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24-epibrassinolide improves cucumber photosynthesis under hypoxia by increasing CO_2 assimilation and photosystem II efficiency

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Abstract

Seedlings of the hypoxia-sensitive cucumber cultivar were hydroponically grown under hypoxia for 7 d in the presence or absence of 24-epibrassinolide (EBR, 2.1 nM). Hypoxia significantly inhibited growth, while EBR partially counteracted this inhibition. Leaf net photosynthetic rate (P_N), stomatal conductance, transpiration rate, and water-use efficiency declined greatly, while the stomatal limitation value increased significantly. The maximum net photosynthetic rate was strongly reduced by hypoxia, indicating that stomatal limitation was not the only cause of the P_N decrease. EBR markedly diminished the harmful effects of hypoxia on P_N as well as on stomata openness. It also greatly stimulated CO_2 fixation by the way of increasing the carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), ribulose-1,5-bisphosphate regeneration, Rubisco activity, and the protection of Rubisco large subunit from degradation. Our data indicated that photosystem (PS) II was damaged by hypoxia, while EBR had the protective effect. EBR further increased nonphotochemical quenching that could reduce photodamage of the PSII reaction center. The proportion of absorbed light energy allocated for photochemical reaction (P) was reduced, while both nonphotochemical reaction dissipation of light energy and imbalanced partitioning of excitation energy between PSI and PSII increased. EBR increased P and alleviated this imbalance. The results suggest that both stomatal and nonstomatal factors limited the photosynthesis of cucumber seedlings under hypoxia. EBR alleviated the growth inhibition by improving CO_2 asimilation and protecting leaves against PSII damage.

Additional key words: brassinosteroids; chlorophyll content; CO_2-response curve; light energy allocation; light-response curve; photosynthesis.

Introduction

Brassinosteroids (BRs), a recently-recognized class of phytohormones, are plant polyhydroxysteroids that play essential roles in plant development. There is an ample evidence that BRs can promote DNA, RNA, and protein synthesis, and can enhance photosynthesis and the activities of various enzymes, such as ATPase and sucrose synthase. Furthermore, BRs have the ability to protect plants from various biotic and abiotic stresses, including

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Abbreviations: AQY – apparent quantum yield; BRs – brassinosteroids; CCP – CO_2 compensation point; CE – carboxylation efficiency; Chl – chlorophyll; C_i – intercellular CO_2 concentration; D – fraction of absorbed photon energy thermally dissipated; E – transpiration rate; EBR – 24-epibrassinolide; ETR – electron transport rate; Ex – photon energy absorbed by PSII antennae and trapped by closed PSII reaction centers; F_0 –minimal fluorescence yield of dark-adapted leaf; F_0' –minimal fluorescence yield of light-adapted leaf; F_m –maximal fluorescence yield of dark-adapted leaf; F_m' –maximal fluorescence yield of light-adapted leaf; F_s –steady-state fluorescence yield; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; J_{max} – maximum RuBP regeneration rates; LCP – light compensation point; L_s – stomatal limitation value; LSP – light saturation point; NPQ – nonphotochemical quenching; P – fraction of photon energy absorbed by PSII antennae trapped by open PSII reaction centers; P_N – net photosynthetic rate; P_{Nmax} – maximum net photosynthetic rate; PS – photosystem; RBCL – Rubisco large subunit; RBCS – Rubisco small subunit; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose-1,5-bisphosphate; V_{cmax} – *in vivo* maximum rate of Rubisco carboxylation; WUE – water-use efficiency; α – proportion of excitation energy captured by PSI; β – proportion of excitation energy captured by PSII; β/α–1 – relative deviation from full balance between the two photosystems; Φ_{PSII} – effective quantum yield of PSII photochemistry.
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those arising from water, heat, heavy metals, and herbicides (Bajguz and Hayat 2009). BRs may improve plant tolerance to various stressors by regulating antioxidant systems (Liu *et al.* 2009, Ogweno *et al*. 2008) and photosynthesis (Holá 2011). The transcript levels of *rca*, *rbcS*, and *rbcL* genes were elevated in EBR-treated plants, but reduced in plants treated with brassinazole, a specific inhibitor of BRs biosynthesis (Xia *et al*. 2009).

Hydroponics is the sophisticated agricultural technology that is used increasingly. Since the root-zone oxygen deficiency is one of the major factors limiting yields in hydroponic culture, the possible method to improve the yield is enhancement of plant root tolerance to low oxygen. Our previous research revealed that EBR could stimulate both photosynthate allocation to the roots and the activities of major glycolytic enzymes, and thus alleviate oxidative damage caused by hypoxic stress in cucumber plants (Kang *et al*. 2009). However, the mechanism of its action in photosynthesis under hypoxic stress needed further investigation. To address this question, we used the hypoxia-sensitive cucumber genotype (*Cucumis sativus* L. cv. Zhongnong 6) to investigate the effect of EBR on photosynthetic performance and to determine its mechanism of action.

