DOI: 10.1007/s11099-017-0753-9 PHOTOSYNTHETICA 56 (3): 942-952, 2018

Combined effects of elevated CO_2 concentration and drought stress on photosynthetic performance and leaf structure of cucumber (*Cucumis sativus* L.) seedlings

B.B. LIU*, M. LI**, Q.M. LI*,**,***,+ , Q.Q. CUI**, W.D. ZHANG**, X.Z. AI*,**, and H.G. BI*,**

*State Key Laboratory of Crop Biology, Tai'an, Shandong 271018, China**
*College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an, Shandong 271018, China***
*Scientific Observing and Experimental Station of Environment Controlled Agricultural Engineering in Huang-Huai-Hai Region, Ministry of Agriculture, Tai'an, Shandong 271018, China****

Abstract

Drought stress is one of the main environmental factors limiting plant growth and productivity of many crops. Elevated carbon dioxide concentration (eCO_2) can ameliorate, mitigate, or compensate for the negative impact of drought on plant growth and enable plants to remain turgid and functional for a longer period. In order to investigate the combined effects of eCO_2 and drought stress on photosynthetic performance and leaf structures, we analyzed photosynthetic characteristics and structure and ultrastructure of cucumber leaves. The decline in net photosynthetic rate under moderate drought stress occurred due to stomatal limitation alone, while under severe drought stress, it was the result of stomatal and nonstomatal limitations. Conversely, eCO_2 improved photosynthetic performance under moderate drought stress, increased the lengths of the palisade cells and the number of chloroplasts per palisade cell under severe drought stress, and significantly increased the grana thickness under moderate drought stress. Additionally, eCO_2 significantly decreased stomatal density, stomatal widths and stomatal aperture on the abaxial surface of leaves under moderate drought stress. In conclusion, eCO_2 can alleviate the negative effects of drought stress by improving the drought resistance of cucumber seedlings through stomatal modifications and leaf structure.

Additional key words: abiotic stress; chloroplasts; electron microscopy; mesophyll; starch accumulation; stomata.

Introduction

Global warming and increasing water shortages are two major environmental issues facing the sustainable development of the world's agriculture. Carbon dioxide is the major anthropogenic greenhouse gas and the atmospheric CO_2 concentration (aCO_2) has increased since 1750 as a result of human activity. The average aCO_2 has increased 1.75 μmol mol^{-1} per year between 1975 and today, reaching an average of 400 μmol mol^{-1} for the first time in April 2015 (NOAA 2015). In the absence of an explicit climate change policy, aCO_2 will reach 800 μmol mol^{-1} by the end of this century. The increasing aCO_2 drives global climate changes, including reducing water availability and elevating temperatures. These factors are expected to influence heavily food production in the coming years (Godfray and Garnett 2014). Drought stress is the main environmental factor limiting plant growth and the productivity of many crops (Araus *et al.* 2002, Chaves 2002). Elevated CO_2 concentration (eCO_2) has been shown

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Received 17 August 2016, *accepted* 29 May 2017, *published as online-first* 19 July 2017.

+Corresponding author; e-mail: gslqm@sdau.edu.cn

Abbreviations: aCO_2 – ambient $[CO_2]$; C – control nutrient solution; C_i – intercellular CO_2 concentration; $[CO_2]$ – CO_2 concentration; E – transpiration rate; eCO_2 – elevated $[CO_2]$; g_s – stomatal conductance; J_{max} – PAR-saturated rate of electron transport; M – moderate drought stress; N_{area} – area-based leaf nitrogen concentration; P_N – net photosynthetic rate; PBS – phosphate buffer saline; P_{Nmax} – CO_2 assimilation maximum rate; S – severe drought stress; TPU – rate of triose phosphate utilization; V_{cmax} – maximum rate of carboxylation by Rubisco.

Acknowledgments: This study was supported by National Natural Science Foundation of China (31471918), Natural Science Foundation of Shandong Province (ZR2013CM008), Colleges and Universities in Shandong Province Science and Technology Projects (J14LF06), and Major Application Technology Innovation Project of Shandong Province. We are very grateful to Berkley J. Walker (Institute for Genomic Biology, University of Illinois at Urbana–Champaign, USA) for helping us to revise our manuscript. We are grateful to the reviewers for their helpful comments and suggestions.

to ameliorate, mitigate, or compensate for some of the negative effects of drought on plant growth, and enable plants to remain turgid and functional for a longer time period (Centrito *et al.* 1999). Stomatal conductance (g_s) regulation is considered a major mechanism responsible for regulating plant responses to water stress because stomatal closure is one of the earliest response to water shortage and often the main limitation to photosynthetic carbon assimilation (Flexas *et al.* 2014). Stomatal control is regulated to optimize both the outward diffusion of water vapor from and the inward diffusion of CO_2 into the leaf during photosynthesis for a given water vapor pressure differential between the leaf and the atmosphere (Hetherington *et al.* 2003). Soil and plant water content decreases with water stress, but less so under eCO_2 compared to aCO_2. eCO_2 also stimulates photosynthesis, increasing the total linear electron transport and allowing greater partitioning of ATP and NADPH towards carbon fixation by minimizing photorespiration. These biochemical and physiological mechanisms allow eCO_2 to offset the risk of damage to the photochemical apparatus, allowing plants to better withstand soil water deficits under eCO_2 (Robredo *et al.* 2010). Despite these biochemical and eco-physiological mechanisms, there is still much unknown concerning how these responses are modified by other factors, such as drought, nitrogen limitation, and other environmental stressors. The combined effects of eCO_2 and drought stress on plant physiology have attracted wide attention (Wang *et al.* 2007). For example, reduced g_s and transpiration combined with the enhancement of carbon assimilation by eCO_2, increased instantaneous and whole plant water-use efficiency in both irrigated and drought-suffering barley plants, which delayed the onset and severity of the water deficit (Robredo *et al.* 2007).

