7.6±0.6a	6.9±0.5ab	7.1±0.4ab	6.0±0.3b	7.2±0.6ab	7.4±0.4a	8.1±0.2a
SAC (μm)	40.2±3.4ab	46.9±1.4ab	43.9±3.4ab	47.1±3.7a	40.2±1.4b	46.0±2.4ab	45.7±3.0ab	45.3±1.2ab
SAA (μm)	89.7±14.3bc	119.4±7.1ab	104.5±16.1abc	111.8±13.7ab	81.6±6.2c	142.5±20.3a	113.6±12.6ab	119.1±6.7ab
SASI (%)	0.23±0.004ab	0.23±0.004a	0.23±0.003a	0.22±0.006b	0.22±0.002b	0.24±0.009a	0.23±0.003a	0.24±0.001a

Note: Values given are means±standard deviation (n = 5) for SD, SAL, SAW, SAC, SAA and SASI. The mean values were compared by the two-way analysis of variance (ANOVA) at $p<0.05$ followed by Duncan's multiple range tests. The different letters indicate statistical differences at $p<0.05$, and the same letters indicate statistical differences at $p>0.05$. SD is the stomatal density (stomatal number per mm^2 leaf area); SAL is the stomatal aperture length (μm); SAW is the stomatal aperture width (μm); SAC is the stomatal aperture circumference (μm); SAA is the stomatal aperture area (μm^2); SASI is the stomatal aperture shape index (%). *Stomatal aperture length (SAL) is the longest dimension, and stomatal aperture width (SAW) is the widest dimension.

Table 2. Effects of $e[CO_2]$ on the morphological traits of stomata on green pepper leaves under water stresses at the anthesis stage.

Stomatal traits	$a[CO_2]$				$e[CO_2]$			
	Full irrigation	Mild stress	Moderate stress	Severe stress	Full irrigation	Mild stress	Moderate stress	Severe stress
Adaxial surface								
SD (No. mm^{-2})	52.5±7.8b	45.3±5.4b	53.8±4.3b	47.3±8.5b	59.8±5.1ab	74.8±2.8a	59.0±5.3ab	53.5±8.5b
SAL (μm)	19.0±1.0a	19.1±1.0a	20.2±1.1a	18.2±0.4a	18.0±1.0a	18.9±0.7a	19.3±1.1a	19.8±0.3a
SAW (μm)	5.6±0.2b	5.9±0.4ab	6.4±0.3ab	6.8±0.3a	5.6±0.4b	5.9±0.4ab	6.3±0.2ab	6.0±0.1ab
SAC (μm)	42.5±1.9a	42.8±2.3a	45.3±2.5a	41.8±1.0a	40.7±2.3a	42.2±1.4a	43.5±2.2a	44.2±0.7a
SAA (μm^2)	82.4±5.3a	90.2±10.7a	101.5±10.4a	93.8±6.8a	84.6±4.8a	85.5±5.8a	93.5±6.8a	90.9±2.9a
SASI (%)	0.21±0.006ab	0.22±0.002ab	0.22±0.002ab	0.23±0.003a	0.20±0.013b	0.22±0.005ab	0.22±0.004a	0.21±0.001ab
Abaxial surface								
SD (No. mm^{-2}) SAL (μm)	168.5±18.8ab 20.1±0.3a	103.0±22.7c 17.9±0.5bcd	125.5±5.5bc 18.4±0.8abc	187.3±9.8a 16.7±0.2cd	186.5±27.5a 16.3±0.9d	184.5±4.6a 18.1±0.6bcd	168.8±32.8ab 17.5±0.9bcd	165.3±6.7ab 18.6±0.3ab
SAW (μm)	7.0±0.2a	6.5±0.4a	6.4±0.2a	6.6±0.2a	6.5±0.9a	7.4±0.5a	6.6±0.2a	6.9±0.3a
SAC (μm)	45.2±0.6a	40.9±1.2bcd	41.8±1.7abc	38.3±0.4cd	37.6±2.5d	42.0±1.2abc	39.9±1.8bcd	42.5±0.6ab
SAA (μm^2)	106.8±4.4a	90.0±6.8a	106.6±19.6a	81.7±2.4a	79.9±14.1a	101.5±7.2a	86.0±5.8a	96.9±3.5a
SASI (%)	0.23±0.003b	0.23±0.003ab	0.23±0.006ab	0.23±0.002ab	0.23±0.007ab	0.24±0.004a	0.23±0.003ab	0.23±0.004ab