Materials and methods

Plants and treatments: All experiments were performed in a greenhouse at Nanjing Agricultural University, China. EBR was purchased from *Sigma-Aldrich* (MO, USA). The seeds of *Cucumis sativus* L. cv. Zhongnong 6, the hypoxia-sensitive cultivar, were surface-sterilized with 0.5% (w/v) NaClO solution for approximately 15 min, and then washed three times with sterile distilled water. The seeds were placed on a filter paper moistened with distilled water in sterile Petri dishes, and allowed to germinate in a thermostatically-controlled chamber at 28 ± 2°C. The germinated seeds were then sown in quartz sand. The average day/night temperatures were 27–30°C/18–20°C. Seedlings were watered with half-strength Hoagland nutrient solution every 2 d. At the 2nd true-leaf stage, the seedlings were transplanted into plastic vessels containing 20 L of half-strength Hoagland nutrient solution (pH 6.3 ± 0.1, electrolytic conductivity of 2.0–2.2 dS m^{-1}, 20–25°C). The seedlings received one of the following 4 treatments in triplicate until a new leaf emerged:

Seedlings	Dissolved oxygen [mg L^{-1}]	Treatment
Control	7.0–8.0	Vigorous aeration 40-min per h
Control + EBR	7.0–8.0	Cultured in half-strength Hoagland nutrient solution with 2.1 nM EBR
Hypoxic	0.9–1.1	Using *Quantum-25* dissolved oxygen controller (*Quantum Analytical Instruments*, USA) and flushing the solution with N_2 instead of air
Hypoxic + EBR	0.9–1.1	Cultured in nutrient solution containing 2.1 nM EBR

The treatments were carried out for 7 d, then growth properties, photosynthetic gas exchange, and chlorophyll (Chl) fluorescence parameters of the leaves were measured. Fifteen plants per treatment were collected for plant growth determination, and at least five independent leaves were used to measure photosynthetic parameters.

Plant growth: The plants were harvested at 0 and 7 d for biomass and leaf-area analysis. The 4th leaf was emerged when the treatments began and its growth was affected by the stress from the very beginning of the treatment, thus, the area, length, and width of the 4th leaf of each plant was measured using the *Epson Expression 1680* tabletop scanner and *WinRHIZO* image analysis software (*Regent Instruments*, Canada).

Chl content was determined following the method of Lichtenthaler *et al.* (1996). Eight leaf discs (0.58 cm^2 per disc) from the 4th leaf of each fresh plant were placed in 15 mL tubes containing 80% acetone. The tubes were kept in darkness for about 48 h until the discs were completely colorless. Absorptions of the extracts at 663 nm, 646 nm, and 470 nm were measured using a *UV-2450* spectrophotometer (*Shimadzu*, Japan). Chl *a*, Chl *b*, Chl (*a*+*b*) concentrations and the Chl *a/b* ratio were calculated according to Lichtenthaler *et al*. (1996).

Gas exchange: Photosynthetic gas-exchange parameters were measured from 8:30–11:30 h using the *LI-6400* portable photosynthesis system (*LI-COR*, USA) equipped with the red/blue LED light source. During measurements, relative air humidity was maintained at about 60% and leaf temperature at 25°C in the leaf chamber. The airflow rate was set to 500 μmol mol^{-1}. The fourth leaf was used for these measurements. The stomatal limitation value (L_s) and the water-use efficiency (WUE) were calculated as follows: $L_s = 1 - C_i/C_a$ (C_a: atmospheric CO_2 concentration, 400 ± 10 μmol mol^{-1}; C_i: intercellular CO_2 concentration); WUE = P_N/E (Bertolde *et al*. 2012). To measure the light-response curve, photosynthetic photon flux density (PPFD) was set at 1,800; 1,500; 1,200; 1,000; 800, 500, 300, 200, 150, 100, 50, 25, and 0 μmol m^{-2} s^{-1}, respectively. To construct the CO_2-response curve, PPFD was set at 1,200 μmol m^{-2} s^{-1} and CO_2 concentration at 50, 100, 120, 150, 200, 380, 600, 800; 1,000; and 1,200 μmol mol^{-1}, respectively. The maximum net photosynthetic rate

(P_{Nmax}), light saturation point (LSP), light compensation point (LCP), *in vivo* maximum rate of Rubisco carboxylation (V_{cmax}), maximum RuBP regeneration rates (J_{max}), CO_2 compensation point (CCP) of the light, and CO_2-response curves were fitted according to the method of Long and Bernacchi (2003).

Rubisco activity: The samples of frozen 4th leaves were ground to a fine powder in liquid N_2 and then extracted in a solution containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM $MgCl_2$, 12.5% (v/v) glycerin, 10% insoluble polyvinylpolypyrrolidone, and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4°C. Total Rubisco activity was assayed after the crude extract was activated in a 0.1 mL activation solution containing 33 mM Tris–HCl (pH 7.5), 0.67 mM EDTA, 33 mM $MgCl_2$, and 10 mM $NaHCO_3$ for 15 min at 25°C. Rubisco activity was measured by coupling the activity to NADH oxidation using phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase according to Xia *et al.* (2009). The oxidation of NADH was followed by changes in absorbance at 340 nm for 90 s.

