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REGULAR PAPER

Effects of 24-epibrassinolide on the photosynthetic characteristics, antioxidant system, and chloroplast ultrastructure in *Cucumis sativus* L. under $Ca(NO_3)_2$ stress

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Abstract The effects of 0.1 μM 24-epibrassinolide (EBL) on plant growth (plant height, leaf area, fresh weight, and dry weight), chlorophyll content, photosynthetic characteristics, antioxidant enzymes, and chloroplast ultrastructure were investigated using cucumber seedlings (*Cucumis sativus* L. cv. Jinyou No. 4) with 80 mM $Ca(NO_3)_2$ to induce stress. The presence of $Ca(NO_3)_2$ caused significant reductions in net photosynthetic rate (P_N), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) of leaves. In addition, $Ca(NO_3)_2$ markedly reduced the chlorophyll content and inhibited photochemical activity, including the actual photochemical efficiency (ΦPSII). In contrast, EBL increased the chlorophyll content, especially chlorophyll *b*, and minimized the harmful effects on photosynthesis caused by the $Ca(NO_3)_2$. The application of EBL to the plants subjected to $Ca(NO_3)_2$-enhanced photochemical activity. EBL protected the photosynthetic membrane system from oxidative damage due to up-regulating the capacity of the antioxidant systems. Microscopic analyses revealed that $Ca(NO_3)_2$ affected the structure of the photosynthetic apparatus and membrane system and induced damage of granal thylakoid layers, while EBL recovered the typical shape of chloroplasts and promoted the formation of grana. Taken together, EBL compensated for damage/losses by $Ca(NO_3)_2$ due to the regulation of photosynthetic characteristics and the antioxidant system.

Keywords 24-Epibrassinolide · $Ca(NO_3)_2$ stress · Photosynthetic characteristics · Antioxidant system · Ultrastructure · *Cucumis sativus* L.

Abbreviations

APX	Ascorbate peroxidase
BRs	Brassinosteroids
CAT	Catalase
Chl	Chlorophyll
EBL	24-Epibrassinolide
Fv/Fm	Maximal quantum yield of PSII photochemistry
GPX	Guaiacol peroxidase
LHC	Light-harvesting complex
PSI	Photosystem I
PSII	Photosystem II
ΦPSII	The actual efficiency of PSII
qN	Non-photochemical quenching
qP	Coefficient for photochemical quenching
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

More than 800 million hectares of land are affected by salt throughout the world (FAO 2012). The majority of these soils are naturally saline, but recently a significant proportion of cultivated land is also being influenced by secondary salinization. Globally, 32 million out of 1.5 billion hectares of cultivated land in a dryland (2 %) are influenced considerably by secondary salinity, a soil condition

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characterized by high concentrations of soluble salts; and 45 million of the 230 million hectares of irrigated land (~20 %) are salt affected (FAO 2012). In China, greenhouse cultivation is the most common vegetables production in farmlands. Secondary salinization of soil is primarily attributable to over-irrigation, intensive farming, lack of rain and concentration of salt through intense evaporation (Ashraf 1994; Liang et al. 2005; Yu et al. 2005). Secondary salinization is an important limitation to crop production in greenhouses in China (Yu et al. 2005).

In China, 40–75 % of the total salt contained in the 0–20 cm layer is concentrated in the soil surface layer (0–5 cm depth). The anions in soil due to secondary salinization are NO_3^-, Cl^-, SO_4^{2-}, and HCO_3^- and the cations are Na^+, K^+, Ca^{2+}, and Mg^{2+}. Among these ions, NO_3^- and Ca^{2+} are usually dominant. More specifically, Ca^{2+} accounts for over 60 % of total cations and NO_3^- accounts for ~67–76 % of the total anions (Wu 2001; Li et al. 2004). According to Tong and Chen (1991), the high level of $Ca(NO_3)_2$ accumulation was one of the main reasons for soil salinization. Excessive $Ca(NO_3)_2$ caused severe oxidative damage and metabolic disorder, suggesting reductions in biomass production in cucumber plants (Sun et al. 2009; Jin et al. 2010) and tomato plants (Zhang et al. 2008). Extensive or definitive research to describe the negative effects of excessive $Ca(NO_3)_2$ has not been conducted.

Brassinosteroids (BRs), a phytohormone, induce physiological and morphological responses in higher plants. When subjected to stress from heavy metal, thermal, pesticide or NaCl, 24-epibrassinolide (EBL) were found to enhance total chlorophyll content (Janeczko et al. 2007; Ali et al. 2008a), stomatal conductance (Ali et al. 2008a; Hu et al. 2010), the capacity of antioxidant system (Xia et al. 2006) and to protect the quantum yield of PSII (Ogweno et al. 2008) in plants. Exogenous EBL, however, showed no significant effect on chlorophyll content or photochemical efficiency of PSII in wheat plants and pepper plants subjected to NaCl stress (Qayyum et al. 2007; Ali et al. 2008b; Houimli et al. 2008).

