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Resistance of spinach plants to seawater stress is correlated with higher activity of xanthophyll cycle and better maintenance of chlorophyll metabolism

J. SUN[*], Y.X. JIA[*,**], S.R. GUO[*,+], J. LI[*], and S. SHU[*]

College of Horticulture, Nanjing Agricultural University; Key Laboratory of Southern Vegetable Crop Genetic Improvement, Ministry of Agriculture, Nanjing–210095, China[*]
College of Resources and Environment, Sichuan Agricultural University, Yaan–625014, China[**]

Abstract

The relationship between the activity of xanthophyll cycle and chlorophyll (Chl) metabolism was investigated using two cultivars, Helan No. 3 (seawater-tolerant cultivar) and Yuanye (seawater-sensitive cultivar), of spinach (*Spinacia oleracea* L.) plants cultured in Hoagland's nutrient solution, with or without seawater (40%). The results showed that, in plants of two cultivars with seawater, the xanthophyll cycle seems to show a principal protection mechanism against photoinhibition under seawater stress. Furthermore, accumulation of reactive oxygen species (ROS) in chloroplasts of two cultivars was enhanced by seawater to lower the activity of porphobilinogen deaminase. Namely, the conversion of porphobilinogen into uroporphyrinogen III involved in Chl biosynthetic processes was inhibited by seawater. In Helan No. 3 spinach plants with seawater, higher activity of xanthophyll cycle in the leaves dissipated more excess light energy, which appeared to lower the levels of ROS in chloroplasts. As a consequence, the Chl biosynthesis in Helan No. 3 leaves with seawater showed only a weak inhibition and the activity of chlorophyllase (Chlase) was not affected by seawater stress. In contrast, a more pronounced accumulation of ROS in chloroplasts of Yuanye leaves, which possess lower xanthophyll cycle activity, severely inhibited Chl biosynthesis and remarkably enhanced the activity of Chlase, which aggravates the decomposition of Chl. These results suggest that higher activity of xanthophyll cycle in seawater-tolerant spinach plays a role in maintaining Chl metabolic processes, probably by decreasing the levels of ROS, when the plants are cultured in the nutrient solution with seawater (40%).

Additional key words: chlorophyll metabolism; photoinhibition; seawater; spinach; xanthophyll cycle.

Introduction

Water resources in the ocean correspond to a capacity of about 1.34×10^{18} m^3, which is equivalent to 96.8% of the total water of the earth. However, the freshwater on land is in short supply. Now, researchers have attempted to cultivate crops for food supply by irrigation using seawater (Sun *et al.* 2008). Spinach (*Spinacia oleracea* L.)

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[+]Corresponding author; phone: +86 25 84395267, e-mail: srguo@njau.edu.cn
Abbreviations: A – antheraxanthin; ALA – δ-aminolevulinic acid; AQY – apparent quantum yield; AsA – ascorbic acid; Chl(s) – chlorophyll(s); Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; Chlase – chlorophyllase; D – dissipative energy; DES – de-epoxidation state; DTT – 1,4-dithiothreitol; EC – electrical conductivity; Ex – excessive energy; F_m – maximal fluorescence in dark-adapted leaves; F_m' – maximal fluorescence in light-adapted leaves; F_o – minimal fluorescence in dark-adapted leaves; F_o' – minimal fluorescence in light-adapted leaves; F_v/F_m – maximal photochemical efficiency of photosystem II; g_s – stomatal conductance; Glu – glutamate; HPLC – high-performance liquid chromatography; LHCII – light-harvesting complexes of photosystem II; MDA – malondiladehyde; Mg-Proto IX – Mg-protoporphyrin IX; MV – methyl viologen; NPQ – non-photochemical quenching; $O_2^{\cdot -}$– superoxide radical; $^1O_2$ – reactive singlet oxygen; P – photochemical energy; PBG – porphobilinogen; PBGD – porphobilinogen deaminase; Pchl – protochlorophyll; PFD – photon flux density of the photosynthetically-active radiation; PSII – photosystem II; P_N – net photosynthetic rate; P_N-PFD – light-response curves of the net photosynthetic rate; Proto IX – protoporphyrin IX; RH – relative humidity; ROS – reactive oxygen species; Uro III – uroporphyrinogen III; V – violaxanthin; VDE – violaxanthin de-epoxidase; Z – zeaxanthin.
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plants are a middle salt-tolerant crop (Shannon and Grieve 1998). Therefore, it is proposed to be possible to cultivate spinach plants by using seawater.

Photosynthesis is essential for growth and development of plants and photoinhibition is a general phenomenon in the photosynthetic process (Anderson *et al.* 1997). When the rate of absorption of light energy by photosynthetic pigments exceeds the rate of its consumption in chloroplasts, the excessive absorbed light energy accelerates the process of photoinhibition (Demmig-Adams and Adams 1992, Melis 1999). Thus, the extent of photoinhibition is enhanced when the consumption of energy during the photosynthetic fixation of CO_2 is limited. Low and high temperatures, drought, and high salinity all strongly limit the photosynthetic fixation of CO_2 and have all been shown to accelerate photoinhibition (Demmig-Adams and Adams 1992, Murata *et al.* 2007). However, several protective mechanisms of plants are developed in the evolution of plants against the potentially damaging effects due to photoinhibition induced by environmental stresses (Anderson *et al.* 1997). Xanthophyll cycle in plants has been recognized as one of the most important regulatory mechanisms operating in plants and other photosynthesizing organisms at the molecular level, to protect the plants from photoinhibition under adversity stress conditions (Latowski *et al.* 2004). Xanthophyll cycle pigments such as zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V) are interconvertible due to epoxidation (Z→A→V) and de-epoxidation (V→A→Z) under certain conditions (Kotabová *et al.* 2008), namely, the violaxanthin de-epoxidase (VDE, EC 1.10.99.3) converts V into Z *via* the intermediate, and A is activated (Latowski *et al.* 2004). According to Mathis and Burkey (1989) and Horton *et al.* (2008), the conformation of light-harvesting complexes of photosystem II (LHCII) is transformed from a light-harvesting state to a dissipative state by Z and pH gradient. As a result, excess energy is dissipated as heat. On the other hand, it has been suggested that protection against photoinhibition requires Z, as a very efficient radical scavenger and chain-breaking antioxidant in peroxyl-radical-mediated peroxidation (Havaux and Niyogi 1999).