Western-blot analysis: The samples of 4th leaves were ground in liquid N_2 with mortar and pestle. Total proteins were extracted with a buffer containing 50 mM Tris–HCl (pH 6.8), 4.5% SDS, 7.5% β-mercaptoethanol, and 9 M urea. For Western-blot analysis, proteins (20 μg from each sample) were separated by SDS-PAGE using 12.5% (w/v) polyacrylamide gels according to the method of Xia *et al.* (2009) and electrophoretically transferred to nitrocellulose membranes (*Millipore*, Saint-Quentin, France). The proteins were detected with rabbit antibodies raised against rice Rubisco. The result was photographed and analysed by *Tanon Gis 1D* software (*Tanon*, China).

Chl fluorescence of the same leaves used for the gas-exchange measurements was measured using a *PAM-2000* portable pulse amplitude modulation fluorometer (*Walz*, Germany). The seedlings were dark-adapted for about 6 h before measurement. The minimum and maximum Chl fluorescence (F_0 and F_m) were measured first, and then the steady-state fluorescence (F_s) was determined under actinic light (450 μmol m^{-2} s^{-1}). Saturating light pulses were applied to obtain the maximum fluorescence under light-adapted conditions (F_m') following each actinic light interval. After removal of actinic light and application of a 3-s far-red light, the minimal fluorescence of the light-adapted state (F_0') was obtained. The maximum quantum yield of PSII photochemistry (F_v/F_m), the effective quantum yield of PSII photochemistry (Φ_{PSII}), nonphotochemical quenching (NPQ), and the photochemical quenching coefficient (q_P) were calculated according to the methods of Schreiber *et al.* (1998). The energy allocated for photochemical reactions (P), the fraction of absorbed photon energy thermally dissipated (D), and the photon energy absorbed by PSII antennae and trapped by closed PSII reaction centers (Ex) were calculated according to the method of Hussain and Reigosa (2011). The relative deviation from full balance between both photosystems, $\beta/\alpha - 1$, was calculated from the equation $(1 - f)/f$ according to Li *et al.* (2003), where β and α represent the photon activity distribution coefficients of PSII and PSI, respectively. In turn, $\beta = 1/(1 + f)$ and $\alpha = f/(1 + f)$, where f is defined as the degree of openness of the PSII reaction center and it was calculated from the equation $f = (F_m - F_s)/(F_m - F_o)$.

Statistical analysis: *Duncan*'s test, as implemented in the *SPSS* statistical software package (*SPSS*, USA), was used to test mean divergence between the treatments.

Results

Effect of EBR on seedling growth: The growth of cucumber seedlings was inhibited significantly after 7 d of hypoxic stress (Table 1). Biomass was 41.7% lower in the hypoxic group compared with the control (normoxic) plants. Hypoxia inhibited the growth of the 4th leaf; the area and width of the leaf decreased significantly. The relative growth rate (RGR) of the plant was reduced by 33.3%. EBR application partially attenuated the stunted growth induced by hypoxic stress, as the biomass was 16.2% greater in EBR-treated hypoxic seedlings compared with the seedlings under hypoxia alone. The leaf area and RGR under hypoxia were also enhanced significantly by EBR. EBR application under normoxic conditions modestly enhanced the growth compared with control plants, but the difference did not reach statistical significance.

Effect of EBR on Chl content: Under hypoxic stress, Chl was degraded significantly (Table 2). EBR increased Chl synthesis, thus the concentrations of Chl *a* and Chl (*a*+*b*) were restored to the control content. Chl *a*/*b* increased under hypoxic conditions and EBR enhanced it further, although the difference between the four treatments was not statistically significant. Under normoxic conditions, EBR also enhanced Chl synthesis, but the effect was not significant.

Effect of EBR on photosynthetic parameters: P_N decreased significantly after 7 d of hypoxia, and it was accompanied by a remarkable reduction in g_s, C_i, E, and WUE (Table 3). L_s was significantly higher than in the control, indicating that the inhibition of photosynthesis under hypoxia was related to stomata closure. EBR attenuated the inhibitory effect of hypoxia on photosynthesis, as P_N, g_s, C_i, and E increased, while L_s decreased significantly, compared with the seedlings grown under hypoxia alone. Photosynthesis of the plants growing under normoxic conditions was also enhanced to a great extent by EBR.

Table 1. Effect of hypoxia and exogenous 24-epibrassinolide on growth and relative growth rate (RGR) of cucumber seedlings exposed to hypoxic stress for 7 d. Means of five replications ± SD. *Different letters* indicate significant differences between treatments (P<0.05) according to *Duncan*'s test. FM – fresh mass.

Treatment	FM [g plant^{-1}]	Biomass [g plant^{-1}]	RGR [g d^{-1}]	Leaf area [m^2]	Leaf length [m]	Leaf width [m]
Control	10.92 ± 0.45^a	0.750 ± 0.035^a	0.0994	0.012 ± 0.003^a	0.167 ± 0.002^a	0.136 ± 0.014^a
Hypoxia	5.37 ± 0.33^c	0.437 ± 0.011^c	0.0663	0.005 ± 0.0004^c	0.103 ± 0.011^b	0.089 ± 0.006^c
Control + EBR	11.71 ± 0.92^a	0.821 ± 0.042^a	0.0997	0.015 ± 0.0003^a	0.195 ± 0.005^a	0.154 ± 0.006^a
Hypoxia + EBR	6.92 ± 0.38^b	0.508 ± 0.019^b	0.0777	0.008 ± 0.0005^b	0.130 ± 0.007^b	0.109 ± 0.004^b

Table 2. Effect of hypoxia and exogenous 24-epibrassinolide on chlorophyll (Chl) *a*, Chl *b*, Chl (*a*+*b*) concentration and ratio of Chl *a* and Chl *b* (Chl *a*/*b*) of the 4th leaves of cucumber seedlings exposed to hypoxic stress for 7 d. Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments (P<0.05) according to *Duncan*'s test.