Little information is currently available in literature regarding the effects of excess $Ca(NO_3)_2$ on the photosynthetic characteristics or the possible alleviation mechanisms of exogenous EBL on the negative effects of $Ca(NO_3)_2$. The purpose of this study is to clarify whether exogenous EBL alleviates the negative growth suppression effect of $Ca(NO_3)_2$ due to changes in photosynthetic characteristics, antioxidant capacity, and the chloroplast ultrastructure in *Cucumis sativus* L.

Materials and methods

Plants

Cucumber (*Cucumis sativus* L., cv. 'Jinyou No. 4') seeds were obtained from Tianjin Kernel Cucumber Research Institute, China. The seeds were germinated on two layers of wet filter paper in Petri dishes in the dark for ~24 h at 29 ± 1 °C. The germinated seeds were sown in sand and moved to a plant growth chamber (Ningbo Jiangnan Instrument Factory, Ningbo, China) at Nanjing Agricultural University, Nanjing, China. The growth chamber was kept at 28 ± 1 °C (day) and 19 ± 1 °C (night) with relative humidity (RH) of 50–60 %. The maximum photosynthetic photo flux density (PPFD) was ~1,200 μmol m^{-2} s^{-1}. When the second leaves were fully expanded, 12 seedlings were transplanted into plastic containers containing half strength Hoagland solution (Zhang et al. 2009). The nutrient solution was aerated using an air pump at an interval of 20 min to maintain the dissolved oxygen concentration at 8.0 ± 0.2 mg l^{-1} during this experiment.

Experimental design

The $Ca(NO_3)_2$ concentration in the nutrient solution was increased at a rate of 40 mM per day until a final concentration of 80 mM was attained. The concentrations of $Ca(NO_3)_2$ and EBL were based on the results of our previous experiment (data not shown). To investigate the effects of EBL (Sigma-Aldrich, USA) sprayed on whole plants grown hydroponically, the cucumber seedlings were divided into four groups. Since the EBL stock solution contained ethanol as a solvent, the nutrient solutions (including distilled water as the control) were prepared with the same ethanol level as the stock solution. After pre-culturing for 3 days, the seedlings were treated as follows:

(a) Cont, 0 mM $Ca(NO_3)_2$ + 0 μM EBL;
(b) CB, 0 mM $Ca(NO_3)_2$ + 0.1 μM EBL;
(c) N, 80 mM $Ca(NO_3)_2$ + 0 μM EBL;
(d) NB, 80 mM $Ca(NO_3)_2$ + 0.1 μM EBL.

The seedlings were sprayed with EBL every 2 days and the nutrient solutions were renewed every 2 days. At 9 days of treatment, photosynthesis and the chlorophyll fluorescence of the plants were measured. At 10 days of treatment, the seedlings were sampled for the determination of morphological parameters, chlorophyll content, oxidative damage, and the activities of antioxidant enzymes. The seedlings were organized in a complete randomized block design.

Morphological analyses

After the shoot length of each plant was measured, the area of the third expanded leaf (from the top) was measured with an Expression 1680 scanner (Epson, Sydney, Australia) and image analysis software (WinRHIZO, Regent Instruments. Inc., Quebec, Canada). After all the leaves

were washed with distilled water and topical moisture removed, their fresh weights were measured; subsequently the dry weights were obtained after drying at 75 °C for 72 h.

Gas exchange parameters

The net photosynthetic rate (P_N), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) of the fully expanded leaves, which were the third leaves from the top, were measured using a portable photosynthesis system (LI-6400, LI-COR Inc., USA). Leaf temperatures were maintained at 25 °C. RH in the assimilation chamber was maintained at 70 %, the external CO_2 concentration remained at 380 ± 10 μmol mol^{-1} and the light intensity was consistent at 1,000 μmol photons m^{-2} s^{-1}.

Chlorophyll fluorescence parameters

Chlorophyll fluorescence of the third fully expanded leaf was measured with a portable fluorometer (PAM 2100, Walz, Germany) after dark adaptation for 30 min according to the method of Kooten and Snel (1990). The maximum quantum yield of PSII (Fv/Fm) was determined after dark adaptation for 30 min. The initial Chl fluorescence yield (Fo) was determined in low-modulated measuring light (<0.1 μmol m^{-2} s^{-1}) and a 0.8-s pulse of saturating white light (8,000 μmol m^{-2} s^{-1}) was applied to obtain the maximum fluorescence yield (Fm). To determine the minimal fluorescence level in a leaf during the illumination (Fo′), black cloth to keep out light was rapidly installed to cover the leaf and the leaf-clip holder in the presence of far-red light to oxidize the PSII centers fully. Immediately after blockage of light, leaf fluorescence dropped to the Fo′ level and rose again within several seconds. The steady-state fluorescence level (Fs) and the maximum fluorescence level (Fm′) during the exposure to light were also measured. Fluorescence levels were used to calculate: (1) the maximum quantum efficiency of PSII, Fv/Fm = (Fm − Fo)/Fm; (2) the photochemical fluorescence quenching coefficient, qP = (Fm′ − Fs)/(Fm′ − Fo′); (3) the non-photochemical quenching, qN = (Fm − Fm′)/(Fm′ − Fo′); and (4) the actual efficiency of PSII, ΦPSII = (Fm′ − Fs)/Fm′.