Many studies have shown that eCO_2 increases the net photosynthetic rate (P_N) in many C_3 species by increasing intercellular CO_2 concentration (C_i), enhancing carboxylation efficiency, and reducing photorespiration (Drake *et al.* 1997, Centritto 2005). Additionally, plants decrease photosynthetic rates during long-term exposure to eCO_2 (Stitt 1999). Since the acclimation occurs often in potted but not in field-grown plants, the root growth restriction or the imbalance in the source–sink relationship of photosynthate has been considered as a major factor causing the acclimation (Arp 1991). Chen *et al.* (2005) reported that the photosynthetic acclimation is not caused by the decreased g_s, photosynthetic acclimation in rice leaves is related to both ribulose-1,5-bisphosphate (RuBP)-carboxylation limitation and RuBP-regeneration limitation (Chen *et al.* 2005). The transcript levels of photosynthesis-related genes decrease in plants acclimated to eCO_2, implying that elevated contents of sugars under eCO_2 may suppress the expression of photosynthesis-related genes, leading to decreases in growth (Cheng *et al.* 1998). Free-air CO_2 enrichment (FACE) experiments have provided ample evidence that the photosynthetic capacity acclimates to eCO_2 in C_3 plants, and the scale of the down-regulation varies with genetic and environmental factors. Although the examination of modulations in stomatal density and aperture were expected to help reveal how plants respond to eCO_2, stomatal responses to eCO_2 were found to differ between plant species (Drake *et al.* 1997). Thus, plant responses to eCO_2 appear to be complicated and species-specific (Sato and Yanagisawa 2014). However, despite the acclimation of the photosynthetic capacity, carbon gain is markedly greater (19–46%) in soybean plants grown at the [CO_2] anticipated for the middle of this century (Leakey *et al.* 2009).

eCO_2 can improve crop photosynthesis, growth, yield, and water-use efficiency due to a significant "fertilization effect" (Curtis and Wang 1998), but the "fertilization effect" of eCO_2 may be reduced under water deficits (Moore *et al.* 1999). The Intergovernmental Panel on Climate Change (IPCC) also noted that increased temperature and decreased soil moisture could result in crop yield reductions and offset the "fertilization effect of CO_2" (Parry *et al.* 2004). Therefore, the effect of eCO_2 on plants and ecosystems are not independent, but are interrelated to temperature, moisture, nutrients, and other environmental factors (Bai and Zhou 2004). There are many reports on the effects of eCO_2 and drought stress on plants in general (Baker and Allen 1993, Drake *et al.* 1997), and our previous research showed that eCO_2 could increase photochemical efficiency under drought stress and be beneficial to cucumber seedlings in a drought environment, thus alleviating or offsetting the negative consequences of global environmental changes (Li *et al.* 2008). Although the relationship between drought and eCO_2 is an area of active research, few studies have addressed the concurrent effects of eCO_2 and drought stress on photosynthetic performance and micro or ultra–structures of cucumber leaves, and how the changes in structure impact leaf functions.

Cucumber is the predominant vegetable grown in solar greenhouses in North China and is very sensitive to water stress. To avoid heat loss, solar greenhouses are seldom ventilated in the winter, so CO_2 deficit frequently occurs during vegetable production, limiting photosynthesis and yields in solar greenhouses (Yuan *et al.* 2009). The objective of this study was to explore the combined effects of eCO_2 and drought stress on photosynthetic performance, and microstructure and ultrastructure of leaves of cucumber seedlings, therefore, provide a theoretical basis for cucumber CO_2 fertilization in solar greenhouses and/or changed climate in the future.

Materials and methods

Plant material and growth conditions: Seeds of cucumber (*Cucumis sativus* L. cv. Jinyou No. 1) were surface-sterilized in 1% (v/v) hypochlorite solution for 15 min. After rinsing in distilled water, seeds were imbibed in water for 6–8 h and germinated for about 48 h in darkness at 26–28°C, and then sown in plastic pots placed in the greenhouse. The pots (785 cm^3, one seed per pot) contained a 3:1:1 (v/v/v) mixture of peat, perlite, and vermiculite. Representative seedlings with two true leaves were transferred and cultured hydroponically in darkened plastic containers (0.013 m^3, eight plants per container). Nine containers were placed randomly in each open-top-greenhouses, and supplied with full-strength Yamazaki cucumber nutrient solutions, which contained 0.5 mM $NH_4H_2PO_4$, 2.0 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 3.2 mM KNO_3, 1.0 mM $MgSO_4 \cdot 7\ H_2O$, and full-strength trace elements, were used. The electrical conductivity and pH of the nutrient solutions were maintained at 2.2–2.5 mS cm^{-1} and 6.8–7.0, respectively. The nutrient solutions were aerated every 2 h by air pumps, supplemented to original volumes every day, and refreshed every 2–3 days.

Treatments and experimental design: The experiment consisted of two [CO_2] and three water treatments, which were arranged in a randomized complete block split-plot design. The main plot was [CO_2], the subplot was water treatments. The CO_2 treatments included an ambient [CO_2] (aCO_2) of ~400 µmol mol^{-1}, designated A, and an eCO_2 of 800 ± 20 µmol mol^{-1}, designated E, each CO_2 treatment was replicated in two OTGs (area of 36 m^2, ridge height of 2.5 m) equipped with an environmental control system (*Auto Company*, Beijing, China) to supply CO_2 from a compressed CO_2 gas cylinder controlled by a solenoid valve. During the treatments, [CO_2] was monitored by a CO_2 dioxide monitor (Telaire 7001; *Hoyt Electrical Instrument Works Inc.*, NH, USA), and CO_2 was automatically injected into the greenhouse to maintain the target concentration. In addition, a *HOBO Data Logger* (*MicroDAQ.com, Ltd.*, NH, USA) was fixed in each greenhouse to record [CO_2] and air temperature every 30 min (Fig. 1S, *supplement available online*). The water treatments included control (nutrient solution, designated C), moderate drought stress (nutrient solution + 5% PEG 6000, $\psi_w = -0.05$ MPa, designated M), and severe drought stress (nutrient solution + 10% PEG 6000, $\psi_w = -0.15$ MPa, designated S). Each water treatment was replicated in 16 pots placed inside each open-top-greenhouse under each CO_2 treatment.

When the third true leaves of cucumber plants fully expanded, two OTGs were supplied CO_2 and maintained at 800 ± 20 µmol mol^{-1}, while the other two were maintained at the ambient [CO_2]. Upon CO_2 enrichment, PEG 6000 was dissolved in the nutrient solution to simulate rhizosphere drought stresses according to the design. In each greenhouse, drought stress treatments were applied randomly.