Treatment	Chl *a* [g m^{-2}]	Chl *b* [g m^{-2}]	Chl (*a*+*b*) [g m^{-2}]	Chl *a*/*b*
Control	213.6 ± 2.9^b	65.8 ± 9.0^a	279.4 ± 10.7^a	3.29 ± 0.43^a
Hypoxia	190.3 ± 8.7^c	54.0 ± 0. 9^b	230.8 ± 21.5^b	3.47 ± 0.12^a
Control + EBR	228.7 ± 8.7^a	67.8 ± 0.8^a	296.5 ± 7.9^a	3.37 ± 0.17^a
Hypoxia + EBR	216.4 ± 6.0^{ab}	60.6 ± 3.6^{ab}	277.0 ± 8.1^a	3.58 ± 0.20^a

Table 3. Effect of hypoxia and exogenous 24-epibrassinolide on net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), transpiration rate (E), stomatal limitation value (L_s), and water-use efficiency (WUE) of cucumber seedlings exposed to hypoxic stress for 7 d. Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments (P<0.05) according to *Duncan*'s test.

Treatment	P_N [μmol m^{-2} s^{-1}]	C_i [μmol mol^{-1}]	g_s [mol(H_2O) m^{-2} s^{-1}]	E [mmol m^{-2} s^{-1}]	L_s	WUE [mol(CO_2) mol^{-1}(H_2O)]
Control	23.5 ± 0.7^b	283 ± 12^{ab}	0.485 ± 0.068^b	5.65 ± 0.36^c	0.256 ± 0.032^{bc}	3.54 ± 0.11^a
Hypoxia	15.9 ± 0.5^c	159 ± 25^c	0.292 ± 0.016^c	3.49 ± 0.62^d	0.582 ± 0.067^a	2.57 ± 0.23^c
Control + EBR	30.8 ± 1.2^a	291 ± 6^a	0.574 ± 0.020^a	6.49 ± 0.17^b	0.233 ± 0.015^c	3.13 ± 0.22^b
Hypoxia + EBR	23.1 ± 0.3^b	267 ± 20^b	0.469 ± 0.040^b	7.42 ± 0.08^a	0.297 ± 0.053^b	2.69 ± 0.03^c

Effect of EBR on photosynthetic CO_2-response curves: In all treatment groups, P_N increased with increasing C_i (Fig. 1*A*). P_{Nmax} was the lowest under hypoxic stress, indicating that hypoxia impaired the photosynthetic capacity of cucumber seedlings. Treating seedlings under hypoxic stress with EBR moderated this reduction.

Parameters fitted from the P_N-C_i response curves indicated that carboxylation efficiency (CE), V_{cmax}, and J_{max} were reduced significantly under hypoxic stress, while CCP had no significant change among all four treatments. Application of EBR under the stress condition could restore CE and V_{cmax} to the control level, meanwhile J_{max} increased remarkably (Table 4). EBR also improved photosynthesis of the seedlings growing under normoxic conditions to a great extent.

Effect of EBR on photosynthetic light-response curves: At light intensities ranging from 0 to 200 μmol m^{-2} s^{-1}, P_N increased steeply with the increasing light intensity (Fig. 1*B*). Hypoxia impaired the ability of plants to utilize light for photosynthesis with P_{Nmax} being 38.4% lower than in the control plants. EBR partially ameliorated the inhibitory effect of hypoxia on photosynthesis, as P_{Nmax} increased significantly compared with that of the seedlings under hypoxia alone. Under hypoxic stress, LSP, LCP, and AQY of the seedlings were reduced remarkably. EBR elevated LSP and AQY to the control level. The seedlings responded to hypoxia by decreasing the respiration rate, which was restored by EBR greatly though it had not reached the control level (Table 5).

Effect of EBR on Rubisco activity and protein content of Rubisco large subunit (RBCL): To further examine how EBR regulated photosynthesis, we analyzed the protein content of RBCL and Rubisco activity under hypoxic stress. Western blotting indicated that RBCL abundance decreased significantly after 7 d of the hypoxic treatment, while EBR could partially lowered this decline (Fig. 2*A*).

As shown in Fig. 2*B*, Rubisco activity was inhibited remarkably after 7 d of hypoxic stress. EBR eliminated this inhibition and Rubisco activity remained similar in the seedlings treated by EBR under hypoxia as that of normoxic plants.