Chlorophyll content

The chlorophyll was extracted from the third fully expanded leaf with a mixture containing acetone, ethanol, and water (4.5:4.5:1, v/v/v). The chlorophyll content was measured on a fresh weight basis according to a modified version of the method of Strain and Svec (1966).

Free radical production and lipid peroxidation

The content of malondialdehyde (MDA) was measured according to the method of Heath and Packer (1968). The concentration was calculated from the value of A_{532}, and non-specific turbidity at the measurements was canceled by subtracting the value of A_{600}.

The superoxide formation rate was measured according to a modified version of the method of Elstner and Heupel (1976). Each leaf sample (1.0 g) was homogenized in 4 mL of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5,000*g* for 10 min. The reaction solution contained 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8), 0.1 mL of 10 mM hydroxylamine hydrochloride, and 1 mL of the supernatant. After the solution was incubated at 25 °C for 20 min, 17 mM sulfanilic acid, and 7 mM α-naphthylamine were added into the solution. The measurement was performed at A_{530}. The $O_2^{\bullet -}$ concentration was calculated from a standard curve of $NaNO_2$.

H_2O_2 content in cucumber leaves was estimated according to the method of Patterson et al. (1984). The assay was based on the absorbance change of the titanium peroxide complex at A_{415}.

Activities of antioxidant enzymes

Ascorbate peroxidase (APX) (EC 1.11.1.1) was assayed according to the method of Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 6.0), 0.1 μM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ascorbate, 1.0 mM H_2O_2, and 50 μL enzyme fraction.

The superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by the method of Giannopolitis and Ries (1977). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 μM EDTA, 13 mM methionine, 75 μM NBT (nitro-blue tetrazolium), 2 μM riboflavin, and 50 μL enzyme fraction.

The guaiacol peroxidase (GPX) (EC 1.11.1.7) activity was determined according to the method of Herzog and Fahimi (1973). The reaction mixture contained 50 mM phosphate buffer (pH 6.0), 25 mM guaiacol, 10 mM H_2O_2, and 0.1 mL enzyme fraction.

The catalase (CAT) (EC 1.11.1.6) activity was measured according to the method of Aebi (1984). The reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 0.1 mL enzyme fraction.

Ultrastructure of chloroplasts

The ultrastructure of the photosynthetic apparatus in chloroplasts was analyzed using electron micrographs. The leaves were cut into pieces of ~1 mm^2. The cut leaves were then immersed in a solution containing 3 %

glutaraldehyde and 1 % formaldehyde in a 0.1 M phosphate buffer (pH 7.4) for 2 h (primary fixation), then immersed in 2 % osmic acid in the same buffer for 2 h (second fixation). After dehydration in acetone and embedding in Durcupan ACM (Fluka), the resulting leaves were cut to obtain ultra-thin sections, stained with uranium acetate and lead citrate in series and examined using a HITACHI transmission electron microscope (Carl Zeiss, Göttingen, Germany) at an accelerating voltage of 80 kV.

Statistical analysis

The data were statistically analyzed with SAS software (SAS Institute, Cary, NC, USA) using Duncan's multiple range test at the $P < 0.05$ level of significance.

Results

Morphological parameters

Under control condition, EBL alone (CB) enhanced the leaf area and dry weight of plants, as compared to the control (Table 1). $Ca(NO_3)_2$ stress (N) significantly inhibited the height, leaf area, fresh weight, and dry weight of the plants to 58.8, 33.2, 46.2, and 60.7 %, respectively. However, EBL in the NB solution alleviated the growth inhibition caused by $Ca(NO_3)_2$ (N) (Table 1).

Chlorophyll content

The EBL (CB) significantly increased the contents of Chl *a*, Chl *b* and Chl $a + b$ in the cucumber leaves (Fig. 1a–c), but did not affect the Chl *a*/*b* ratio (Fig. 1d). The contents of Chl *a*, Chl *b* and Chl $a + b$ decreased with the salt treatment (N) by 19.8, 17.7, and 19.3 %, respectively, compared to the control. However, the Chl *a*/*b* ratio did not significantly change from the control. In the case of NB, the contents of the Chl *a*, Chl *b*, and Chl $a + b$ were significantly increased, whereas the ratio of Chl *a*/*b* showed a tendency to decline (Fig. 1).

Gas exchange parameters

In the cucumber plants treated with EBL alone (CB), the gas exchange parameters indicated a significant increase in

Table 1 Effects of Ca(NO3)2 and/or EBL treatment on plant height, leaf area, fresh weight and dry weight in cucumber plants

Treatments	Plant height (cm)	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
Cont	19.4 ± 0.29 ab	181.6 ± 5.85 b	27.3 ± 1.66 a	1.91 ± 0.07 b
CB	23.6 ± 1.85 a	211.8 ± 0.84 a	29.6 ± 2.13 a	1.88 ± 0.83 a
N	11.4 ± 0.27 c	60.3 ± 4.08 d	12.6 ± 2.05 b	1.16 ± 0.16 c
NB	17.7 ± 0.12 b	142.7 ± 3.61 c	27.8 ± 1.52 a	2.33 ± 0.16 ab

Values represent the mean ± SE ($n = 3$). Letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests

Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)^2$ added into nutrient solution; NB, both EBL and Ca(NO3)2 treatments