Chls are responsible for receiving solar energy in photosynthetic antenna systems and for charge separation and electron transport within reaction centers (Tanaka and Tanaka 2006). Reduced photosynthesis with increasing salinity has been attributed to a decline in Chls (Kolchevskii *et al.* 1995). However, the level of Chls is dependent on homeostasis between synthesis and degradation in the Chl metabolic processes of plants (Yu *et al.* 2006). Regulation of the levels of Chl and its derivatives, such as protochlorophyll (Pchl) or protoporphyrin IX (Proto IX), is extremely important, because these molecules are strong photosensitizers; that is, when present in excess, they will generate ROS. ROS, in turn, promote growth retardation or cell death. Therefore, to maintain healthy growth, plants must finely control the entire Chl metabolic process. Chl metabolism is a highly coordinated process that is executed *via* a series of cooperative reactions catalyzed by numerous enzymes (Barry 2009). Environmental stresses such as high salinity aggravate photoinhibition and further induce photooxidization over a long period to accumulate ROS in chloroplasts. According to Tanaka and Tanaka (2006), cell-death phenotypes induced by ROS have been observed in various plant species, in which genes encoding Chl biosynthetic enzymes are impaired. Thus, the normal process of Chl metabolism is disturbed by environmental-stress-induced ROS.

Enhanced salinity is experienced as a stress condition in plants, since it provokes changes in the photosynthetic apparatus, by affecting both its structure and function. NaCl-imposed ionic and oxidative stress on chloroplasts causes structural alterations in thylakoid membranes that induce significant loss in the functional ability of thylakoid membranes, such as to perform photochemical electron transport (Parida *et al.* 2003), and leads to the formation of ROS in chloroplasts (Sibole *et al.* 1998). Gruszecki *et al.* (2006) suggested that the higher xanthophyll-dependent non-photochemical quenching (NPQ) seems to suppress the risk of salt-induced generation of ROS leading to a damage of photosynthetic organisms. However, how the xanthophyll cycle in plants affects the Chl metabolic process under environmental stress such as high salinity has not been clarified. Seawater adversity was caused by excess ions as a dominant factor of stress, and it also aggravated photoinhibition of plants (Sun *et al.* 2008). Our earlier results have proven that the level of Chls in leaves of spinach plants was suppressed by seawater stress (Sun *et al.* 2008). In the present study, our purpose is to clarify the relationship between xanthophyll cycle and Chl metabolism, using spinach plants cultured under the seawater hydroponics. This should help to understand the physiological mechanisms of plant tolerance to seawater.

Materials and methods

Plant materials and conditions of cultivation: The experiment was carried out in an environment-controlled greenhouse. Two cultivars (cv. Yuanye and cv. Helan No. 3) of spinach (*Spinacia oleracea* L.) were used in this study. According to Sun *et al.* (2008), Yuanye is a seawater-sensitive cultivar and Helan No. 3 is a cultivar comparatively tolerant to seawater. The seeds were germinated on wet filter paper in a chamber at 18°C in the dark with 90% relative humidity (RH). The germinated seeds were cultured hydroponically in rock wool brick (one plant per brick) which was inserted in 72 holes of plastic trays. After the fifth leaf expanded,

seedlings grown uniformly in the bricks were transplanted to plastic boxes containing 50 L of full strength Hoagland's nutrient solution [pH 6.5±0.1, electrical conductivity (EC) 2.0–2.2 mS cm^{-1}]. The plants were cultured in a greenhouse at 26°C during day (10 h), average photon flux density of the photosynthetically active radiation (PFD) of 300 μmol m^{-2} s^{-1} and 10°C at night with 65–90% RH. Nutrient solution was aerated using an air pump at an interval of 20 min at 18–20°C. After preculture for 15 days, the seedlings were cultured in the nutrient solution with or without seawater (40%). According to Sun *et al.* (2008), difference in tolerance to seawater between two spinach cultivars such as Yuanye and Helan No. 3 under concentration of seawater (40%) was the greatest. Seawater (a total salt: 26.64 g L^{-1}, pH 7.8) was taken from the sea area of Dongtai, Jiangsu province, China, the composition of ions in the seawater sample was 349.98 mM of Na^+, 410.14 mM of Cl^-, 40.20 mM of Mg^{2+}, 21.15 mM of SO_4^{2-},7.61 mM of Ca^{2+}, and 6.83 mM of K^+.

Experimental treatments: On the 2nd day after supplying seawater, spinach plants of the two cultivars were divided into 4 groups as follows: No. 1 was sprayed with 0.05% (v/v) Tween 20 or distilled water on the plants cultured in the Hoagland's nutrients solution without or with seawater (CK or S, respectively). No. 2 was sprayed with 0.001 mM methyl viologen (MV) [solved by 0.05% (v/v) Tween 20] (CK+MV or S+MV) to induce oxidative stress with molecular oxygen to produce superoxide radical ($O_2^{\cdot -}$) in the stroma of chloroplasts, No. 3 with 5 mM ascorbic acid (AsA) (CK+AsA or S+AsA) to eliminate $O_2^{\cdot -}$ and H_2O_2 in plants to inhibit peroxidation of membrane lipids, No. 4 with 0.001 mM MV at zero time, and with 5 mM AsA at 24 h (CK+MV+AsA or S+MV+AsA). At 72 h after spraying on No. 1 (*i.e.* on the 6th day after supplying seawater), the 4th or 5th leaves from the top of nonstressed and stressed plants were collected to measure the contents of Chl precursors, activity of porphobilinogen deaminase (PBGD, EC 4.3.1.8) and chlorophyllase (Chlase, EC 3.1.1.14). Furthermore, intact chloroplasts were isolated for measuring other correlative index such as $O_2^{\cdot -}$ generation rate, hydrogen peroxide (H_2O_2) contents and malondialdehyde (MDA) contents.