Leaf gas-exchange: After 7-d treatments, the uppermost fully expanded leaves were measured with six replications to measure the P_N, transpiration rate (E), g_s, and C_i between 09:00 and 10:00 h using a *Li-6400XT* portable photosynthesis system (*LiCor Inc.*, Lincoln, NE, USA) with the following settings: CO_2 reference = 400 µmol mol^{-1}; light = 1,100 µmol m^{-2} s^{-1}; leaf temperature = 25°C; and flow rate of 400 µmol s^{-1}.

Response of photosynthesis to C_i: Using a portable *LI-6400* system (*Li-Cor Inc.*, Lincoln, NE, USA), we measured P_N/C_i curves of the uppermost fully expanded leaves (about fifth or sixth true leaves developed during treatments), ambient CO_2 concentration of the cuvette (C_a) in the open gas-exchange chamber was reduced from 400 to 250, 200, 150, 100, and 50 mmol(CO_2) mol^{-1}, then increased from 50 to 250, 400, 600, 800; 1,000; and 1,200 mmol(CO_2) mol^{-1} at a constant saturating light intensity of 1,500 µmol(photon) m^{-2} s^{-1}. The P_N/C_i curves were fitted according to Sharkey *et al.* (2007), then assimilation rate at saturating CO_2 (P_{Nmax}), maximum rate of carboxylation by Rubisco (V_{cmax}), PAR-saturated rate of electron transport (J_{max}), and rate of triose phosphate use (TPU) were calculated by Sharkey *et al.*'s *Microsoft EXCEL* spreadsheet-based software (Sharkey *et al.* 2007).

Embedding and sectioning: Leaf embedding and sectioning were performed according to the method of Liu *et al.* (2010), with minor modifications. Healthy third fully expanded true leaves were selected and cut into 1-cm^2 squares at 18:00 p.m., and immediately put into 1% glutaraldehyde (using phosphate buffer saline, PBS, pH 6.8) under vacuum for 5 min. After the tissue sunk, they were fixed with 4% glutaraldehyde at 4°C for ~3 h. Samples were washed four to six times, each wash being 30 min, in PBS and post-fixed for 2 h with 1% osmium tetroxide at 4°C. Samples were then rinsed four to six times, each rinse being 30 min, with PBS to wash away excess fixative and then serially dehydrated for 30 min with 30, 50, 70, 80, 90, and 95% acetone. They were finally dehydrated with three washes of 100% acetone. After dehydration, they were penetrated with acetone: embedding medium mixtures of 3:1, 1:1, and 1:3 for ~3 h, 5 h, and 12 h, respectively, and then embedded into pure embedding medium twice for 24 h [embedding medium: 10 mL of epoxy resin *Epon 812* + 4 mL of dodecenyl succinic anhydride + 7 mL of methanmetamine + 0.3 mL of 2,4,6-tris (dimethylaminomethyl) phenol]. Polymerization was conducted for 12 h at 30°C, and then 48 h at 60°C.

Light microscope: After polymerization and trimming, the semithin slices (1,100 nm) were sectioned using a *Leica* ultramicrotome (*Leica Microsystems GmbH*, Wetzlar, Germany). Sections were dried at 60°C, stained for 25 s with 1% toluidine blue, then rinsed with distilled water and dried. The upper and lower epidermises, palisade, and spongy tissue were imaged by multifunction microscope (*BX51TR-32-FB3-E01*; *Olympus Co., Ltd.*, China) and the images were analyzed using *Image-Pro Plus* image processing and analysis software (*Media Cybernetics, Inc.*, MD, USA). Photos of three typical leaves were taken for semithin sections of each treatment at 1,000 × magnification.

Transmission electron microscope: Polymerized blocks were cut into ultrathin slices of 70 nm thickness using a *Leica* ultramicrotome and stained with uranyl acetate and lead citrate, then imaged using transmission electron microscopy (*JEM–1230*; *JEOL Ltd.*, Tokyo, Japan). To determine the chloroplast number, starch grain size, grana thickness, and grana layers, *Image–Pro Plus* image processing and analysis software (*Media Cybernetics, Inc.*, MD, USA) was used. Thirty representative images were selected for each treatment.

Scanning electron microscope: Healthy third fully expanded true leaves were cut into tissue blocks (2 × 2 mm) avoiding the main veins, and immediately put into 1% (w/v) glutaraldehyde (using PBS, pH 6.8) for 5 min for air drying. After the tissue blocks sunk, they were fixed with 4% (w/v) glutaraldehyde for ~3 h at 4°C, then washed by PBS four to six times, with each wash lasting 30 min, the gradient dehydrated for 30 min with 30, 50, 70, 80, 90, and 95% acetone, and then finally dehydrated with 100% acetone three times for 30 min. After being dehydrated with 100% acetone, the solution was replaced with isoamyl acetate twice for 30 min, and then the blocks were dried with a *Hitachi HCP–2*-type critical point dryer. Samples were gold-,coated and the abaxial surface imaged using a *JSM–6360LV* scanning electron microscope (*JEOL Ltd.*, Tokyo, Japan). Stomatal densities, sizes, and stomatal aperture (degree of stomatal opening) were measured using *Smile View* software. Thirty representative images were selected for each treatment.

Statistical analysis: For the overall mean comparisons between treatments for the parameters, analyses of variances (factorial) were performed using *SPSS 13.0* for *Windows* (*SPSS Inc.*) with a univariate command of the general linear model process. *Duncan*'s post-hoc test was used to denote the significant differences at $P<0.05$ for the interactions of all of the factors.

Results

Effects of eCO$_2$ on the photosynthesis: Drought stresses decreased P_N, E, and g_s of cucumber leaves significantly under both aCO$_2$ and eCO$_2$. But eCO$_2$ increased the P_N significantly by 16.9, 26.1, and 31.5% under C, M, and S drought stress conditions, respectively, therefore, offset some of the impact of drought in P_N. eCO$_2$ also decreased E significantly through decreased g_s, reduced by 17.2, 22.2, and 22.0%, respectively, under three different water conditions (Fig. 2S, *supplement available online*).

Biochemical capacity for photosynthesis: Increased drought stress decreased P_{Nmax}, V_{cmax}, J_{max}, and TPU significantly. Additionally, there were significant differences between the different water stress conditions in the response to eCO$_2$ (Fig. 1; Fig. 5S, *supplement available online*). Under C conditions, eCO$_2$ significantly enhanced P_{Nmax}, V_{cmax}, J_{max}, and TPU by 12.7, 12.0, 14.7, and 9.9%, respectively, compared with aCO$_2$. Under mild and severe drought stresses, eCO$_2$ increased the values of those parameters slightly but not significantly.