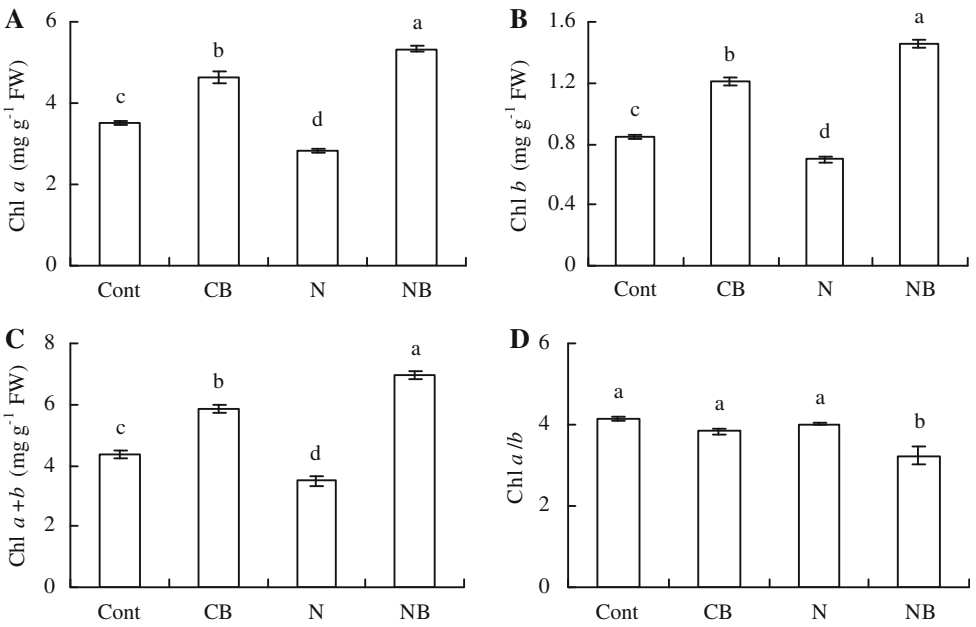

Fig. 1 Effects of $Ca(NO_3)_2$ and/or EBL on Chl *a*, Chl *b*, Chl $a + b$ and Chl *a*/*b* in cucumber plants. Values represent the mean ± SE. ($n = 3$). *Letters* indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests. Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

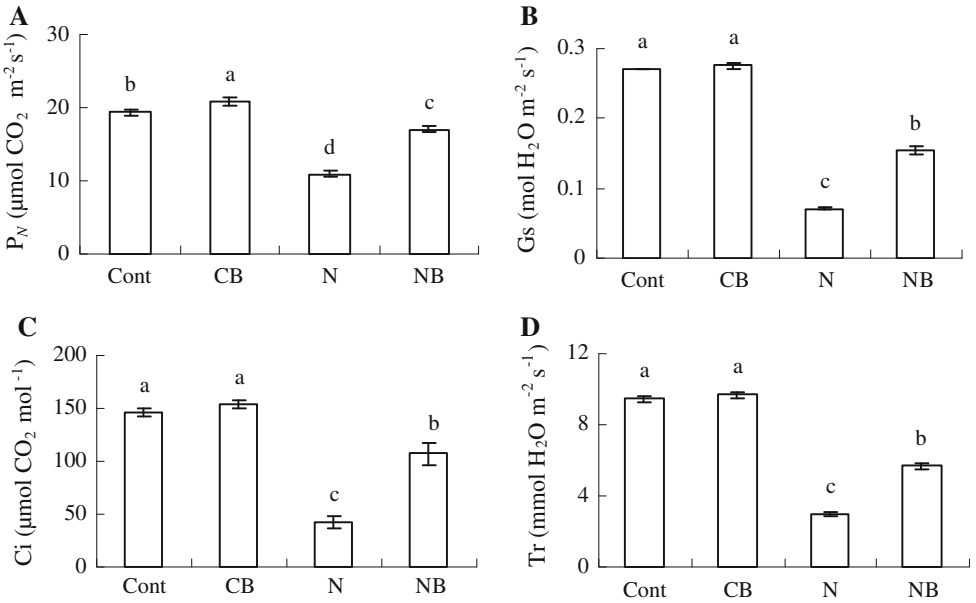

Fig. 2 Effects of $Ca(NO_3)_2$ and/or EBL on net photosynthetic rate (P_N), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr) in cucumber plants. Values represent the mean ± SE ($n = 3$). *Letters* indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests. Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

the P_N (Fig. 2a), but showed no significant influence on the Gs (Fig. 2b), Ci (Fig. 2c) or Tr (Fig. 2d). P_N in the plants subjected to $Ca(NO_3)_2$ stress (N) showed significantly lower values than the control plants; 11.0 and 19.3 μmol CO_2 m^{-2} s^{-1}, respectively. The Gs, Ci, and Tr also decreased by the $Ca(NO_3)_2$ treatment (N). The EBL in the NB solution enhanced the values of photosynthetic parameters that were suppressed by $Ca(NO_3)_2$, although they remained below the levels measured in the control plants.