The 4th or 5th leaves from the top of nonstressed and stressed plants without MV, AsA or MV+AsA were collected to measure the light response curves of the net photosynthetic rate (P_N-PFD) and apparent quantum yield (AQY). Chl fluorescence parameters were measured, at one time, petioles and nerves were removed from the 4th or 5th leaves before freezing in liquid nitrogen for measurement of xanthophyll cycle pigments. In addition, the younger leaves with petioles were dipped in full-strength Hoagland's nutrient solution with or without seawater (40%). The Hoagland's nutrient solutions were composed of CK and S with or without AsA (20 mM, pH 6.25, AsA enhances de-epoxidation) (*i.e.* CK + AsA or S + AsA), with or without 1, 4-dithiothreitol (DTT, 3 mM; an inhibitor of de-epoxidation) (*i.e.* CK + DTT or S + DTT), respectively, and the leaves were incubated under controlled environmental conditions in a growth chamber at 25°C with 65% RH. Light in the chamber was irradiated at 30 μmol m^{-2} s^{-1} for 3 h and then at 2,000 μmol m^{-2} s^{-1} for 1 h. After the incubation, the Chl fluorescence parameters and xanthophyll cycle pigments were measured with five replicates.

Chl fluorescence: Chl fluorescence was measured using a pulse amplitude-modulated fluorometer (*PAM 2000, Heinz Walz GmbH,* Effeltrich, Germany) on fresh leaves sampled at an interval of 1 h from 8:30 to 16:30 in an environment-controlled greenhouse. Minimum fluorescence (F_o) was determined by illuminating the leaf with a dim red light (<0.1μmol m^{-2} s^{-1}) modulated at 0.6 kHz after keeping the leaf for 30 min in darkness. Maximum fluorescence of dark-adapted leaf (F_m) was obtained during a subsequent saturating light pulse (8,000 μmol m^{-2} s^{-1}, 0.8 s). The leaf was then continuously illuminated with actinic light at an intensity of 150, 200, 350, 600, 1,400; 1,300; 900, 300 and 80 μmol m^{-2} s^{-1} which were equivalent to the growth of spinach plants at an interval of 1 h from 8:30 to 16:30 h in the greenhouse. The steady-state fluorescence (F_s) was thereafter recorded, and a second saturating pulse of white light (8,000 μmol m^{-2} s^{-1} for 0.8 s) was imposed to determine the maximum fluorescence level in the light-adapted state (F_m'). The actinic light was put out and then the minimal fluorescence level in the light-adapted state (F_o') was determined by illuminating the leaf with a far-red light for 3 s. The following parameters were estimated to characterize the fluorescence induction kinetics: (*1*) non-photochemical quenching (NPQ) was calculated as $F_m/F_m' - 1$, (*2*) dissipative energy (D) was calculated as $1 - (F_v'/F_m')$, (*3*) photochemical energy (P) was calculated as $(F_m' - F_s)/F_m'$, (*4*) excessive energy (Ex) was calculated as $F_v'/F_m' \times (1 - q_P)$ (Demmig-Adams *et al.* 1996), and (5) the percentages of D, P, and Ex in total were done. The P, D and Ex were obtained from Chl fluorescence at 10:30.

***P*$_N$-PFD and AQY**: Light-response curves of the net photosynthetic rate were measured using portable photosynthesis system (*Li-6400, LI-COR Inc.,* Lincoln, NE, USA) at ambient CO_2 concentration [360 μmol(CO_2) mol^{-1}] on attached leaves after 30 min of dark adaptation in the morning (between 09:00 and 10:00). The greenhouse for measuring photosynthesis was controlled to maintain the leaf temperature at 22°C and the relative humidity at 60–70%. The leaves were exposed for 5 min to increase the incident PFD (0, 25, 50, 100, 150, 200, 400, 600, 800, 1,000; 1,200; 1,500; and 1,600 μmol m^{-2} s^{-1}) to measure gas-exchange parameters such as net photosynthetic rate (P_N) and stomatal conductance (g_s).

Increasing incident PFD (0, 25, 50, 100, 150, 200, and 250 μmol m^{-2} s^{-1}) was provided to measure gas-exchange parameters, AQY was estimated from the initially slopes of P_N-PFD curves.

Pigment extraction and its separation by HPLC: Extraction of xanthophyll cycle pigments was performed according to the method of Park *et al.* (2008). The pigments were estimated by gradient reverse-phase high-performance liquid chromatography (HPLC) by a modified version of the method of Gilmore and Yamamoto (1991). Pigments were separated at 25°C on a spherisorb column (250 × 4 mm) preceded by a spherisorb guard column (4 × 4 mm), using an injection volume of 20 μL. The isocratic elution for 0–4 min with the solvent composed of acetonitrile, methanol, and 50 mM Tris (72:8:3, v/v/v) was followed by a 2.5-min linear gradient into a second solvent composed of methanol and *n*-hexane (5:1, v/v) at a flow rate of 1.5 mL min^{-1}. The peak areas at 440 nm were integrated using *ChromQuest software* (*ThermoQuest,* San Jose, CA, USA) and compared to a calibration curve of purified standards of V, Z, and A (*Sigma-Aldrich,* Saint Louis, MO, USA). The de-epoxidation state (DES) of the xanthophyll cycle pigments was calculated as (Z+0.5A)/(V+A+Z), which is represented as the activity of the xanthophyll cycle.

Isolation of intact chloroplasts, measurements of $O_2^{\cdot -}$ generation rate, H_2O_2, and MDA contents: Isolation of intact chloroplasts was done at room temperature according to the method of Takeda *et al.* (1982). The intactness of chloroplasts was estimated to be 90% by the ferricyanide method of Yamamoto *et al.* (1972b). The measurement of $O_2^{\cdot -}$ generation rate of isolated chloroplasts dispersed with 50 mM sodium phosphate buffer (pH = 7.8) was carried out by monitoring the A_{530} (*JH-752, Jinghua Inc.*, Shanghai, China) to detect the hydroxylamine reaction due to the method of He *et al.* (2005). A standard curve with NO_2^- was used to estimate the generation rate of $O_2^{\cdot -}$.Chloroplasts suspension was diluted with 0.2 N perchloric acid to one third. The diluted suspension was neutralized with KOH and then centrifuged at 10,000 × *g* for 5 min. An aliquot of the supernatant was used for the quantification of H_2O_2 according to the modified method of Okuda *et al.* (1991), the amount of H_2O_2 was calculated using a standard graph of known concentrations. And the remaining supernatant was diluted with 5% trichloroacetic acid for the estimation of the level of lipid peroxidation from the relative content of thiobarbituric acid reactive products (TBARPs), calculated as the absorption difference between A_{532} and A_{600} (*JH-752, Jinghua Inc.*, Shanghai, China) with an extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Vlckova *et al.* 2006).