Leaf blade thickness: Drought stresses decreased palisade layers thickness significantly, but eCO$_2$ increased the thickness significantly under all water treatments (Table 1). Drought stress significantly reduced the spongy layers thickness under aCO$_2$ and eCO$_2$, while eCO$_2$ significantly increased the spongy layers thickness only under M stress. Thus, the reduced blade thickness under drought stress was caused by the decreased thickness of both palisade and spongy layers, while the significant increase in the blade thickness under eCO$_2$ was mainly owing to the significant increase in the palisade layers thickness (Table 1, Fig. 2).

Mesophyll cell size: The lengths and widths of palisade cells and sponge cells gradually decreased as drought stress got severer. Both M and S stress significantly reduced cell lengths, while cell widths were reduced significantly only under S stress. The lengths of palisade cells significantly increased, while the widths and the sponge cell sizes did not significantly changed under eCO$_2$ (Table 1S, *supplement available online*).

Chloroplast number in mesophyll cells and starch grain number per chloroplast: The numbers of chloroplasts in palisade and spongy cells were reduced significantly under drought stress. More severe the stress, the lesser number of chloroplasts. While M and S stress had no significant effect on chloroplast number in spongy cells under eCO$_2$, eCO$_2$ increased the numbers of chloroplasts in palisade and spongy cells significantly in the absence of water stress, and also significantly increased the number of chloroplasts in palisade cells under S stress. There was significant increase in the starch grain numbers per chloroplast under the condition of drought stress and eCO$_2$ (Table 2).

Chloroplast size and starch grain size: Drought stress and eCO$_2$ did not only affect the numbers of chloroplasts and starch grains, but also their sizes. M stress reduced chloroplast lengths significantly, while S stress simultaneously reduced the lengths and widths significantly. eCO$_2$ had significant effect on chloroplast lengths only under S stress. Drought stress increased starch grain size significantly, while eCO$_2$ increased starch grain length significantly under S stress, and increased starch grain width and starch content under all water status (Table 2S, *supplement available online*; Fig. 3; Fig. 6S, *supplement available online*). Under S stress, grana thickness and the number of stroma lamella decreased significantly, while grana thickness was significantly increased by eCO$_2$ under C and M stress. The number of stroma lamella under C conditions were also significantly increased by eCO$_2$ (Table 3S, *supplement available online*; Fig. 4).

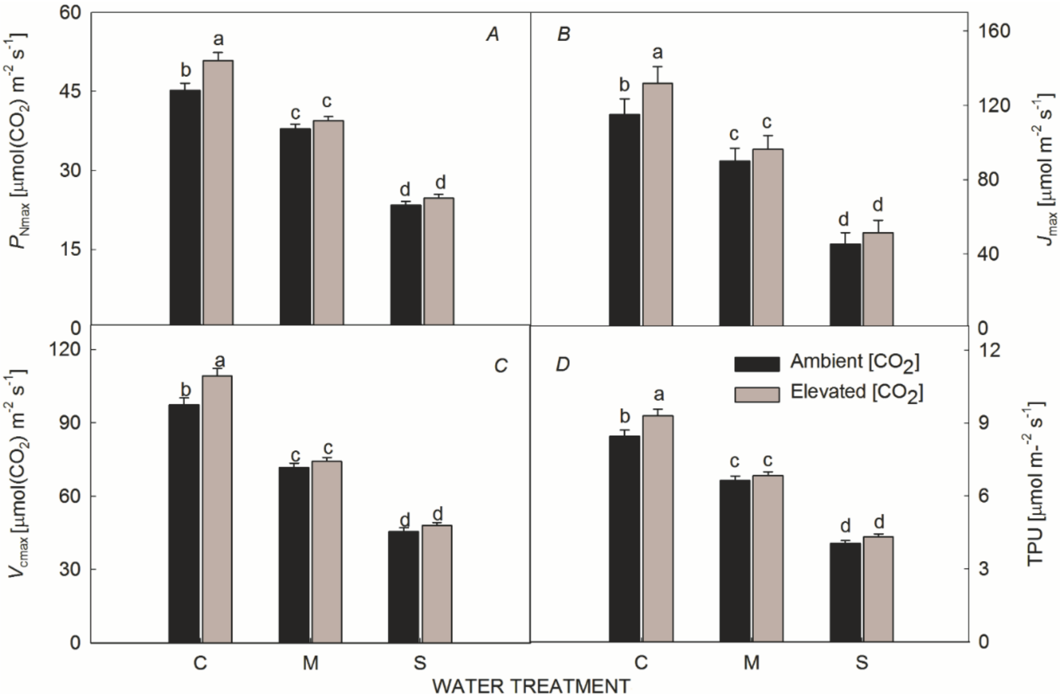

Fig. 1. Effect of drought stress and elevated CO$_2$ on the P_N/C_i curve and relative parameters of cucumber seedlings. (*A*) Assimilation rate at saturating CO$_2$ (P_{Nmax}), (*B*) PAR-saturated rate of electron transport (J_{max}), (*C*) maximum rate of carboxylation by Rubisco (V_{cmax}), and (*D*) rate of triose phosphate use (TPU).

Table 1. Effect of drought stress and elevated CO$_2$ on the blade thickness of cucumber seedling leaves. Different *lowercase letters* are statistically different at the $P<0.05$ level. EC, AC, EM, AM, ES, and AS represent treatments, where A – ambient CO$_2$ concentration; E – elevated CO$_2$ concentration; C – control; M – medium drought stress; S – severe drought stress.