Note: Values given are means±standard deviation (n = 5) for SD, SAL, SAW, SAC, SAA and SASI. The mean values were compared by the two-way analysis of variance (ANOVA) at $p<0.05$ followed by Duncan's multiple range tests. The different letters indicate statistical differences at $p<0.05$, and the same letters indicate statistical differences at $p>0.05$. SD is the stomatal density (stomatal number per mm^2 leaf area); SAL is the stomatal aperture length (μm); SAW is the stomatal aperture width (μm); SAC is the stomatal aperture circumference (μm); SAA is the stomatal aperture area (μm^2); SASI is the stomatal aperture shape index (%). *Stomatal aperture length (SAL) is the longest dimension, and stomatal aperture width (SAW) is the widest dimension.

Table 3. Effects of $e[CO_2]$ on the morphological traits of stomata on green pepper leaves under water stresses at the maturation stage.

Stomatal traits	$a[CO_2]$				$e[CO_2]$			
	Full irrigation	Mild stress	Moderate stress	Severe stress	Full irrigation	Mild stress	Moderate stress	Severe stress
Adaxial surface								
SD (No. mm^{-2})	45.5±2.4cd	37.3±1.5d	41.8±4.1d	58.3±4.9bc	79.5±4.4a	51.5±2.9bcd	60.8±10.8bc	62.8±6.5b
SAL (μm)	20.1±0.4ab	18.7±0.7ab	20.6±0.6a	18.4±0.8b	23.3±1.6ab	20.5±1.0ab	19.1±0.6ab	20.0±0.8ab
SAW (μm)	6.6±0.7ab	6.2±0.4ab	7.2±0.1a	5.6±0.3b	5.7±0.5b	5.4±0.3b	6.0±0.2b	6.1±0.4b
SAC (μm)	44.9±0.9ab	42.0±1.3ab	46.6±1.2a	41.1±1.8b	44.9±3.2ab	44.9±2.2ab	42.5±1.3ab	44.5±1.8ab
SAA (μm^2)	99.4±8.4ab	89.0±6.0b	113.9±4.6a	80.1±6.7b	102.0±10.3ab	86.5±8.2b	88.9±4.7b	95.6±8.5ab
SASI (%)	0.22±0.007ab	0.22±0.007a	0.23±0.001a	0.22±0.003ab	0.21±0.009ab	0.21±0.003b	0.22±0.002ab	0.22±0.004ab
Abaxial surface								
SD (No. mm^{-2}) SAL (μm)	149.3±4.5bc 20.6±0.4a	151.0±2.3bc 18.0±0.7b	126±13.6c 17.6±0.5b	176.3±2.4abc 17.1±0.6b	173.8±5.4ab 22.3±1.0b	191.5±27.3ab 18.6±0.4ab	150.5±24.0bc 16.7±0.9b	201.0±22.9a 17.4±1.0b
SAW (μm)	7.7±0.3a	6.6±0.4bc	7.1±0.2ab	6.1±0.3bc	6.0±0.3c	7.7±0.3a	6.6±0.4bc	6.7±0.3bc
SAC (μm)	46.6±0.9a	40.6±1.5bc	40.7±0.9bc	38.6±1.3bc	36.9±2.1c	42.5±1.2ab	38.4±2.0bc	39.6±2.0bc
SAA (μm^2)	118.2±5.3a	88.7±7.4bc	94.3±2.9bc	79.4±6.1c	119.2±2.6c	104.8±7.7ab	84.8±7.7bc	88.6±7.4bc
SASI (%)	0.23±0.003ab	0.23±0.002ab	0.24±0.003ab	0.23±0.004ab	0.22±0.014b	0.24±0.001a	0.24±0.003a	0.23±0.002ab

Note: Values given are means±standard deviation (n = 5) for SD, SAL, SAW, SAC, SAA and SASI. The mean values were compared by the two-way analysis of variance (ANOVA) at $p<0.05$ followed by Duncan's multiple range tests. The different letters indicate statistical differences at $p<0.05$, and the same letters indicate statistical differences at $p>0.05$. SD is the stomatal density (stomatal number per mm^2 leaf area); SAL is the stomatal aperture length (μm); SAW is the stomatal aperture width (μm); SAC is the stomatal aperture circumference (μm); SAA is the stomatal aperture area (μm^2); SASI is the stomatal aperture shape index (%). *Stomatal aperture length (SAL) is the longest dimension, and stomatal aperture width (SAW) is the widest dimension.