Determination of Chl precursors: Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) were estimated spectrophotometrically using pigment fraction extracted by 80% acetone with a small amount of $MgCO_3$ (Lichtenthaler 1987). Protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX) and protochlorophyll (Pchl) were extracted using a mixture of acetone:ammonia (1%) (4:1), extracts were followed by measuring the absorbance at 575 nm, 590 nm, and 628 nm (Hodgins and van Huystee 1986). Fresh leaves were iced-homogenized using Tris-HCl (pH 7.2), the homogenates were centrifuged at 5,000 × *g* for 15 min at 4°C, uroporphyrinogen III (Uro III) content in supernatants was determined by the method of Bogorad (1962). Leaf samples were homogenized with Tris-HCl buffer (pH 8.0) containing 100 mM Tris, 50 mM mercaptoethanol, homogenates were centrifuged at 8,000 × *g* for 15 min at 4°C. Specifically, 2 mL of supernatants or standard were mixed with 2 mL of freshly prepared Ehrlich's reagent and after 30 min the mixture was used to determine A_{555} for the determination of porphobilinogen (PBG) by the method of Bogorad (1962). Fresh leaves were homogenized with acetic sodium buffer (pH 4.6), homogenates were extracted in boiling water bath for 15 min and centrifuged at l0,000 × *g* for 20 min at 4°C, the supernatants were pooled to determine δ-aminolaevulinic acid (ALA) content by the method of Richard (1975). Content of ALA was calibrated using the standard of ALA-HCl (*Sigma-Aldrich, Saint Louis,* MO, USA).

Assay of activities of PBGD and Chlase: Fresh leaves were homogenized with Tris-HCl buffer (pH 8.0) containing 50 mM Tris, 5 mM mercaptoethanol, 2 mM phenylmethyl sulfonyl fluoride, 2 mM EDTA-Na_2 at 4°C. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was allowed to stand at 65°C for 20 min and then cooled in an ice bath. The cooled supernatant was centrifuged again as described above and used to determine $A_{405.5}$ (*JH-752, Jinghua Inc.*, Shanghai, China) for the estimation of PBGD activity by the method of Riminton (1960).

Preparation of substrates, enzyme extraction, and assay of Chlase activity were performed according to the method of Costa *et al.* (2005). Protein extraction buffer contained: 100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 0.2% (v/v) Triton X-100, 30 g L^{-1} polyvinylpolypyrrolidone, 1 mM phenyl methyl sulfonyl fluoride, 5 mM cysteine, pH 6.0. Chls of spinach leaves were used as a substrate. 6 g of fresh mass (FM) of spinach leaves were homogenized in an omnimixer with 60 ml of acetone:water (80:20, v/v) solution at 4°C. The suspension was centrifuged at 9,000 × *g* and the supernatant was added with 40 ml of petroleum ether to extract the Chls. After this, the ether was evaporated under N_2 and the Chls dissolved in 4–5 ml of acetone. The Chls concentration in the assays was kept 1.0 mg mL^{-1}. The following reaction mixture was used: 100 mM sodium phosphate buffer (pH 7.0) with 0.15 % (v/v) Triton X-100, 0.01 mM Chls, 16% (v/v) acetone and 2 mL of enzymatic extract in a

total volume of 13 mL. The mixture was incubated at 40°C and duplicate samples of 2 mL were taken after the beginning of the reaction, and poured into 5 mL of mixture of hexane:acetone (7:3) precooled in ice-water. The mixtures were vigorously stirred until emulsion formation, and then allowed to stand in the dark at 4°C and centrifuged at 6,000 × *g* for 5 min at 4°C. The upper phase contained the remaining Chl, while the lower phase contained the chlorophyllide. The progress of the Chlase activity was followed by measuring the absorbance at 663 nm (*JH-752, Jinghua Inc.*, Shanghai, China) in the lower phase. The enzyme activity was expressed as the increment of optical density at 663 nm per min under the test conditions.

Protein contents in the enzyme extract were determined by the method of Bradford (1976) using bovine serum albumin (*Sigma-Aldrich,* Saint Louis, MO, USA) as the standard.

Statistical analysis: All data were expressed as the means ± SD (n = 5). Variance analysis was performed on all experimental data and statistical significance of the means of five replicates was judged by least significance difference (LSD) test at P<0.05 using *SPSS 11.0 for Windows* (*SPSS Inc.*, Chicago, IL, USA).

Results

Allocation of light absorbed by leaves: P between leaves of two cultivars such as Yuanye and Helan No. 3 was shown to have a tendency to be somewhat suppressed and in contrast, D seemed to be somewhat enhanced. However, Ex in Yuanye spinach plants was shown to have a tendency to be enhanced and that in Helan No. 3 spinach plants was shown to have no effect, compared with spinach leaves without seawater stress (Fig. 1). Under seawater stress, suppression of P in leaves of both cultivars seems to induce photoinhibition, and the enhancement of D seems to depend on thermal dissipation.

***P*$_N$-PFD and AQY**: Net photosynthetic rate values in both spinach plants (Yuanye and Helan No. 3) without seawater reached a plateau at a PFD of 800–1,200 μmol m^{-2} s^{-1}, but PFDs more than 1,200 μmol m^{-2} s^{-1} caused small values of P_N. In the case of spinach plants with seawater, P_N values in Yuanye and Helan No. 3 peaked at 800 μmol m^{-2} s^{-1}, and declined above this value (Fig. 2*B*). P_N and g_s (Fig. 2*A,C*) were measured again for PFDs between 0 to 250 μmol m^{-2} s^{-1} and AQY was estimated (Fig. 2*D*). We found that P_N, g_s, and AQY were suppressed in both cultivar leaves with seawater. These suppressions of Yuanye spinach leaves, were shown to be large, compared with Helan No. 3 spinach leaves.