Chlorophyll fluorescence parameters

EBL alone (CB) showed no effect on the Chl fluorescence parameters, as compared to the control. $Ca(NO_3)_2$ (N) significantly decreased the ΦPSII and qP in cucumber plants. However, both of them were significantly enhanced by EBL in the NB solution (Table 2).

Chl fluorescence analysis showed differences in the dissipation of excitation energy among the treatments (Table 2). The qN was significantly decreased in the plants subjected to $Ca(NO_3)_2$ and the EBL in the NB solution enhanced it. $Ca(NO_3)_2$ (N) slightly reduced the Fv/Fm ratio, while the EBL in the NB solution alleviated this decline.

MDA content, $O_2^{\bullet-}$ formation rate, and H_2O_2 content

EBL (CB) showed no effect on the MDA content (Fig. 3a) and $O_2^{\bullet-}$ formation rate (Fig. 3b). In contrast, it significantly decreased the H_2O_2 content (Fig. 3c). $Ca(NO_3)_2$ (N) significantly increased the MDA content, $O_2^{\bullet-}$ formation rate and H_2O_2 content, and showed the highest levels among all treatments. However, EBL in the NB solution induced low levels of the MDA content, $O_2^{\bullet-}$ formation rate and H_2O_2 content to 76.9, 72.4, and 80.8 %, respectively, as compared to the salt-stressed plants (N).

Activities of antioxidant enzymes

EBL in the CB solution significantly enhanced the activities of APX (Fig. 4a) and GPX (Fig. 4c). In contrast, the activities of SOD (Fig. 4b) and CAT (Fig. 4d) were not influenced by EBL in the CB solution. The $Ca(NO_3)_2$ stress

Table 2 Effects of $Ca(NO_3)_2$ and/or EBL on actual photochemical efficiency in the light-adapted state (ΦPSII), coefficient for photochemical quenching (qP), non-photochemical quenching (qN), and maximal quantum yield of PSII photochemistry (Fv/Fm) in cucumber plants

Treatments	ΦPSII	qP	qN	Fv/Fm
Cont	0.59 ± 0.005 b	0.80 ± 0.011 b	0.51 ± 0.020 b	0.83 ± 0.007 a
CB	0.60 ± 0.009 b	0.82 ± 0.006 b	0.52 ± 0.011 b	0.83 ± 0.003 a
N	0.53 ± 0.006 c	0.76 ± 0.005 c	0.46 ± 0.018 c	0.80 ± 0.001 b
NB	0.66 ± 0.027 a	0.85 ± 0.002 a	0.56 ± 0.007 a	0.82 ± 0.003 a

Values represent the mean ± SE. ($n = 3$). Letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests

Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

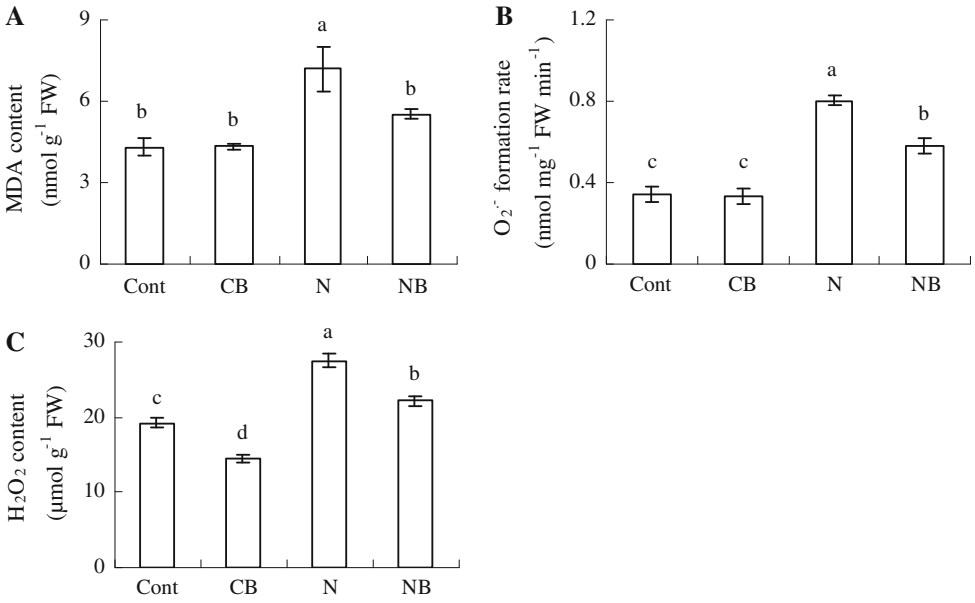

Fig. 3 Effects of $Ca(NO_3)_2$ and/or EBL on MDA content, O_2^- formation rate and H_2O_2 content in leaf of cucumber plants. Values represent the mean ± SE ($n = 3$). *Letters* indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests. Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

(N) significantly reduced the activities of APX, SOD, GPX and CAT to be 80.8, 80.2, 86.3, and 63.5 %, respectively, as compared to the control. Whereas, EBL in the NB solution greatly enhanced the activities of APX, SOD, GPX, and CAT, as compared to the activities of the enzymes in the plants (N) with salt stress (Fig. 4).