Table 4. Interactive effects of [CO_2] and water on stomatal parameters between leaf surfaces of green pepper at the early anthesis stage.

Stomatal traits	SD (No. mm^{-2})	SAL (μm)	SAW (μm)	SAC (μm)	SAA ($μm^2$)	SAAI (%)
[CO_2]	<0.05	0.363	0.983	0.372	0.325	0.611
Water	0.317	0.787	0.522	0.809	0.582	0.160
Leaf	<0.05	<0.05	<0.05	<0.05	0.437	<0.05
[CO_2] × Water	<0.05	<0.05	0.459	<0.05	0.228	0.230
[CO_2] × Leaf surface	0.207	0.474	0.257	0.704	0.832	0.126
Water × Leaf surface	0.094	0.633	0.135	0.509	0.643	0.430
[CO_2] × Water × Surface	0.212	0.453	0.414	0.360	0.161	0.535

Table 5. Interactive effects of [CO_2] and water on stomatal parameters between leaf surfaces of green pepper at the anthesis stage.

Stomatal traits	SD (No. mm^{-2})	SAL (μm)	SAW (μm)	SAC (μm)	SAA ($μm^2$)	SAAI (%)
[CO_2]	<0.05	0.363	0.983	0.372	0.325	0.611
Water	0.317	0.787	0.522	0.809	0.582	0.160
Leaf	<0.05	<0.05	<0.05	<0.05	0.437	<0.05
[CO_2] × Water	<0.05	<0.05	0.459	<0.05	0.228	0.230
[CO_2] × Leaf surface	0.207	0.474	0.257	0.704	0.832	0.126
Water × Leaf surface	0.094	0.633	0.135	0.509	0.643	0.430
[CO_2] × Water × Surface	0.212	0.453	0.414	0.360	0.161	0.535

Table 6. Interactive effects of [CO_2] and water on stomatal parameters between leaf surfaces of green pepper at the maturation stage.

Stomatal traits	SD (No. mm^{-2})	SAL (μm)	SAW (μm)	SAC (μm)	SAA ($μm^2$)	SAAI (%)
[CO_2]	<0.05	0.479	0.052	0.321	0.133	0.227
Water	<0.05	0.186	0.119	0.255	0.091	0.113
Leaf surface	<0.05	<0.05	<0.05	<0.05	0.794	<0.05
[CO_2] × Water	0.824	<0.05	<0.05	<0.05	<0.05	0.356
[CO_2] × Leaf surface	0.383	0.055	0.202	0.106	0.895	0.223
Water × Leaf surface	<0.05	0.710	0.246	0.665	0.209	0.558
[CO_2] × Water × Surface	0.721	0.176	0.089	0.136	0.057	0.301

effects of [CO_2] and water on the stomatal traits were detected at the early anthesis stage of green pepper plants (all $p>0.05$; Table 4). Moreover, [CO_2] × water × leaf surface significantly changed the stomatal aperture shape index (SASI) ($p<0.05$), whereas barely affected the other stomatal features of green pepper leaves at the early anthesis stage (all $p>0.05$; Table 4). Elevated [CO_2] barely affected the stomatal opening regardless of water status at the anthesis stage (Table 2) and maturation stage (Table 3 and Fig. 1). We found significant difference in SAL and SAW (all $p<0.05$) between the adaxial and abaxial leaf surfaces, while the stomatal aperture area was barely changed by [CO_2] at the anthesis ($p>0.05$; Table 2) and maturation stage ($p>0.05$; Table 3 and Fig. 1).