NPQ and DES: On the sixth day after supplying seawater, the daily dynamics of NPQ (Fig. 3*A*) and DES (Fig. 3 *B*) in both spinach cultivar leaves seemed to have a similar effect as a daily dynamics of photosynthetically active radiation (data not shown), and showed the peak at 12:30. Multinomial correlation between daily dynamics of NPQ and DES in both cultivar leaves, with or without seawater stress, was shown by recursive analysis. The correlative coefficients were significant. Furthermore, NPQ (Fig. 3*C*) and DES (Fig. 3*D*) of both spinach cultivars with seawater stress were higher than those without that stress, which was greater in Helan No. 3 than that in Yuanye. De-epoxidation was associated with NPQ (Demmig-Adams 1998), which was also promoted by AsA (Veljovic-Jovanovic *et al.* 2001), but particularly restrained by low concentration of DTT, an inhibitor of de-epoxidation (Yamamoto and Kamite 1972a). Ascorbic acid also had a tendency to enhance NPQ and DES, as well as seawater. However, DTT suppressed NPQ and DES of both cultivar leaves, without or with seawater.

Effects of MV and AsA on $O_2^{\cdot -}$ generation rate, H_2O_2 and MDA content in chloroplasts: MV is often used to induce oxidative stress with molecular oxygen to produce superoxide radical $O_2^{\cdot -}$ in stroma of chloroplasts

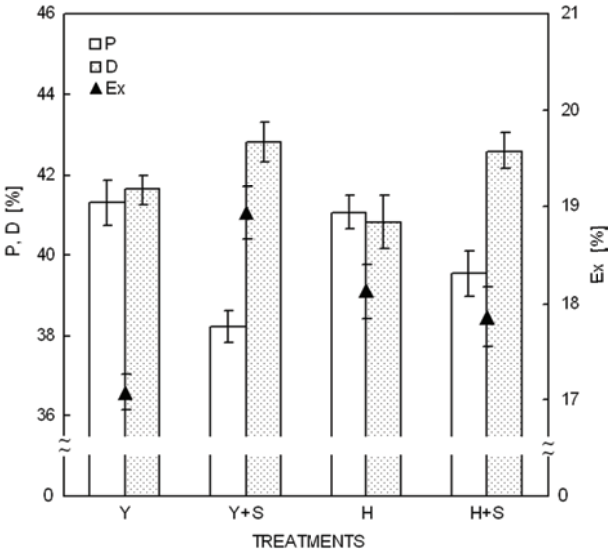

Fig. 1. Effects of seawater stress on allocation of fraction that is consumed for photochemical reaction (P), fraction of light absorbed in the PSII antennae that is dissipated thermally (D) and fraction that remained in the photosystem II (PSII) reactive center (Ex) of spinach leaves. P was calculated as $(F_m' - F_S)/F_m'$, D as $1 - (F_v'/F_m')$ and Ex as $F_v'/F_m' \times (1 - q_P)$. Chlorophyll fluorescence was determined at sixth day after supplying seawater (40%). *Vertical bars* represent the means ± SD (n = 5). Y – Yuanye spinach without supplying seawater; H – Helan No. 3 spinach without supplying seawater; Y+S – Yuanye spinach with supplying seawater; H+S – Helan No. 3 spinach with supplying seawater.

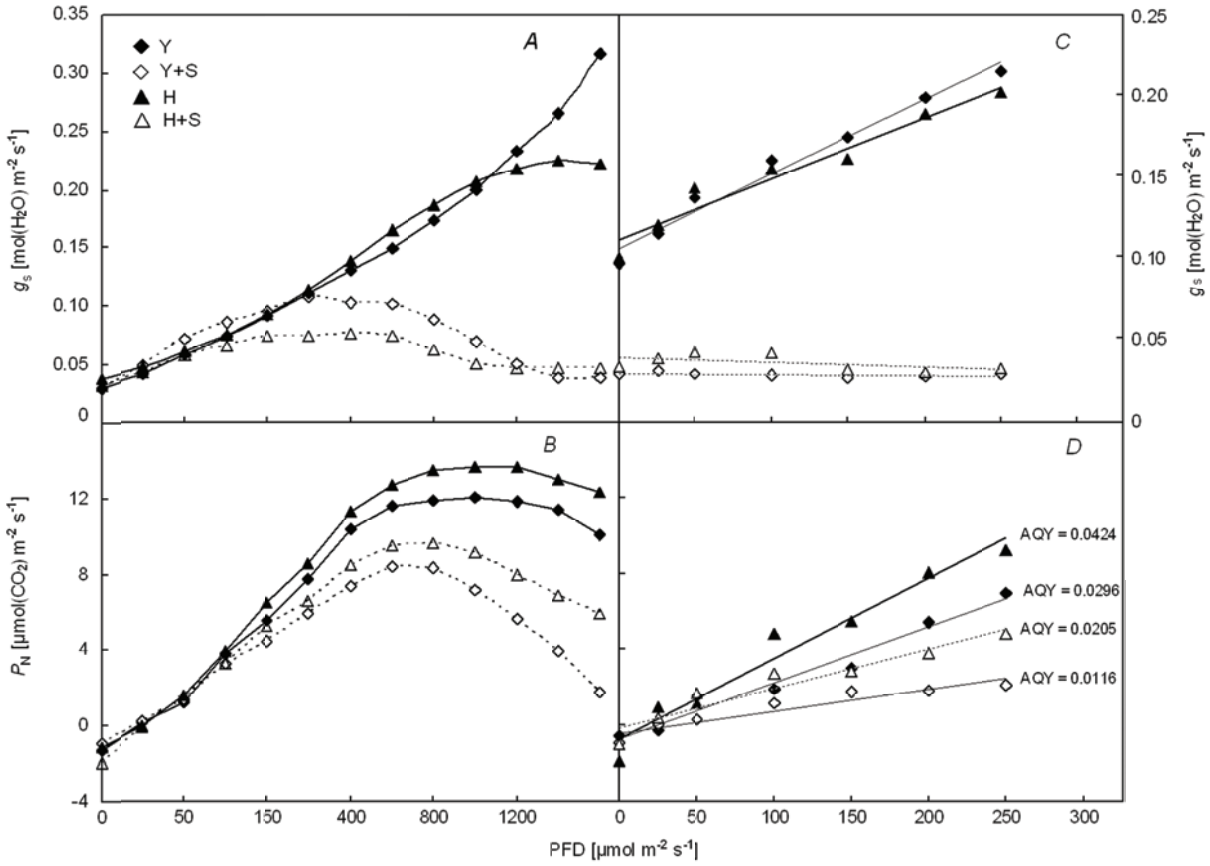

Fig. 2. Effects of seawater stress on the stomatal conductance (g_s) (*A,C*), light-response curves of the net photosynthetic rate (P_N-PFD) (*B*) and apparent quantum yield (AQY) (*D*) of spinach leaves. The leaves at sixth day after supplying seawater (40%) were irradiated for 5 min to enhance incident photosynthetically active photon flux density (PFD) after keeping dark adaptation in the morning for 30 min and P_N in the leaves were determined. The greenhouse for measuring photosynthesis was controlled to maintain the leaf temperature at 22°C and the relative humidity at 60–70%. For Y, Y+S, H, and H+S, *see* Fig. 1.