Treatment	Upper epidermis thickness [μm]	Lower epidermis thickness [μm]	Palisade tissue thickness [μm]	Spongy tissue thickness [μm]	Blade thickness [μm]
EC	19.39 ± 0.83^a	18.88 ± 0.85^a	117.90 ± 5.07^a	94.60 ± 3.84^a	250.77 ± 10.77^a
AC	19.28 ± 0.85^a	18.82 ± 0.79^a	107.49 ± 4.39^b	89.20 ± 4.41^{ab}	234.79 ± 10.12^b
EM	19.04 ± 0.79^a	18.70 ± 0.88^a	96.70 ± 4.47^c	84.00 ± 5.57^b	218.44 ± 11.34^c
AM	18.93 ± 0.99^a	18.61 ± 0.93^a	86.30 ± 6.23^d	78.80 ± 6.21^c	202.64 ± 12.07^d
ES	18.76 ± 0.91^a	18.50 ± 0.91^a	78.80 ± 5.58^e	71.70 ± 3.39^{cd}	187.76 ± 10.88^e
AS	18.71 ± 0.89^a	18.46 ± 0.97^a	70.00 ± 6.27^f	67.50 ± 4.35^d	174.67 ± 12.46^f

Stomatal properties: There were different responses in stomata density to drought stress and eCO$_2$, on the adaxial and abaxial leaf surface of cucumber seedlings. When drought stress got severer, significant increases were observed in stomata density on adaxial and abaxial leaf surface, while the stomata density on abaxial leaf surfaces was significantly decreased by eCO$_2$ under C and M conditions. There was no difference observed in the stomata length on the adaxial leaf surfaces between treatments, but drought stress significantly affected the stomata lengths on the abaxial leaf surfaces under aCO_2, but not under eCO_2. eCO_2 only reduced stomata lengths on the abaxial leaf surface under C conditions. Drought stress significantly reduced the stomata widths of adaxial and abaxial leaf surfaces, while eCO_2 only significantly reduced the stomata width on the abaxial leaf surface. Drought stresses significantly reduced stomata aperture on adaxial and abaxial leaf surfaces, and eCO_2 significantly decreased the stomata aperture of both epidermises under C conditions, whereas eCO_2 only decreased the stomata aperture significantly on the abaxial leaf surface under M stress (Table 3, Fig. 3S, *supplement available online*; Fig. 5).

Discussion

Photosynthesis: In this study, the decrease of P_N in cucumber leaves resulted from mainly stomatal limitations under M stress, while the decrease of P_N under S stress was attributed to combined consequence of stomatal and nonstomatal limitations. The nonstomatal limitation could mostly owe to larger starch grain size or more starch grains in chloroplast. In the short term, C_3 plants appear to respond to eCO_2 exclusively through the direct effects of increased carboxylation by Rubisco and decreased stomatal opening (Long *et al.* 2004). eCO_2 maintained a suitable C_i or improved the activity of Rubisco and carboxylation direction. It is consistent with the reduction in g_s reported for 28 species growing at eCO_2 and that a reduction in the g_s improves the water balance, which delays the onset of midday water stress, extends the most active photosynthesis period, and increases the soil water content (Drake *et al.* 1997).

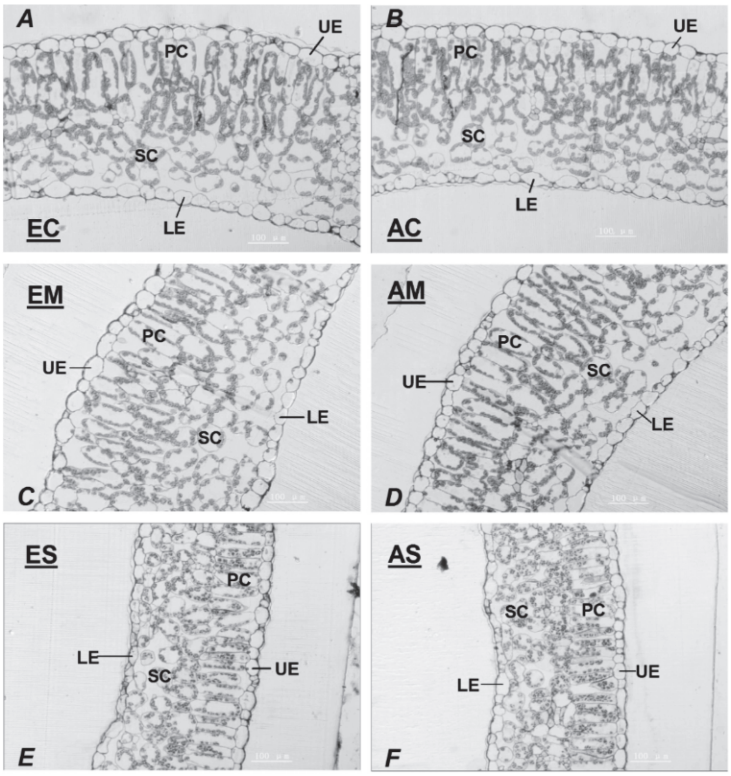

Fig. 2. Effect of drought stress and elevated CO_2 on cucumber leaf microstructure. UE – upper epidermis cell; LE – lower epidermis cell; PC – palisade cell; SC – spongy cell. *A, B, C, D, E*, and *F* represent EC, AC, EM, AM, ES, and AS treatments, where A – ambient CO_2, E – elevated CO_2, and C – control; M – medium drought stress; S – severe drought stress. 1,000×, scale bars = 100 µm.

Table 2. Effect of drought stress and elevated CO_2 on chloroplast numbers in mesophyll cells and starch grain numbers per chloroplast in cucumber seedling leaf cells. EC, AC, EM, AM, ES, and AS represent treatments, where A – ambient CO_2 concentration; E – elevated CO_2 concentration; C – control; M – medium drought stress; S – severe drought stress.

Treatment	Palisade cell number	Spongy cell number	Starch grains number per chloroplast
EC	12.70 ± 0.55^a	9.90 ± 0.22^a	2.90 ± 0.11^d
AC	10.80 ± 0.47^b	7.40 ± 0.31^b	2.40 ± 0.09^e
EM	9.10 ± 0.42^c	6.50 ± 0.37^c	4.20 ± 0.12^b
AM	8.30 ± 0.46^c	6.00 ± 0.25^{cd}	3.40 ± 0.13^c
ES	7.80 ± 0.24^c	5.40 ± 0.23^d	5.40 ± 0.11^a
AS	6.60 ± 0.31^d	4.80 ± 0.31^d	4.50 ± 0.18^b