Ultrastructure of the photosynthetic apparatus

Under control condition, the chloroplasts showed thylakoids with grana (Fig. 5). The ultrastructure of the photosynthetic apparatus seemed to show no difference between the control plants and plants treated with EBL alone (CB). However, $Ca(NO_3)_2$ (N) caused remarkable structural changes. Specifically, internal lamellae of the stromal thylakoids were damaged to induce partly granal thylakoid layers to loosen. Furthermore, the chloroplast envelopes (membranes) were damaged by salt (N) and became indistinct. EBL in the NB solution apparently enhanced recovery of the chloroplast shape in that the stromal lamellae seemed to form formally the grana ultrastructure and the chloroplasts were observed to make distinct envelopes (membranes).

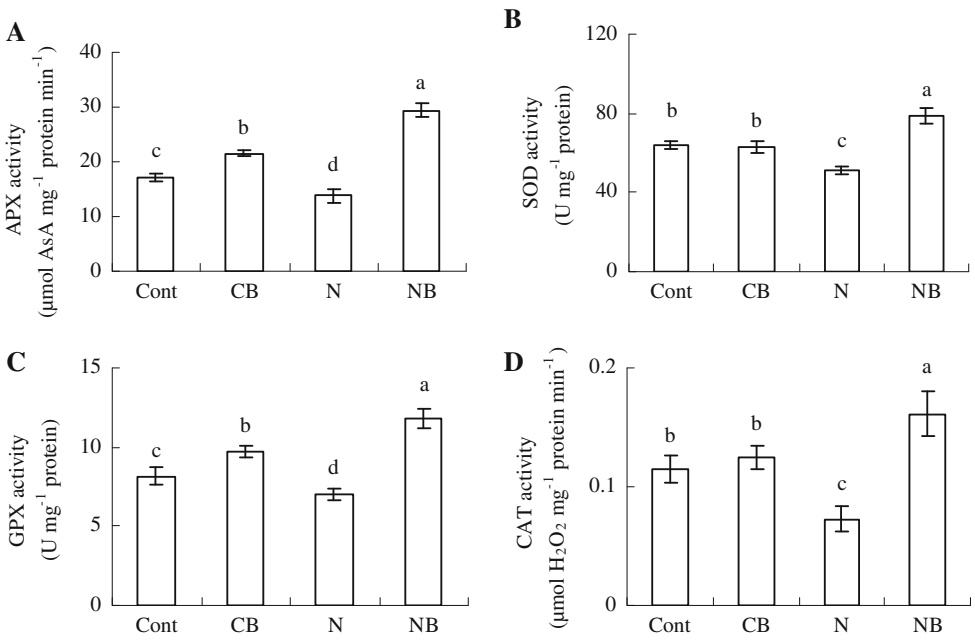

Fig. 4 Effects of $Ca(NO_3)_2$ and/or EBL on APX, SOD, GPX, and CAT activities in leaf of cucumber plants. Values represent the mean ± SE ($n = 3$). *Letters* indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests. Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