(Farrington *et al.* 1973). The AsA plays a role as a scavenger of $O_2^{\cdot -}$ and H_2O_2 in plants to inhibit peroxidation of membranes (Asada 1999). MDA, a decomposition product of polyunsaturated fatty acids, is an indicator of membrane damage (Fadzilla *et al.* 1997). As shown in Fig. 4, $O_2^{\cdot -}$ generation rate (Fig. 4*A*), H_2O_2 (Fig. 4*B*) and MDA (Fig. 4*C*) contents in chloroplasts of both cultivar leaves were remarkably enhanced by supplying seawater and MV, compared with control (CK). The values of Yuanye leaves were higher than those of Helan No. 3 leaves. However, $O_2^{\cdot -}$ generation rate, H_2O_2 and MDA contents in chloroplasts of both cultivar leaves with treatments designated as S+AsA, CK+MV+AsA and S+MV+AsA showed lower values, compared with treatments of S, CK+MV and S+MV, respectively. These results suggest that supplying seawater as a function of MV induces ROS accumulation in chloroplasts to disrupt normal metabolism of plants through oxidation of membrane lipids.

Effects of MV and AsA on content of Chl precursors: It is well known that biosynthesis of Chl can be blocked at many points. As a result, precursors before the blocked site are accumulated and then decreased (Yu *et al.* 2006). The contents of Chl *b*, Chl *a*, Pchl, Mg-Proto IX and Uro III of both spinach cultivar leaves were remarkably suppressed, and, in contrast, contents of PBG and ALA were significantly enhanced by supplying seawater and MV, compared with control (CK) (Table 1); and those precursors in leaves of both spinach cultivars with respective treatments designated as CK+MV+AsA and S+MV+AsA were enhanced. In contrast, PBG and ALA were suppressed by AsA, compared with treatments of CK+MV and S+MV, respectively. These data suggest that biosynthesis of Chl is blocked at the site of the conversion of PBG into Uro III by seawater-induced ROS in chloroplasts of spinach cultivars, and the blockage is greater in cv. Yuanye than that in cv. Helan No. 3.

Effects of MV and AsA on the activities of PBGD and Chlase: Decreasing PBGD activity is probably related to inhibition of Chl biosynthesis (Cheng *et al.* 2006), and the first enzyme action in the pathway of Chl degradation is the removal of phytol by Chlase (Fang *et al.* 1998).