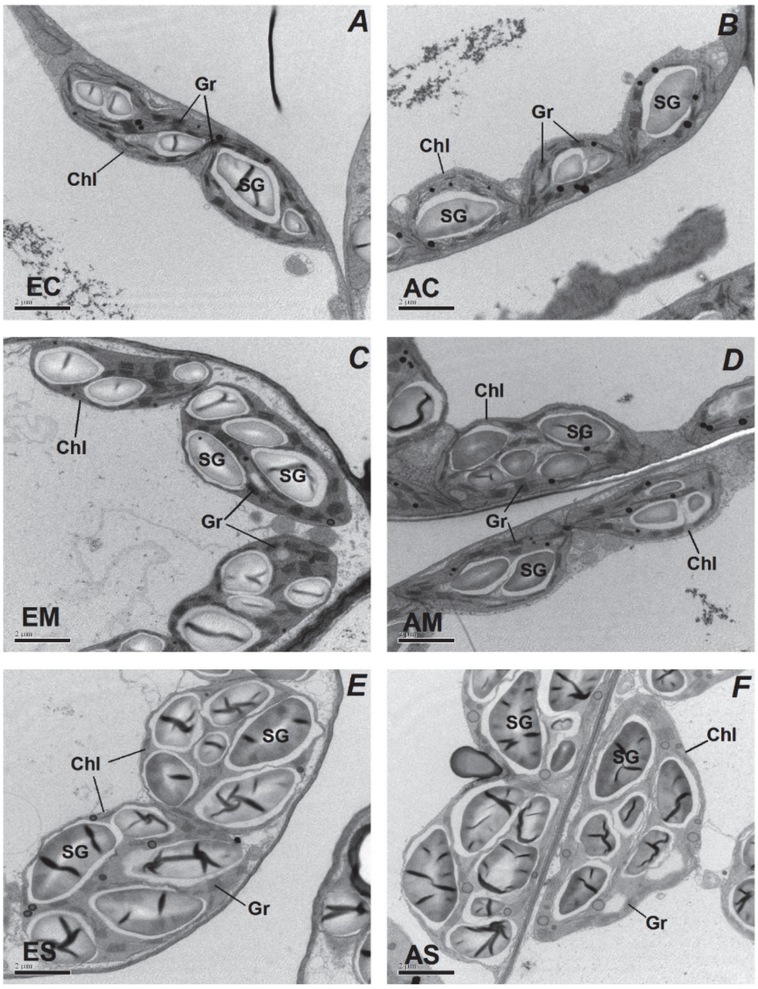

Fig. 3. Effect of drought stress and elevated CO_2 on the submicroscopic structure of cucumber seedling leaf cells. *A, B, C, D, E*, and *F* represent EC, AC, EM, AM, ES and AS treatments, where A – ambient CO_2, E – elevated CO_2, and C – control; M – medium drought stress; S – severe drought stress. Chl: chloroplast; Gr: grana; SG: starch grain. 10,000×, scale bars = 2 µm.

Photosynthesis is determined by the rate of RuBP regeneration and increases as [CO_2] increases because lower contents of ATP and NADPH are diverted into the photorespiratory metabolism and, therefore, more is available for CO_2 assimilation (Long *et al.*2004). We found that eCO_2 enhanced P_{Nmax}, V_{cmax}, J_{max}, as well as TPU of cucumber leaves under control condition, which means that eCO_2 can improve P_N by promoting the electron transport and photophosphorylation under no drought stress condition, while there were no differences under drought stresses. Free-air CO_2 enrichment (FACE) experiments on various C_3 plant species had shown that eCO_2 stimulated leaf photosynthesis and photosynthetic carbon gain (Long *et al.* 2004, Leakey *et al.* 2009). However, the stimulation of photosynthesis and yield is much lower than expected, owing to plant acclimation responses to eCO_2 (Long *et al.* 2004). Long-term exposure to eCO_2 can cause photosynthetic down-regulation, typically a decrease in V_{cmax}, accompanied by a reduction in area-based leaf nitrogen concentration (N_{area}) and an accumulation of carbohydrates, especially when nitrogen supply is limited or rooting volumes are restricted (Sage 1994, Drake *et al.* 1997, Sims *et al.* 1998, Stitt and Krapp 1999). Photosynthetic acclimation in rice leaves to eCO_2 is related to both RuBP carboxylation limitation and RuBP regeneration limitation (Chen *et al.* 2005), while in wheat leaves it is largely due to RuBP carboxylation limitation (Zhang *et al.* 2009). Kitao *et al.* (2007) reported that the offsetting responses—reduced V_{cmax} plus increased C_i at eCO_2 and increased V_{cmax} plus reduced C_i under drought conditions—resulted in a narrow range of susceptibility to photoinhibition at the growth [CO_2] in Japanese white birch seedlings grown in various water availability × [CO_2] treatment combinations (Kitao *et al.* 2007). We speculate that the differences mainly due to our treatments were short-term (7-d treatment) exposure to eCO_2, that the seedlings were not yet acclimated to it. Our previous study showed that eCO_2 can increase the maximal quantum yield of PSII, and reduce nonphotochemical quenching under all of the water conditions to alleviate the negative effects of drought stress on cucumber (Li *et al.* 2008). Therefore, it is required to investigate further cucumber seedlings under longer-term exposure to eCO_2.

Stomatal regulation is one of the key mechanisms of plant adaptation to drought stress (Guan *et al.* 1995). Plant stomata display a wide range of short-term behavioral and long-term morphological responses to [CO_2]. Changes in stomatal number changed the P_N and water-use efficiency of plants (Berryman *et al.* 1994), thus any change in stomatal number caused by the eCO_2 affects photosynthesis (Douville *et al.* 2000). This study showed that drought stress increased the stomatal density due to the reduced leaf area (Fig. 4S, *supplement available online*), but reduced the stomatal size and the stomatal aperture. Yang *et al*. (1997) reported that doubled [CO_2] increased