Our three-way ANOVA results showed that [CO_2] × water significantly affected the stomatal aperture length (SAL, $p<0.05$), stomatal aperture circumference (SAC, $p<0.05$), whereas barely affected the stomatal opening of green pepper leaves at the anthesis stage ($p>0.05$; Table 5). However, the interactive effects of [CO_2] × leaf surface, water × leaf surface and [CO_2] × water × leaf surface on the stomatal traits were not detected at the anthesis stage ($p>0.05$; Table 5). Three-way ANOVA results showed that [CO_2] × water substantially affected the SAL, SAW, SAC, and SAA (all $p<0.05$; Table 6) at the maturation stage, whereas barely changed the SASI of green pepper leaves ($p>0.05$; Table 6).

Several studies have claimed that plants usually maintained their water use efficiency by reducing

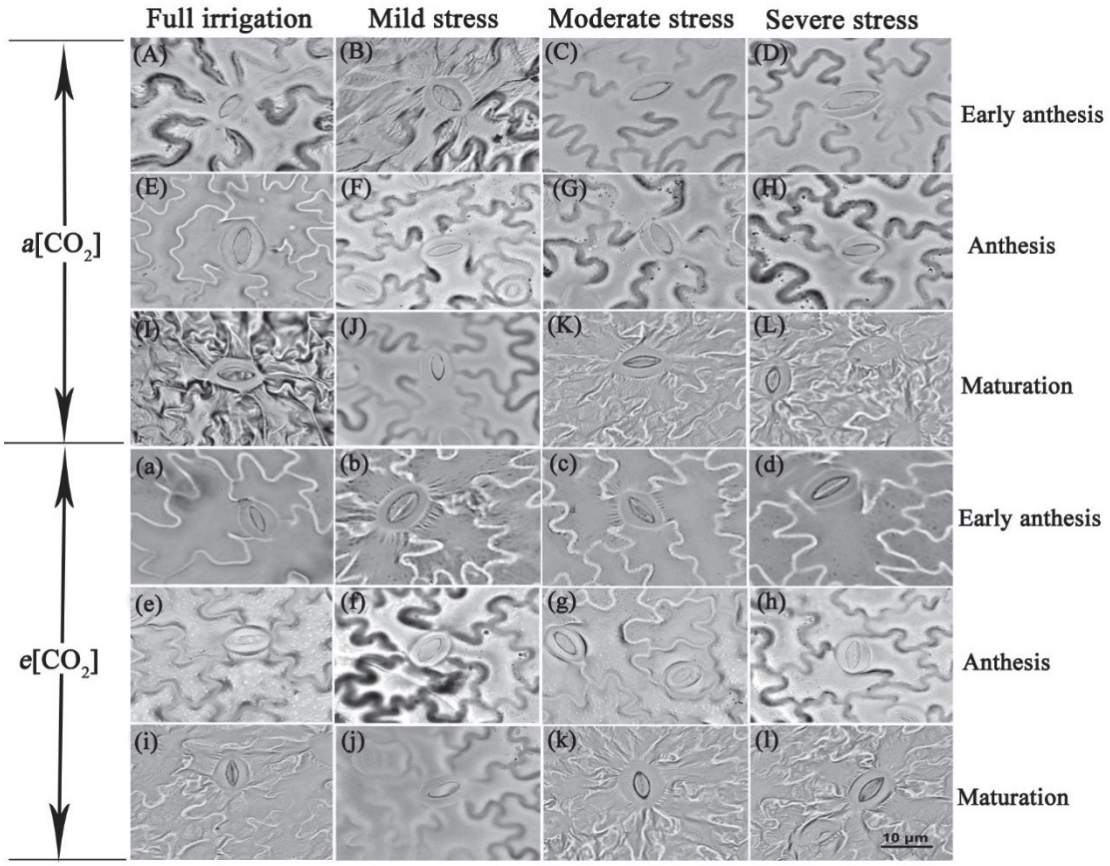

Fig. 1. Effect of water stress on the morphological traits of individual stoma of green pepper plants under *a*[CO_2] (A-L) and *e*[CO_2] (a-l). Note that water stress resulted in larger stoma on green pepper leaves at the early anthesis stage (A-D) regardless of [CO_2] (a-d), especially the mild water stress dramatically increased stomatal opening under *e*[CO_2]. However, elevated [CO_2] barely affected stomatal opening regardless of water status at the anthesis and maturation stages of green pepper plants.