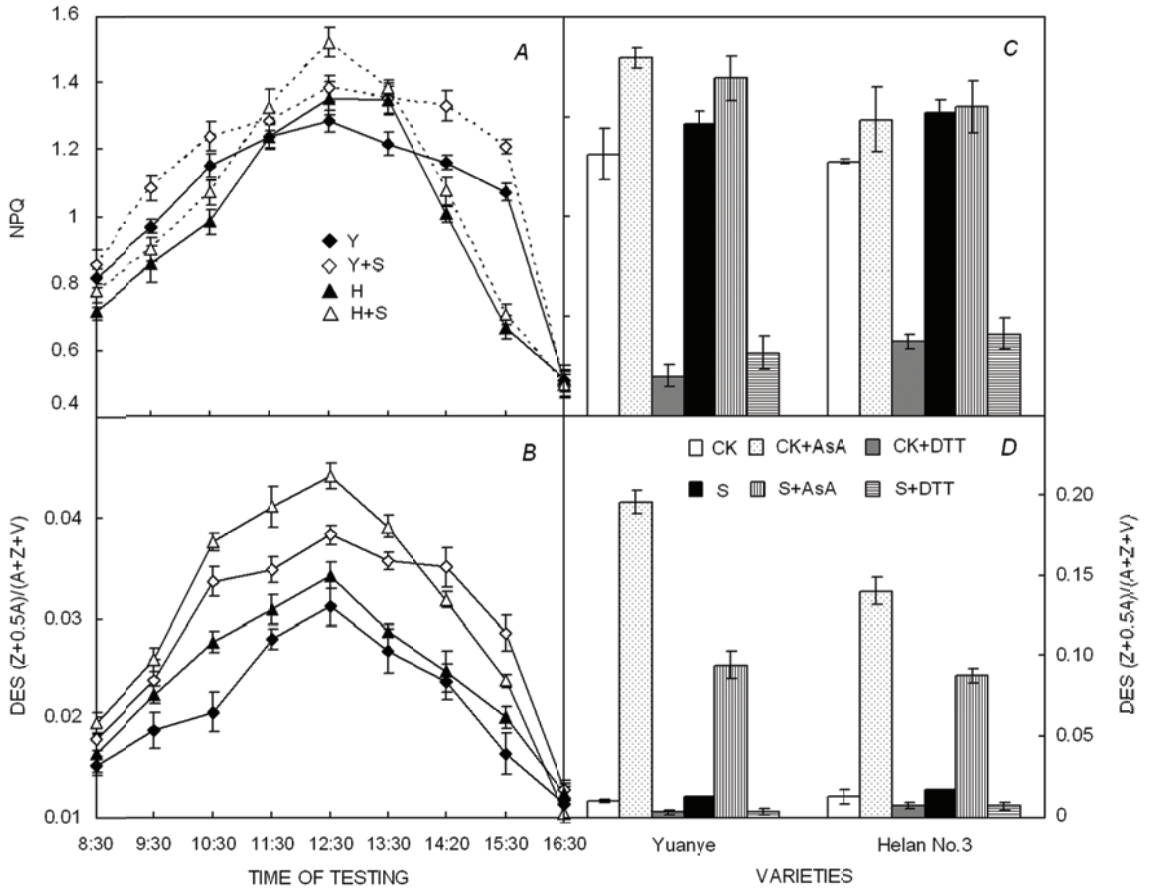

Fig. 3. Daily dynamics of non-photochemical quenching (NPQ) (*A*), de-epoxidation state (DES) of the xanthophyll cycle pigments (*B*) and effects of ascorbic acid (AsA) and 1,4-dithiothreitol (DTT) on NPQ (*C*) and DES (*D*) of spinach leaves. NPQ and DES were determined using the same leaves at sixth day after supplying seawater (40%). The NPQ parameter was calculated as $(F_m–F_m')/F_m'$, the DES as (Z+0.5A)/(A+Z+V). For pigment analysis, leaf discs were frozen in liquid nitrogen for pigment extraction. *Vertical bars* represent the means ± SD of results from five replicates. For Y, Y+S, H and H+S, *see* Fig. 1. CK – control (without supplying seawater); CK+AsA – control with supplying AsA; CK+DTT – control with supplying DTT; S – with supplying seawater; S+AsA – with seawater and AsA; S+DTT – with seawater and DTT; A – antheraxanthin; V – violaxanthin; Z – zeaxanthin.

As shown in Fig. 5*A*, PBGD activities of Yuanye and Helan No. 3 leaves under saline condition are lower than those under control conditions, which is greater in cv. Yuanye than that in cv. Helan No. 3. PBGD activity of both cultivar leaves with respective treatments designated as S, CK+MV, and S+MV, except for (S+AsA)-treated leaves of cv. Helan No. 3., show lower values, compared with treatments of S+AsA, CK+MV+AsA and S+MV+AsA, respectively. Chlase activity of Yuanye leaves is remarkably enhanced by seawater and MV, compared with control, and the enhancement is retarded by AsA. However, Chlase activity of Helan No. 3 leaves is not affected by seawater, MV and AsA, compared with control (Fig. 5*B*). These results suggest that seawater-induced ROS accumulation in chloroplasts suppresses PBGD activity in leaves of both cultivars and enhances Chlase activity of Yuanye leaves, but does not affect Chlase activity of Helan No. 3 leaves.

Discussion

Light energy absorbed by plant leaves plays three roles such as photochemical energy (P), dissipative energy (D), and excessive energy (Ex) as Chl fluorescence parameters which are useful to understand allocation of light energy absorbed (Costa *et al.* 2005). In the leaves of 2 cultivars with supplying seawater, suppression of P (Fig. 1) seems to induce photoinhibition, and enhancement of D (Fig. 1) may depend on thermal dissipation stimulated by seawater stress to play a crucial role for regulation between the absorbed light and electron transport in PSII. However, Ex (Fig. 1) increasing in Yuanye leaves with seawater stress seems to induce more reactive singlet oxygen ($^1O_2$) in reaction centers and LHCII (Asada 1999, Zhou *et al.* 2004).

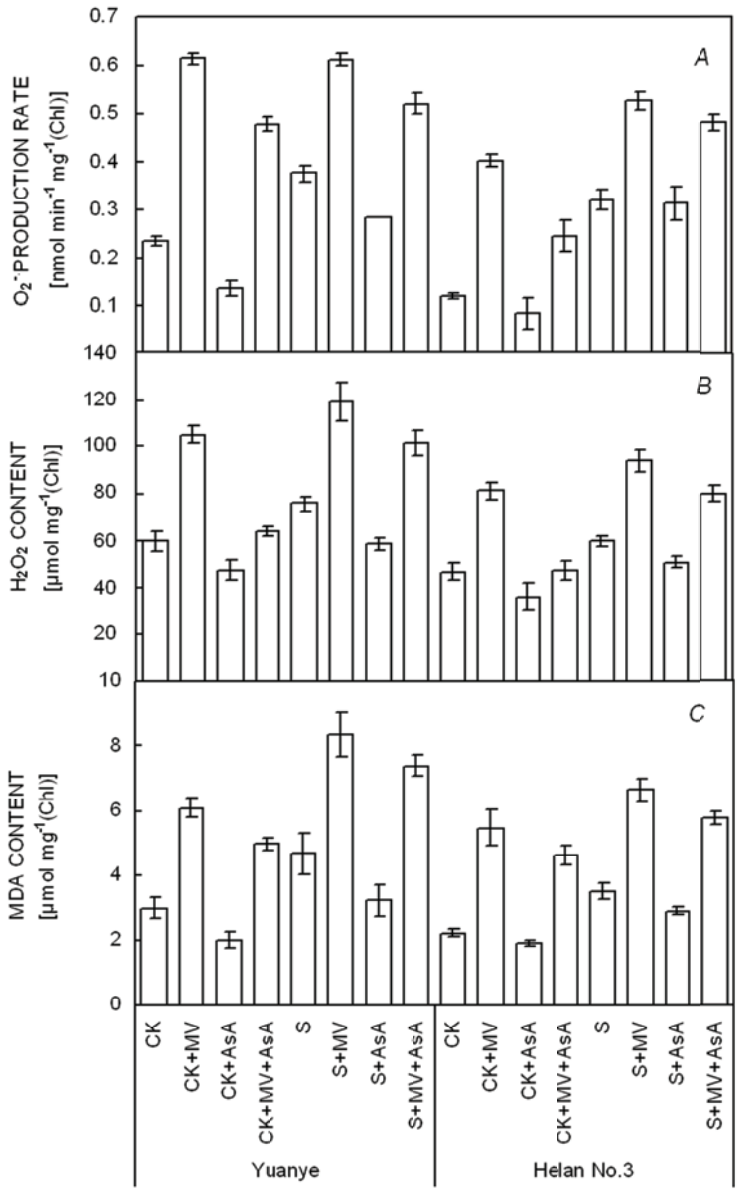

Fig. 4. Effects of methyl viologen (MV) and ascorbic acid (AsA) on $O_2^{\cdot-}$ generation rate (*A*), hydrogen peroxide (H_2O_2) (*B*) and malondialdehyde (MDA) (*C*) in chloroplasts of spinach leaves. Those values were obtained using the intact chloroplasts in the 4th or 5th leaves from the top of plants at sixth day after supplying seawater (40%). CK – control; CK+MV – control with supplying MV; CK+AsA – control with AsA; CK+MV+AsA – control with MV and AsA; S – with supplying seawater; S+MV – with supplying seawater and MV; S+AsA – with supplying seawater and AsA; S+MV+AsA – with supplying seawater, MV and AsA.