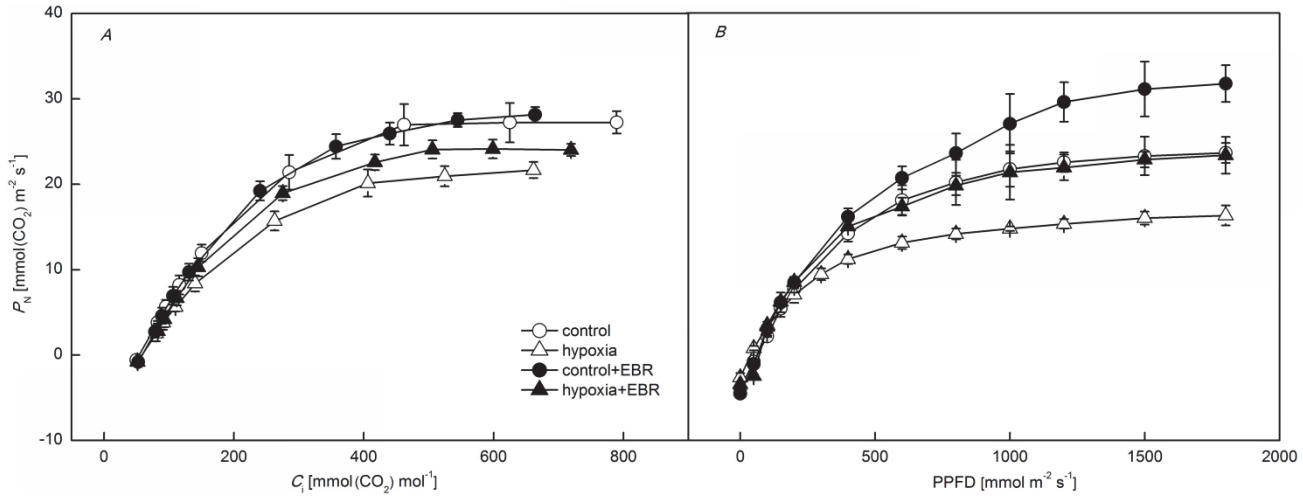

Fig. 1. The net photosynthetic rate (P_N) *vs*. intercellular CO_2 concentration (C_i) (*A*) and photosynthetic photon flux density (PPFD) (*B*) of cucumber seedlings after growing under hypoxia in the presence or absence of 24-epibrassinolide (EBR, 2.1 nM) for 7 d. P_N-PPFD curves were measured with values of an ambient CO_2 concentration of 400 μmol mol^{-1}, while P_N-C_i curves were taken at the PPFD of 1,200 μmol m^{-2} s^{-1}. Symbols indicate mean ± SD, n = 5.

Table 4. Effect of hypoxia and exogenous 24-epibrassinolide on indices derived from CO_2-response curves of cucumber seedlings exposed to hypoxic stress for 7 d: carboxylation efficiency (CE), CO_2 compensation point (CCP), *in vivo* maximum rate of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) carboxylation (V_{cmax}), maximum RuBP regeneration rates (J_{max}). Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments (P<0.05) according to *Duncan*'s test.

Treatment	CE	CCP [μmol mol]	V_{cmax} [μmol m^{-2} s^{-1}]	J_{max}
Control	0.120 ± 0.009^{ab}	55.5 ± 2.7^{a}	54.0 ± 1.60^{b}	50.5 ± 3.08^{c}
Hypoxia	0.099 ± 0.012^{c}	60.5 ± 5.8^{a}	46.6 ± 3.12^{c}	43.7 ± 2.59^{d}
Control + EBR	0.131 ± 0.010^{a}	56.7 ± 3.0^{a}	65.8 ± 3.63^{a}	68.5 ± 2.75^{a}
Hypoxia + EBR	0.108 ± 0.014^{bc}	59.4 ± 4.9^{a}	55.9 ± 2.32^{b}	55.9 ± 2.77^{b}

Table 5. Effect of hypoxia and exogenous 24-epibrassinolide on indices derived from light-response curves of cucumber seedlings exposed to hypoxic stress for 7d: light compensation point (LCP), light saturation point (LSP), apparent quantum yield (AQY), respiration rate, and maximum net photosynthetic rate (P_{Nmax}). Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments (P<0.05) according to *Duncan*'s test.

Treatment	LSP [μmol m^{-2} s^{-1}]	LCP [μmol m^{-2} s^{-1}]	AQY	Respiration rate [μmol m^{-2} s^{-1}]	P_{Nmax} [μmol m^{-2} s^{-1}]
Control	1,378 ± 38^{b}	62 ± 5.1^{a}	0.066 ± 0.002^{a}	3.99 ± 0.40^{b}	28.6 ± 0.9^{b}
Hypoxia	952 ± 36^{d}	48 ± 5.0^{b}	0.053 ± 0.006^{b}	2.73 ± 0.11^{d}	17.6 ± 0.7^{d}
Control + EBR	1,743 ± 26^{a}	65 ± 2.3^{a}	0.069 ± 0.002^{a}	4.53 ± 0.06^{a}	34.6 ± 1.2^{a}
Hypoxia + EBR	1,261 ± 44^{c}	49 ± 2.3^{b}	0.062 ± 0.005^{a}	3.42 ± 0.08^{c}	25.5 ± 0.8^{c}

Effect of EBR on PSII photochemical activity: F_v/F_m was reduced significantly under hypoxia, but it was restored to near-control levels by EBR. Φ_{PSII} decreased to 77.7% of the control. EBR application under hypoxic stress increased Φ_{PSII} by 18.8%. After 7 d of hypoxic stress, NPQ increased significantly. EBR further enhanced NPQ of the seedlings under hypoxic stress. The electron transport rate (ETR) changed in the same manner as Φ_{PSII} under hypoxic stress. The excitation pressure of PSII ($1 - q_P$) was enhanced 1.45-fold by hypoxia compared with the controls, while EBR treatment decreased it significantly (Table 6).