stomatal density in response to long-term water stress and decreasing stomatal aperture size to quickly respond short-term water changes [15, 18, 19]. Our results showed that the stomatal aperture area and stomatal density on the abaxial surface was substantially decreased under water stress and *a*[CO_2] at the maturation stage, suggested that stomatal aperture size may be the most important factor to potentially affect the maximum stomatal conductance and transpiration rate, and in turn regulates water use efficiency of green pepper plants. Moreover, it is noted that stomatal opening, also known as stomatal movement, may be an alternative mechanism for plants in response to water changes with preventing water loss and protecting physiological damage from drought stress [21]. However, we also found that the stomatal opening of green pepper was increased under water stress at the early anthesis and maturation stages, indicating that stomatal aperture size of individual stoma may feature high plasticity in response to different water conditions during development stages, and thus improve their adaptability to different water situations through coordinated and compensatory variation in stomatal density and size of green pepper plants.

Elevated atmospheric CO_2 concentrations can benefit most of C_3 plant species, which is generally associated with biochemical process of photosynthesis [11], and thus highly dependent on the stomatal morphology and stomatal distribution pattern [14]. However, elevated CO_2 concentration generally reduces stomatal opening through depolarizing the guard cell membrane potential due to the changes in organic solution and ion concentration [22]. Elevated CO_2 concentration substantially decreased the stomatal opening under moderate water stress and full irrigation during the maturation stage in this study, suggesting elevated CO_2 concentration may reduce leaf transpiration by inducing stomatal closing.

Effects of [CO_2] and Water Stress on Stomatal Distribution Patterns

We found that stomatal distribution patterns were highly scale dependent during the three development stages (Fig. 2). The stomatal distribution pattern spatially followed a regular pattern [Lhat(d) values below the lower 95% boundary] at small scales and a random distribution pattern [Lhat(d) values between the lower 95% and upper 95% boundaries] at larger scales