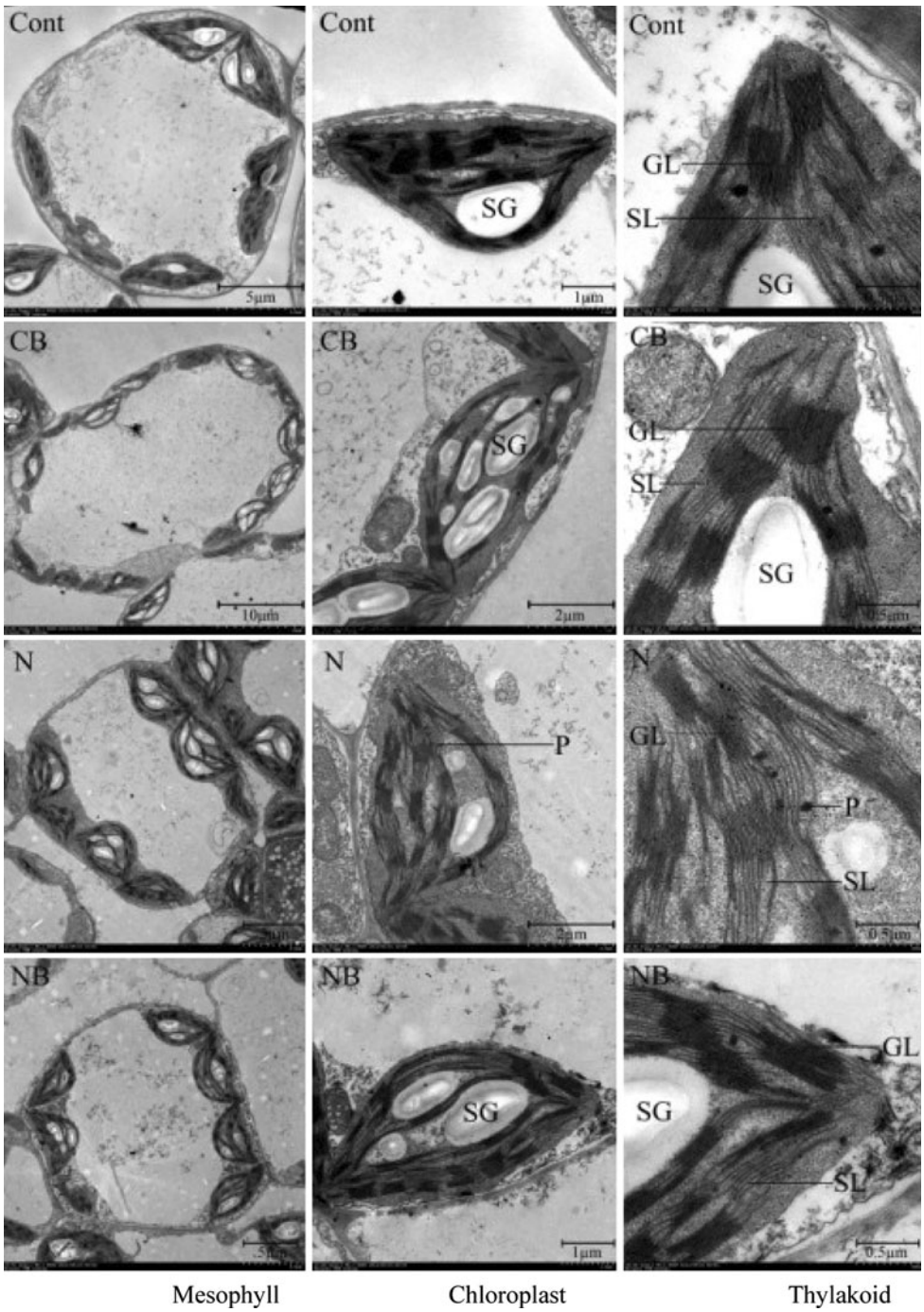

Fig. 5 Changes in ultrastructure of mesophyll cell, chloroplast and thylakoid of cucumber plants. *SG* starch globule, *GL* grana lamellae, *SL* stroma lamellae, *P* plastogloblus. Cont, control; CB, 0.1 μM EBL sprayed on leaves; N, 80 mM $Ca(NO_3)_2$ added into nutrient solution; NB, both EBL and $Ca(NO_3)_2$ treatments

Discussion

According to Ali et al. (2008a) and Hayat et al. (2010), BRs can ameliorate the detrimental effect of NaCl stress on P_N, Gs, Ci, and enhance the antioxidant system in plants. Current literature provides little evidence for the effects of EBL on plants under $Ca(NO_3)_2$ stress. In this study, the exogenous EBL enhanced plant growth and photosynthetic characteristics, alleviated oxidative damage and supported the recovery of chloroplast ultrastructure in plants when subjected to $Ca(NO_3)_2$ stress. These phenomena appear to result in physiological/metabolic alteration due to EBL as described below.

EBL in the CB solution caused a significant increase in the P_N in cucumber plants. The levels of chlorophyll content (Fig. 1) enhanced by EBL seemed to contribute to an increase in the P_N (Fig. 2). Moreover, this phenomenon may result in activation and/or induction of enzymes in chloroplasts by EBL. EBL also enhances the capacity of CO_2 assimilation in the Calvin cycle (Yu et al. 2004; Xia et al. 2009).

EBL in the NB solution significantly enhanced the P_N and induced a higher biomass accumulation, as compared to plants stressed with salt (Table 1). The enhancement of P_N may be attributed to activation of stomatal and/or non-stomatal ability, because the enhancement also seemed to activate Gs and Ci, as shown in Fig. 2. In this work, $Ca(NO_3)_2$ stress (N) caused reduction of P_N, Gs and Ci in cucumber plants (Fig. 2a–c). These phenomena appeared to be due to a deterioration of stomatal condition caused by $Ca(NO_3)_2$. The transpiration stream has a very important function with the transport of nutrients, such as inorganic elements in soil, from the soil to the roots (Lambers et al. 2008). Therefore, Tr reduced by salt (N) (Fig. 2d) seemed to decrease the absorption and utilization of water and nutrients in cucumber. In contrast, EBL in the NB solution caused significant increases in the Gs, Ci, and Tr, enhancing the photosynthesis as shown in Fig. 2. This phenomenon suggested that the influence by EBL on photosynthesis was more pronounced in plants stressed by $Ca(NO_3)_2$ than in the control. Understanding the causes of this differential response will require further investigation.

In this work, EBL in the NB solution significantly increased the Chl *a* and Chl *b* contents, but it decreased the ratio of Chl *a*/*b* (Fig. 1). These results suggested that the increases in the contents of chlorophyll by EBL in the NB solution are different between Chl *b* and Chl *a*. Specifically, in the salt-stressed plants, EBL caused a greater increase in Chl *b* than in Chl *a*. Horn et al. (2007) demonstrated that Chl *b* combined with the LHC more slowly and more tightly than Chl *a*. Moreover, they proposed that the Chl *b*-combining form finally stabilized the conformation of the LHC. The present study found that $Ca(NO_3)_2$ treatment darkened the green color of the leaves, which indicated that the total of chlorophyll content, on an area basis, was higher than in the control plants (data not shown). However, the chlorophyll content on a fresh weight basis in the salt-treated cucumber plants was lower than in the control (Fig. 1), suggesting that $Ca(NO_3)_2$ promoted the thickening of the leaves.