Effect of EBR on allocation of absorbed light energy and excitation energy in the photoreaction system: Under normoxic conditions, of the total light energy absorbed by PSII in cucumber leaves, 54.4% was used for P, 26.6% was dissipated by the antenna, and 19% was dissipated nonphotochemically. After 7 d of hypoxic stress, the light energy used for P decreased significantly and it was only 69.7% of the control, while D changed only slightly. Ex increased by 64.4% compared with the control seedlings. EBR application increased P by 15.8% and reduced Ex by 11.3% compared with the hypoxic seedlings not treated by EBR (Fig. 3*A*).

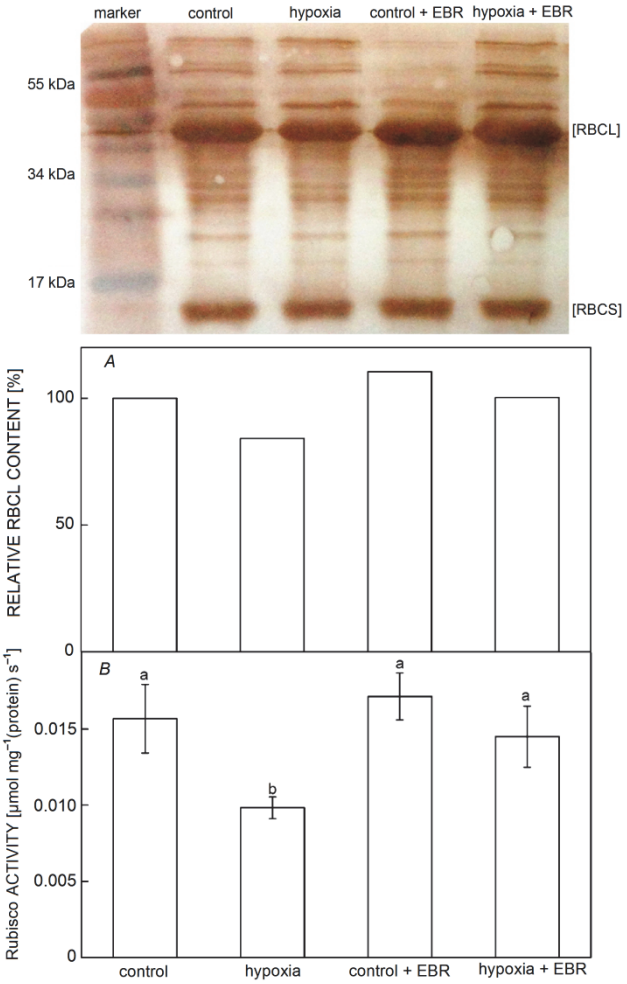

Fig. 2. Effect of hypoxia and exogenous EBR on the protein levels of Rubisco large subunit (RBCL) (*A*) and Rubisco activity (*B*) of cucumber seedlings exposed to hypoxic stress for 7 d. Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments ($P<0.05$) according to *Duncan*'s test.