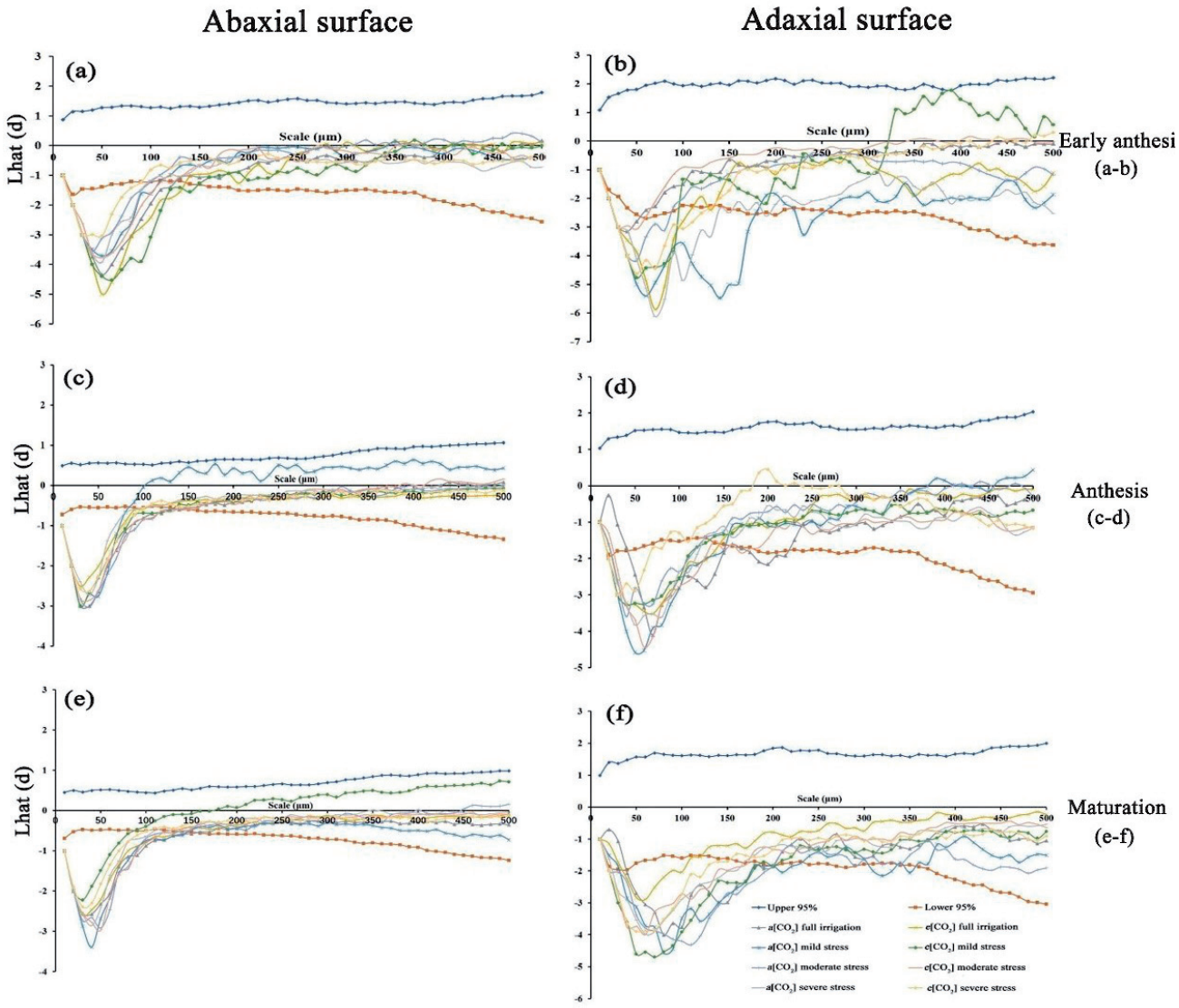

Fig. 2. Point pattern analyses of stomata on the adaxial leaf surface (a, c and e) and the abaxial leaf surface (b, d and f) on the green pepper plants under CO_2 and water treatments during the early anthesis (a-b), anthesis (c-d), and maturation stages (e-f). Note that the more regular distribution pattern of stomata featured with a lower Lhat(d) value. Moreover, if the stomata are randomly distributed on the leaf surface at a given scale of d, then the calculated Lhat(d) value should be located within the upper and lower 95% boundaries. In addition, if the Lhat(d) value is smaller than the lower 95% boundary, then the stomata will follow are regular distribution at the scale. Otherwise, the stomata would follow a cluster distribution at that scale if the Lhat(d) value is larger than the upper 95% boundary. The Lhat(d) value was obtained by averaging the Lhat(d) values of five sampled leaves at each CO_2 and water treatment (n = 5). The upper and lower 95% boundaries were obtained by Monte Carlo simulation of 1000 replicates.

on leaf surfaces regardless of CO_2 concentration and water treatments (Fig. 2). Stomata on the adaxial leaf surface were more regular than those on the abaxial surface as evidenced by the lower Lhat(d) values on the adaxial surface at same scales under water treatments at the early anthesis stage (Fig. 2). We also found spatial distribution pattern of stomata on the adaxial and abaxial surfaces showed different trends to elevated CO_2 concentration among water treatments. Elevated CO_2 concentration made the regular distribution pattern at a larger scale range on the abaxial surface from *c.*50 μm to *c.*100 μm under full irrigation (Fig. 2a). However, water deficit decreased the minimum Lhat(d) values and meanwhile increased the scale ranges of the regular distribution on the abaxial surface under elevated CO_2 concentration (Fig. 2a).

Similarly, our results showed that stomata followed a regular distribution pattern [Lhat(d) values below the lower 95% boundary] at small scales (<90 μm) on the abaxial leaf surface of green pepper treated with ambient [CO_2] and mild water stress at the anthesis stage (Fig. 2c). We found that green pepper under water deficits features the most regular distribution pattern at the spatial scale of *c.*40 μm with the minimum Lhat(d) value of -2.87 on the abaxial surface (Fig. 2c) and at the scale of *c.*60 μm with a minimum Lhat(d) value of -3.70 on the adaxial surface (Fig. 2d) at the anthesis stage. Additionally, elevated CO_2 concentration made

the distribution pattern of green pepper more regular with moderate water deficits because the minimum Lhat(d) values were decreased on the adaxial surface at the anthesis stage (Fig. 2d).