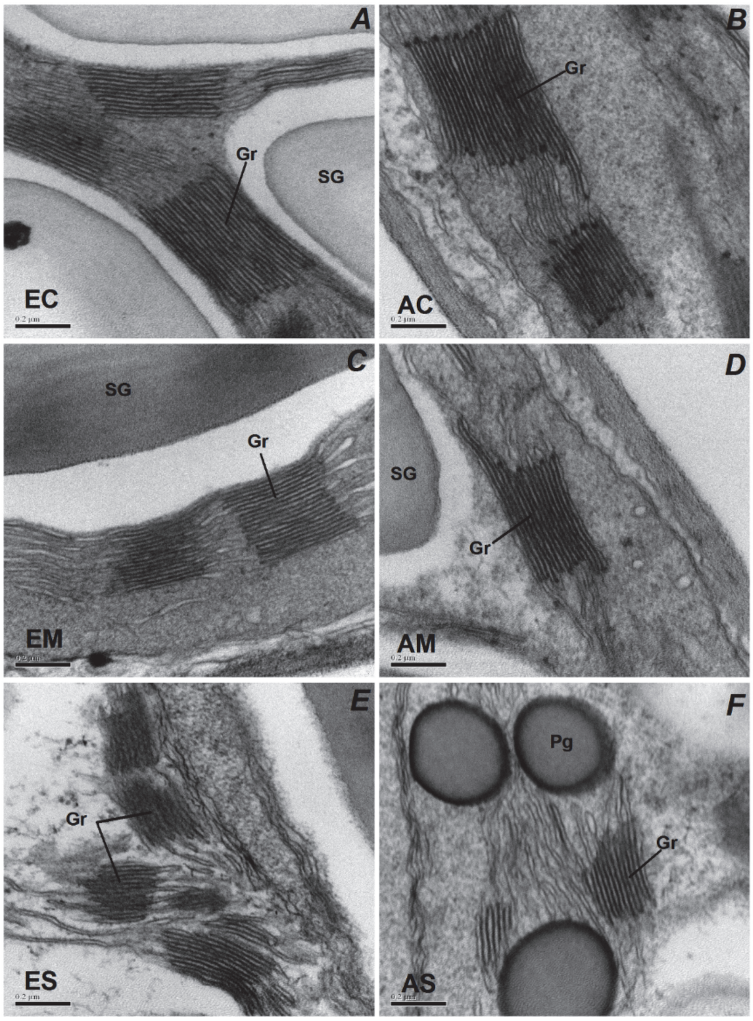

Fig. 4. Effect of drought stress and elevated CO_2 on the ultrastructure of cucumber seedling leaf cells. *A, B, C, D, E*, and *F* represent EC, AC, EM, AM, ES, and AS treatments, where A – ambient CO_2, E – elevated CO_2, and C – control; M – medium drought stress; S – severe drought stress. Gr: grana; SG: starch grain; Pg: plastoglobule. 100,000×, scale bars = 0.2 µm.

Table 3. Effect of drought stress and elevated CO_2 on the stomatal properties of cucumber seedling leaf cells. EC, AC, EM, AM, ES, and AS represent treatments, where A – ambient CO_2 concentration; E – elevated CO_2 concentration; C – control; M – medium drought stress; S – severe drought stress.

Leaf surface	Treatment	Stomatal density [No. mm^{-2}]	Stomata length [µm]	Stomata width [µm]	Stomatal aperture [µm]
Adaxial surface of leaves	EC	289.71 ± 22.46^c	20.91 ± 0.90^a	12.83 ± 0.55^{ab}	1.36 ± 0.06^b
	AC	300.00 ± 22.90^c	21.06 ± 0.91^a	13.38 ± 0.48^a	1.44 ± 0.06^a
	EM	317.30 ± 23.64^b	20.21 ± 0.89^a	12.17 ± 0.49^b	1.23 ± 0.05^c
	AM	324.00 ± 23.91^b	20.54 ± 0.87^a	12.46 ± 0.51^b	1.28 ± 0.06^c
	ES	351.79 ± 19.14^a	20.09 ± 0.81^a	11.82 ± 0.55^c	1.05 ± 0.07^d
	AS	358.00 ± 19.38^a	20.16 ± 0.92^a	11.91 ± 0.56^c	1.09 ± 0.11^d
Abaxial surface of leaves	EC	564.00 ± 21.27^d	19.05 ± 0.91^b	12.53 ± 0.49^{ab}	2.20 ± 0.09^b
	AC	600.11 ± 24.81^c	19.42 ± 0.83^a	13.02 ± 0.51^a	2.46 ± 0.08^a
	EM	613.90 ± 21.40^c	18.90 ± 0.87^{bc}	11.43 ± 0.53^c	1.74 ± 0.08^c
	AM	663.00 ± 20.51^b	18.99 ± 0.94^b	12.23 ± 0.54^b	2.11 ± 0.10^b
	ES	724.27 ± 21.14^a	18.68 ± 0.92^c	10.40 ± 0.54^d	1.51 ± 0.08^d
	AS	752.00 ± 22.34^a	18.70 ± 0.87^c	11.12 ± 0.48^c	1.62 ± 0.07^{cd}

leaf thickness and number of chloroplasts, and decreased average stomatal density and stomatal index significantly (Yang *et al*. 1997). We found that eCO_2 decreased stomatal density of abaxial surface of cucumber leaves (Table 6; Figs. 7, 8), suggests that [CO_2] can control stomatal opening (Berryman *et al.* 1994) and the number of stomata (Woodward 1987). The improved growth rate of plants with substantially decreased stomatal density perhaps reflects a combination of improved tissue water status from lower *E*, improved CO_2 assimilation rate from favorably higher leaf temperature, and lower metabolic cost from developing and operating less guard cells (Franks *et al.* 2009). A genetic component of stomatal development responding to doubled [CO_2] has been identified (Gray *et al.* 2000), and the control of the [CO_2] through stomata occurs in the early stages of leaf development (Donnelly *et al.* 1999). Nevertheless, further work is required to establish any relationship between the degree of stomatal density response and stomatal external [CO_2] sensitivity in plants grown across a range of [CO_2]. The relationship between the degree of stomatal aperture control to eCO_2 and the extent of any reduction in stomatal density may suggest the co-ordination of physiological and morphological responses of stomata to [CO_2] in the optimization of water-use efficiency.