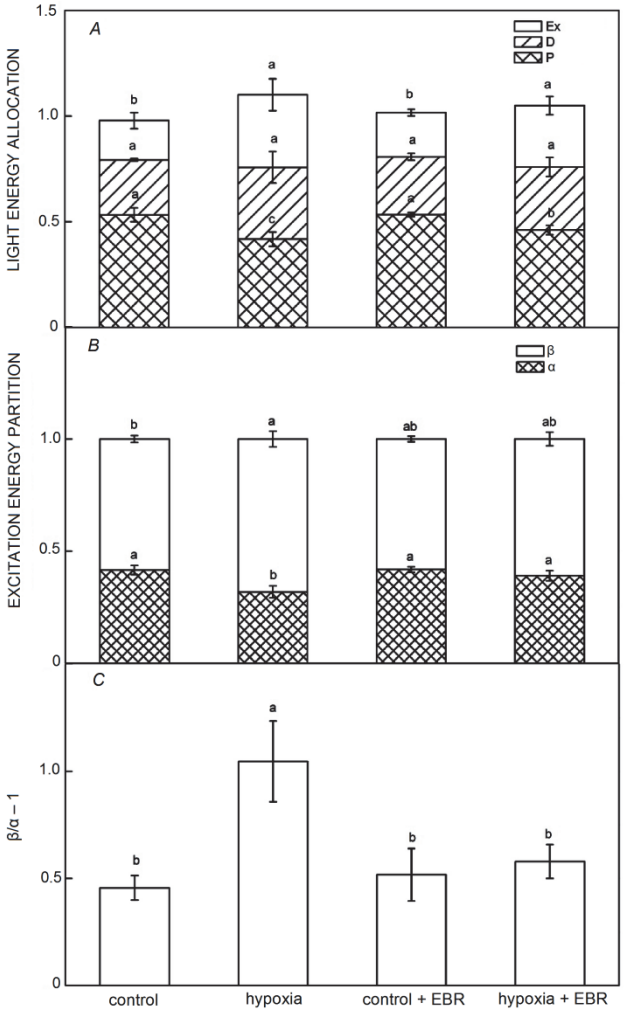

Fig. 3. Effect of hypoxia and exogenous EBR on the partitioning of light energy absorbed by PSII (*A*) and excitation energy (*B*) and deviation from full balance (*C*) of cucumber seedlings exposed to hypoxic stress for 7 d. Ex – photon energy absorbed by PSII antennae and trapped by closed PSII reaction centers; D – absorbed photon energy that was thermally dissipated; P – photon energy absorbed by PSII antennae trapped by open PSII reaction centers. α – excitation energy captured by PSI; β – excitation energy captured by PSII. β/α – 1 is the relative deviation from full balance between the two photosystems. Means of five replications ± SD. *Different letters* indicate significant differences between treatments ($P<0.05$) according to *Duncan*'s test.

Under hypoxic stress, the proportion of excitation energy captured by PSI (α) decreased significantly, while that captured by PSII (β) increased significantly compared with the seedlings grown under normoxic conditions. Thus, hypoxic stress hindered ETR between both two photosystems. EBR lessened the hypoxia-induced increase of α (Fig. 3*B*).

After 7 d of hypoxic stress, β/α–1 increased significantly (Fig. 3*C*). EBR reduced β/α–1 to the control level.

Discussion

Hypoxia is a common abiotic stress experienced by plants in poorly drained soils or during flooding. Roots are the plant organs that suffer most frequently from low oxygen stress. Under oxygen-deficient conditions, the root cell energy pool is greatly reduced (Sairam *et al*. 2008). Energy is necessary for the uptake of mineral nutrients by active processes in plants. Hence, the uptake of nutrients such as potassium, magnesium, and phosphate declines (Morard *et al*. 2004). Hypoxia resulted in a disruption of aquaporin functioning and in the decrease of root hydraulic

Table 6. Effect of hypoxia and exogenous 24-epibrassinolide on fluorescence parameters of cucumber seedlings exposed to hypoxic stress for 7 d: maximal quantum yield of PSII photochemistry (F_v/F_m), effective quantum yield of PSII photochemistry (Φ_{PSII}), nonphotochemical quenching (NPQ), excitation pressure on PSII ($1 - q_P$) and electron transport rate (ETR). Means of 5 replications ± SD. *Different letters* indicate significant differences between treatments ($P<0.05$) according to *Duncan*'s test.

Treatment	F_v/F_m	Φ_{PSII}	NPQ	$1 - q_P$	ETR [μmol m^{-2} s^{-1}]
Control	0.823 ± 0.012^{b}	0.512 ± 0.011^{a}	0.488 ± 0.052^{c}	0.216 ± 0.018^{b}	157 ± 5.1^{a}
Hypoxia	0.798 ± 0.004^{c}	0.398 ± 0.030^{b}	0.761 ± 0.099^{ab}	0.529 ± 0.054^{a}	141 ± 3.4^{b}
Control + EBR	0.835 ± 0.007^{a}	0.477 ± 0.064^{a}	0.547 ± 0.030^{bc}	0.257 ± 0.059^{b}	171 ± 10.4^{a}
Hypoxia + EBR	0.813 ± 0.002^{bc}	0.473 ± 0.013^{a}	0.814 ± 0.169^{a}	0.290 ± 0.039^{b}	167 ± 3.6^{a}

conductance (Tournaire-Roux *et al.* 2003). The lack of oxygen in the nutrient medium induced the 42% reduction of water consumption by excised tomato roots (Morard *et al*. 2004). Disturbances linked to root hypoxia affected the aerial part of plants: growth and development were reduced (Table 1). One of the physiological consequences of root hypoxia seemed to be the leaf stomata closure (Table 3). ABA may act as the signal of hypoxic stress to close stomata (Olivella *et al*. 2000, Bai *et al.* 2013). Stomata closure reduced the supply of CO_2 and led to the downregulation in photosynthesis (Table 3). Besides the stomatal factor, photosynthetic inhibition induced by hypoxia can be attributed to other nonstomatal limitation factors, such as Chl degradation, a reduction in the capacity of RuBP carboxylation, and RuBP regeneration (Pezeshki 1994). In the present study, Chl content decreased meanwhile V_{cmax} and J_{max} were inhibited greatly under hypoxic stress (Tables 2, 4). Hypoxia aggravated the degradation of RBCL (Fig. 2*A*) and inhibited Rubisco activity to a great degree (Fig. 2*B*). P_{Nmax} decreased significantly under the saturating light intensity, as AQY, LSP, and LCP did indicating that light utilization was suppressed under hypoxia (Table 5). Protecting plants from full sunlight could limit the extent of damage caused by hypoxia on photosynthesis (Jans *et al*. 2012). The rate of Chl degradation was strongly dependent on the light intensity in bean leaf discs under low oxygen (Hildbrand *et al*. 1994). Hence the downregulation of these light utilization efficiency parameters under hypoxia might be the photoprotective mechanism for the plants.