In addition to the early anthesis stage and anthesis stage, our results showed that the stomata on green pepper characterized regularly stomatal distribution pattern (at the scale of *c.*180 μm) and randomly spatial distribution pattern at larger scales (>200 μm) on the adaxial leaf surface under different water treatments during the maturation stage (Fig. 2f). However, stomata on the adaxial surface are more regular than those on the abaxial surface (Fig. 2e), because the scale range of the regular distribution on the adaxial surface (<180 μm) was larger than that on the abaxial surface (<150 μm). Moreover, the adaxial leaf surface had lower Lhat(d) values than that on the abaxial leaf surface at same scales under full irrigation at the maturation stage (Figs 2(e, f)). Water deficits at the maturation stage resulted in the most regular spatial pattern of stomata occurring at the scales of *c.*90 μm on the adaxial surface and *c.*40 μm on the abaxial surface with average minimum Lhat(d) values of -4.03 and -2.87, respectively (Figs 2(e, f)).

Most previous studies only focused on the changes in stomatal frequency [14] or the behaviors of an individual stoma, namely stomatal movement [9, 23] in response to water stress, mainly due to the difficulty in estimating stomatal distribution pattern of leaves [24]. In the current study, we employed a geostatistical method to examine the changes of spatial distribution pattern of stomata and found that water deficits made stomatal distribution pattern more regular than that treated with full irrigation during the early anthesis, anthesis, and maturation stages. These results suggested that green pepper plants in response to water stress may also improve the regular pattern of stomata distributed on leaf surfaces for enhancing water use efficiency, due to the more regular stomatal distribution pattern, which normally features with higher leaf gas exchange efficiency with the shortest CO_2 diffusion distance from individual stoma to other stomata [11]. Previous studies have reported that the more regular stomatal distribution pattern may partially contribute to the higher stomatal conductance and transpiration rate of plants [14, 23, 25].

Many climate models have projected that elevated atmospheric CO_2 concentration was usually confounded by other climatic factors such as precipitation, nitrogen deposition, temperature, and ozone concentration in the coming future decades [2], whereby the CO_2 fertilization effect on plant growth may be reduced or even canceled out through modifying the morphological traits of individual stoma and the spatial distribution pattern of stomata [11]. The increased atmospheric CO_2 concentration generally leads to climate warming through the greenhouse effect and meanwhile results in global precipitation distribution uneven both temporally and spatially [2]. As a result, simultaneous elevated CO_2 concentration and water stress on plant growth often occur due to a decline in precipitation and freshwater availability for irrigation in summer in many arid and semi-arid ecosystems [26-27].

Water stress usually induces negative impacts on the growth and production of crops, and thus crops may also benefit from elevated CO_2 concentration through promoting the drought resistance [28] with enhancing both the photosynthetic capacity and water use efficiency [14,19]. Previous studies have reported that stomata usually set the limit for maximum stomatal conductance [6], which has been widely used to quantify the efficiency of leaf gas exchange [29]. We found that elevated CO_2 concentration substantially increased the stomatal density and stomatal opening of green pepper plants grown under water stress, indicated that plants subjected to water stress may benefit from elevated CO_2 concentration through modifying their stomatal traits to increase leaf carbon gain [15, 16], and thus enhance the water use efficiency [26-28]. In addition to morphological traits of individual stoma, elevated CO_2 concentration also improved the regularity of stomatal distribution under elevated CO_2 concentration, as evidenced by the more regular stomatal distribution pattern, which is one of the important factors controlling the leaf gas exchange efficiency [11, 14].

Conclusions

We found that the morphological traits of individual stoma in response to elevated [CO_2] and water deficits strongly depended on vegetative growth stages, and green pepper plants subjected to water stress may benefit from elevated CO_2 concentration through modifying the stomatal density and stomatal aperture size to enhance leaf carbon gain and water use efficiency. In addition to morphological traits of individual stoma, elevated CO_2 concentration also dramatically promoted the regularity of stomatal distribution pattern on leaves of green pepper under water stress as evidenced by the more regular stomatal distribution patterns on both leaf surfaces with smaller Lhat (d) values than those plants subjected to water stress. Overall, our results suggest that green pepper plants in response to elevated CO_2 concentration and water stress through modifying the number and opening of individual stoma and adjusting the spatial distribution pattern of stomata.

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Conflict of Interest

The authors declare no conflict of interest.

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