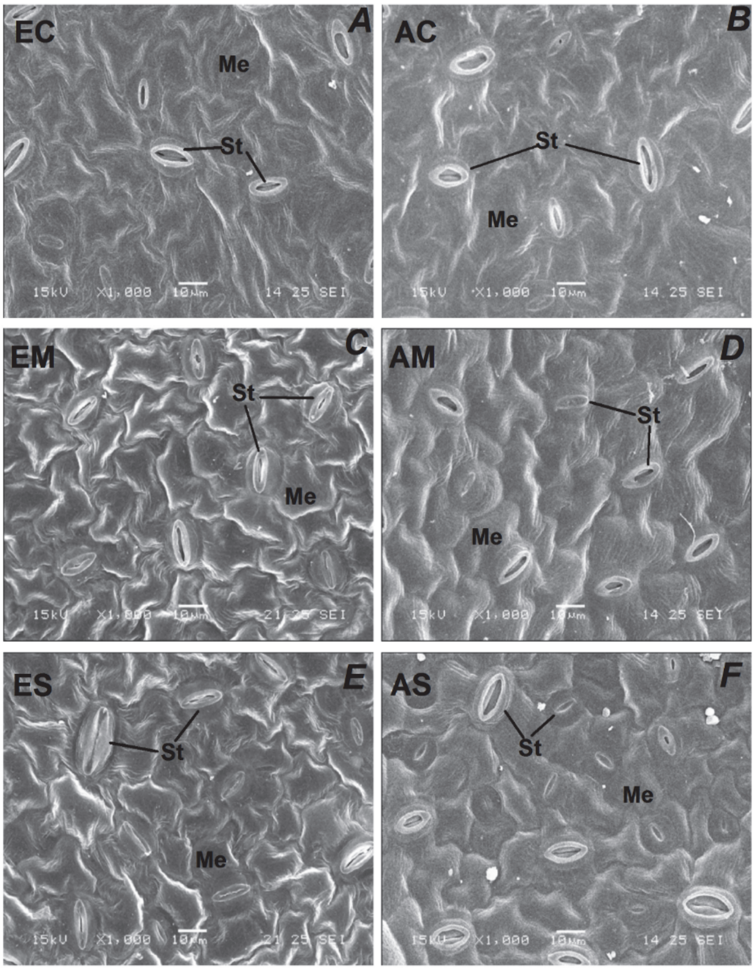

Fig. 5. Effect of drought stress and elevated CO_2 on abaxial surface of cucumber seedling leaves. *A, B, C, D, E*, and *F* represent EC, AC, EM, AM, ES, and AS treatments, where A – ambient CO_2, E – elevated CO_2, and C – control; M – medium drought stress; S – severe drought stress. St: stomata; Me: mesophyll cell. 1,000×, scale bars = 10 µm.

Response of cucumber leaf structures to drought stress and eCO_2: Leaf anatomy is often altered by eCO_2, and increases in the total leaf area per plant, single-leaf area, and leaf thickness have been reported for many species (Pritchard *et al.* 1999). There are different crop-specific adaptive strategies to eCO_2 involving leaf structure; cell division and expansion are likely to be the causes of these changes. Our results in cucumber showed that the thickness of cucumber seedling leaves was reduced under drought stress mainly because of the simultaneous decrease of palisade and spongy layers thickness; eCO_2 increased leaf thickness mainly due to the increase of palisade layers thickness. This is consistent with the report that the increase in soybean leaf thickness was caused by the well-developed palisade cell layer and an increase in the cell number (Vu *et al.* 1989). The lower leaf surface of soybean grown under eCO_2 environment was covered with a wax layer of cuticle surface, the leaf became thicker owing to an additional layer of palisade mesophyll tissue, and the stomatal density decreased (Lin and Hu 1996). But

Radoglou and Jarvis (1992) reported that eCO_2 increased the leaf thickness of kidney bean, mainly due to changes in the spongy tissue, however, the ratio of palisade and spongy tissues did not change (Radoglou and Jarvis 1992). The divergence may be attributed to different responding mechanisms in diverse species of crop growing under varied environmental conditions.

Levitt (1980) pointed out that drought stress reduced chloroplast activity, probably owing to the denaturation of chloroplast membrane proteins, leading to the destruction of thylakoids. Wu and Li (2001) reported that drought stress can cause swelling of the chloroplast thylakoid in wheat, and severe soil drought stress can disintegrate the thylakoid gradually, resulting in the chloroplast envelope being ruptured and the matrix is spilling out, thus abolishing chloroplast activity (Wu and Li 2001). eCO_2 promoted the development of chloroplast thylakoid membrane systems in alfalfa, increased grana size, and the thylakoid membranes of grana and stroma being arranged in ordered structures, which provides a theoretical basis for how eCO_2 increase P_N, biomass yields, and grain yields of the majority of C_3 plants (Zuo *et al.* 1996). We found that grana thickness and lamellae numbers decreased significantly as drought stress got sever, while eCO_2 had a significant increase effect on the thickness of grana under control and moderate drought stress, as well as the lamellae number under control. The P_N of cucumber leaves increased under eCO_2, which can manufacture and accumulate more carbohydrates, laying the basis for the development of the leaf structures (Wei *et al.* 2002). Long-term growth under eCO_2 also causes excessive accumulation of leaf starch, which can distort grana or directly damage chloroplasts (Wulff and Strain 1982). Continuous and excessive starch grain accumulation can destroy plants by causing leaf chlorosis, necrosis, and premature aging. Carbohydrate accumulation in leaves when there is an imbalance between source and sink at the whole plant level can lead to decreased expression of photosynthetic genes and accelerated leaf senescence (Paul and Foyer 2001). Although eCO_2 increased the P_N while drought stress reduced the P_N (Li *et al.* 2008), this study showed that starch grains accumulated significantly under drought stress as well as eCO_2 conditions. eCO_2 enrichment increased the numbers and sizes of starch grains in tomato leaves, while thylakoid deformation was limited to a few chloroplasts in the phloem cells (Yelle *et al.* 1989). The numbers and sizes of starch grains in millet and alfalfa increased under eCO_2, which affected the grana size, distribution, and lamella number in chloroplasts (Zuo *et al.* 1996). Changes in the microscopic and ultramicroscopic structures of cucumber leaves under eCO_2 may be adaptive mechanisms to eCO_2, enhancing light absorption and both water- and CO_2-use capacity under drought stress. Because the influences of eCO_2 on the transcriptome, metabolome, and nitrogen assimilation are dependent on the plant's developmental stage, further studies are required to comprehensively clarify the adaptive mechanism of cucumber plants exposed to drought stress and eCO_2 in the whole growth period.

Conclusion: Under drought stress conditions, eCO_2 can change leaf tissue structures, improve photosynthetic performance, ameliorate water status and improve drought resistance of cucumber seedlings, thus alleviating the negative effects of drought stress.

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