EBR enhances plant tolerance to various environmental stress by improving photosynthesis (Ogweno *et al*. 2008, Piñol and Simón 2009). In plants under hypoxia and in the CO_2-enriched medium, EBR inhibited the oxidative degradation of lipids in biological membranes and prevented the disruption of membrane structures (Ershova and Khripach 1996). EBR recovered the typical shape of chloroplasts and promoted the formation of grana in plants under the salt stress (Yuan *et al.* 2012). It regulated the glutathione redox state in the chloroplasts and enhanced the stability and the activity of redox-sensitive photosynthetic enzymes through post-translational modifications (Jiang *et al.* 2012). EBR could also increase the capacity of CO_2 assimilation in the Calvin cycle (Yu *et al*. 2004) and enhanced activation of Rubisco and expression of photosynthetic genes (Xia *et al*. 2009). In this paper, we found that EBR reduced greatly the hypoxia-mediated photosynthetic inhibition in cucumber seedlings. It alleviated Chl degradation (Table 2) and reversed the limitation on photosynthetic efficiency imposed by stomata closure in cucumber plants under hypoxia (Table 3). The results that LSP and AQY were restored to the control level (Table 5) indicated that EBR improved light-utilization efficiency of seedlings under hypoxic conditions. On the other hand, EBR upregulated photosynthetic capacity under hypoxia by improving the capacity of Rubisco carboxylation and RuBP regeneration (Table 4). It protected RBCL from considerable degradation (Fig. 2*A*) and attenuated the inhibiton effect of hypoxia on Rubisco activity to a great degree (Fig. 2*B*). As a result, photochemical energy utilization under hypoxia was improved by EBR.

Environmental stress had been shown to accelerate photoinhibition (Takahashi and Murata 2008). Cucumber seedlings under hypoxia were photoinhibited and xanthophyll cycle was the primary heat dissipation route (Jia *et al.* 2011). Smethurst *et al.* (2005) found that reduced capacity for CO_2 assimilation in waterlogged plants was associated with inhibition of PSII. Root hypoxia reduced both PSI and PSII reaction center complexes (Ladygin 2004). Light utilization decreased under environmental stress, thus absorption of excess light energy by the photosynthetic machinery resulted in the generation of reactive oxygen species (ROS) (Danon 2012). In particular, ROS inhibited synthesis of the D1 protein, a component of the reaction center of PSII (Nishiyama *et al.* 2001). Hypoxia enhanced the production of ROS (Kumutha *et al.* 2009). In our study, photosynthetic efficiency of cucumber seedlings decreased under hypoxia. At the same time, F_v/F_m and Φ_{PSII} decreased while NPQ increased, indicating that PSII reaction center was disturbed under hypoxia (Table 6).

EBR had been shown to improve function of PSII and reduced the toxic effect of Cd on photochemical processes by diminishing damage to photochemical reaction centers and oxygen evolution complexes and by maintaining efficient photosynthetic electron transport (Janeczko *et al*. 2005). The protective role of EBR on photosynthesis may be associated with improving antioxidant system activity (Fariduddin *et al.* 2011). In the current study, F_v/F_m was

restored to control levels and Φ_{PSII} increased significantly in EBR-treated hypoxic plants. NPQ increased under hypoxia, but increased even more in the presence of EBR (Table 6). In summary, these results suggested that EBR protect the PSII reaction center of cucumber seedlings under hypoxic stress.

Plants have evolved a set of mechanisms to defend against photodamage, including heat dissipation of excess excitation energy, regulation of PSII light-capturing efficiency, and shifting between PSI and PSII dominance (Gray *et al.* 1996, Havaux and Kloppstech 2001, Ivanov *et al*. 2008). Excitation pressure on PSII ($1 - q_P$) is the index the redox balance of the intersystem electron transport chain (Gray *et al.* 1996). Higher excitation pressure indicates greater PSII reaction center closure, which leads to inhibition of electron transport due to excess excitation energy. Plants adapt to stress by repartitioning the excitation energy between PSI and PSII. A higher $\beta/\alpha - 1$ reflects the unbalanced partitioning of excitation energy between PSI and PSII (Braun *et al*. 1991). In this paper, PSII excitation pressure increased 1.45-fold under hypoxia, indicating that hypoxia caused more PSII reaction centers were closed (Table 6). EBR reduced the excitation pressure on PSII and restored $\beta/\alpha - 1$ to the control level, indicating that EBR greatly relieved both oxidation of primary quinone acceptor of PSII (Q_A) and the imbalanced partition of excitation energy between both two photosystems under hypoxic stress (Fig. 3*B*, *C*). Under hypoxia, the proportion of light energy absorbed by PSII and used for P was reduced, while D and Ex increased. EBR reduced hypoxia-caused injury of photosynthesis by allocating more energy to PSII than to PSI, with 15.8% more light energy being used by the photochemical pathway (Fig. 3*A*).

From the study, we might bring up a working hypothesis: hypoxia impeded water absorption of root and stomata closure was induced by a signal such as ABA. For this reason, CO_2 input decreased and photosynthetic CO_2 carboxylation was downregulated. As a result, PSII and CO_2 carboxylation was damaged by excessive excitation energy. The application of exogenous EBR alleviated stomata closure and improved the capacity of CO_2 carboxylation under hypoxia. It also protected photosynthetic apparatus against PSII damage. Hence, the tolerance of the cucumber seedlings to hypoxia was improved.

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