EBL in the NB solution played a role in the protection of PSII against excess energy through enhancing the thermal dissipation of the excitation energy (Table 2). ΦPSII, decreased by $Ca(NO_3)_2$ stress, was involved with changes in the photochemical conversion efficiency of PSII, which was regarded as injury to PSII complexes and the possible photoinhibition of photosynthesis. The decreases in the ΦPSII and qP by $Ca(NO_3)_2$ stress (N) (Table 2) suggested that the electron transport capacity in PSII was reduced. Salinity was also known to decrease qN, indicating that the radiant energy dissipation process was affected by $Ca(NO_3)_2$ stress. Furthermore, the Fv/Fm decreased by $Ca(NO_3)_2$ stress seemed to damage the reaction centers. EBL in the NB solution enhanced the ΦPSII, qP, and qN, and alleviated the decline of Fv/Fm. In addition, EBL might accelerate the repair process of pre-D1, release the photoinhibition of PSII and enhance thermal dissipation, thus avoiding the damage caused by excess energy excitation in the reaction centers. This can be illustrated as an increased qN (Table 2).

According to Mittler (2002), EBL treatment effectively protected plants from the oxidative stress induced by salt through enhancing the capacity of the antioxidant system. ROS can trigger peroxidative reactions and cause major damage to essential macromolecules, such as proteins involved in various enzymes of photosynthesis and in the systems of photosynthetic membranes (Foyer et al. 1994). Yabuta et al. (2002) reported that the overexpression of tAPX cDNA enhanced the level of thylakoid membrane-bound APX to maintain a higher ascorbic acid content which was engaged in maintaining cellular redox homeostasis under stresses. In this work, $Ca(NO_3)_2$ stress induced the accumulation of ROS (Fig. 3) and decreased the antioxidant enzyme activities (Fig. 4) in the cucumber leaves. The inactivation of SOD appeared to be due to excess Ca^{2+}, which might inhibit the absorption of coupling factors such as Fe, Mn, Cu, or Zn ions. The mechanism of the repression of the antioxidant enzyme genes or the transcription/translation of the enzyme proteins by excess Ca^{2+} remains to be investigated. The addition of EBL significantly increased the activities of APX, SOD, GPX, and CAT in the salt-stressed plants. These phenomena seemed to decrease the levels of ROS by the EBL (Fig. 3). EBL also regulates the expression of the peroxidase-encoding genes, ATP2 and ATP24a, in Arabidopsis (Goda et al. 2002). Therefore, the enhanced activities of antioxidant enzymes seemed to play an important role in order to scavenge the ROS (Fig. 3b, c). As a result, the activation of the enzymes will probably reduce the inhibitory effect of H_2O_2 on the synthesis of pre-D1 and protect the photosynthetic apparatus.

The activated behavior of antioxidant system in this study (Fig. 4) suggested that EBL acted to normalize the shape of thylakoid, promoted the formation of thylakoid grana and maintained the integral chloroplast envelopes in the leaves of cucumber plants treated with $Ca(NO_3)_2$. According to Parida et al. (2003), Sam et al. (2003), and Yamane et al. (2003), NaCl stress induced extreme damage on the ultrastructure of chloroplasts and thylakoids. However, few studies are reported on the harmful effects of $Ca(NO_3)_2$ on the ultrastructure of the photosynthetic apparatus in higher plants. The microscopic analysis in this study revealed that $Ca(NO_3)_2$ stress caused the blurred/loose shapes of the part of internal lamellae thylakoids as shown in Fig. 5, although most of granal thylakoids were still maintained. The chloroplasts and thylakoids envelope were shown to be degraded to varying degrees by the

oxidative stress (cf. the higher MDA content in Fig 3). The thylakoid plays an important role in the function of light absorption, electron transport, and energy conversion. Moreover, its structure also plays a functionally important role in the photosynthetic apparatus (Ioannidis and Kotzabasis 2007). Therefore, the oxidative damage of the thylakoids layers is considered to cause the blockage of electron transport, which leads to the decline of the photosynthetic rate. The results in this study suggested that EBL in the NB solution relieved this structural damage by protecting the photosynthetic membrane system from oxidative stress. Thylakoids and chloroplasts in cucumber plants by EBL were maintained their typical shapes similar to those of the control.

In conclusion, $Ca(NO_3)_2$ suppressed plant height, leaf area, biomass and physiological actions of cucumber plants. $Ca(NO_3)_2$ caused oxidative damage to the photosynthetic apparatus and resulted in the decline of the net photosynthetic rate and a slight change in chlorophyll fluorescence. The EBL positively regulated the content of the photosynthetic pigments, enhancing the capacity of the antioxidant system and protecting the reaction centers from photoinhibition. These results indicated that EBL played an important role in the relief of the harmful effect by $Ca(NO_3)_2$ and promotion of plant growth. Although the application of EBL (CB) under normal conditions also promoted plant growth and enhanced net photosynthetic rate and capacity of antioxidant system, the effects of EBL in plants under the salt-stressed condition were more prominent than those under the condition without salt. The mechanism of these phenomena by EBL remains obscure. These issues are currently being investigated from viewpoints of biochemistry and molecular biology.

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