Photoinhibition of plants seems to be reflected by decreasing photochemical efficiency of PSII (F_v/F_m), AQY, and P_N at full light intensity (Björkman and Demmig-Adams 1994). In this study, F_v/F_m (Sun *et al.* 2008), AQY, and P_N at full light intensity of the leaves of both cultivars with supplying seawater were all suppressed (Fig. 2*B,D*). This suppression suggests that photoinhibition of the two cultivars is enhanced by seawater stress. Higher plants have developed several strategies to avoid or minimize damage due to photoinhibition, and nonradiative dissipation of the excess excitation in the antenna, detected as NPQ, is the major protective mechanism against destruction of the photosynthetic apparatus by excessive radiation (Härtel and Lokstein 1995), but this phenomenon depends on the differences between varieties (Hong and Xu 1997). Thermal dissipation dependent on the xanthophyll cycle seems to be similar to the principal protective mechanism for wheat plants, but this shows the reversible inactivity of PSII reactive center for soybean plants (Jia *et al.* 2000). The positive correlation between NPQ and de-epoxidation is well established (Gilmore 2001). However, this result was not confirmed by other researchers (Horton *et al.* 1996). In this study, the correlation between daily dynamics of NPQ (Fig. 3*A*) and DES (Fig. 3*B*) in leaves between both cultivars is shown. Besides, in leaves between two cultivars without and with seawater, NPQ and DES are promoted by AsA, but suppressed by DTT (Fig. 3*C,D*), proving that xanthophyll cycle plays a role of nonradiative dissipation of the excess excitation in the plants of spinach. The activity of VDE (the key enzyme of de-epoxidization) is strictly regulated by AsA (Mathis and Burkey 1989), and the AsA regulation is probably connected to a competition for AsA between ascorbic

Table 1. Effects of methyl viologen (MV) and ascorbic acid (AsA) on the contents of chlorophyll (Chl) precursors in the leaves of spinach plants cultured in Hoagland's nutrient solution with seawater. The contents of Chl precursors in leaves were determined using the 4th or 5th leaves below the top of plants at sixth day after supplying seawater (40%). Values are represented as the means of results from five replicates. *Different letters* indicate significant differences at $P<0.05$ according to *Duncan*'s multiple range tests. For Y and H, *see* Fig. 1. For CK, CK+MV, CK+AsA, S, S+MV, S+AsA and S+MV+AsA, *see* Fig. 4. ALA – δ-aminolevulinic acid; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; Mg-Proto IX – Mg-protoporphyrin IX; PBG – porphobilinogen; Pchl – protochlorophyll; Proto IX – protoporphyrin IX; Uro III – uroporphyrinogen III; FM – fresh mass.

Treatments	Chl *b* [mg g^{-1}(FM)]		Chl *a* [mg g^{-1}(FM)]		Pchl [mg g^{-1}(FM)]		Mg-Proto IX [mg g^{-1}(FM)]		Proto IX [mg g^{-1}(FM)]		Uro III [mg g^{-1}(FM)]		PBG [mg g^{-1}(FM)]		ALA [mg g^{-1}(FM)]	
	Y	H	Y	H	Y	H	Y	H	Y	H	Y	H	Y	H	Y	H
CK	0.202^{b}	0.350^{a}	0.537^{b}	0.815^{a}	0.379^{b}	0.475^{b}	0.250^{b}	0.265^{b}	0.646^{b}	0.639^{b}	13.423^{b}	14.831^{a}	10.558^{d}	10.161^{f}	0.782^{d}	0.835^{f}
CK+MV	0.144^{d}	0.250^{d}	0.428^{e}	0.695^{d}	0.263^{d}	0.402^{d}	0.141^{d}	0.197^{d}	0.398^{d}	0.520^{d}	3.624^{e}	6.607^{e}	12.806^{c}	12.145^{d}	3.454^{a}	3.811^{c}
CK+AsA	0.253^{a}	0.358^{a}	0.648^{a}	0.804^{a}	0.418^{a}	0.480^{b}	0.309^{a}	0.267^{b}	0.721^{a}	0.753^{a}	15.613^{a}	15.079^{a}	11.346^{d}	10.825^{ef}	2.483^{b}	1.546^{d}
CK+MV+AsA	0.157^{c}	0.301^{c}	0.467^{cd}	0.772^{b}	0.301^{c}	0.470^{b}	0.192^{c}	0.245^{c}	0.488^{c}	0.553^{cd}	6.593^{d}	9.409^{d}	11.219^{d}	11.484^{de}	3.031^{ab}	3.432^{c}
S	0.165^{c}	0.296^{c}	0.482^{c}	0.727^{bc}	$0.265d$	0.436^{c}	0.141^{d}	0.241^{c}	0.365^{e}	0.578^{c}	10.022^{c}	12.977^{b}	13.633^{bc}	13.600^{c}	1.523^{c}	1.298^{e}
S+MV	0.106^{e}	0.256^{d}	0.322^{f}	0.679^{d}	0.209^{e}	0.303^{f}	0.118^{f}	0.211^{d}	0.316^{f}	0.467^{e}	4.321^{e}	7.220^{e}	15.716^{a}	17.832^{a}	3.560^{a}	5.543^{a}
S+AsA	0.193^{b}	0.338^{b}	0.556^{b}	0.759^{b}	0.253^{d}	0.551^{a}	0.139^{e}	0.286^{a}	0.382^{d}	0.642^{b}	12.868^{b}	14.758^{a}	14.836^{ab}	13.972^{bc}	2.448^{b}	1.564^{d}
S+MV+AsA	0.143^{d}	0.291^{c}	0.456^{d}	0.719^{c}	0.247^{d}	0.389^{e}	0.149^{d}	0.248^{b}	0.360^{e}	0.553^{cd}	6.593^{d}	10.162^{c}	13.997^{b}	14.526^{b}	2.515^{b}	4.948^{b}

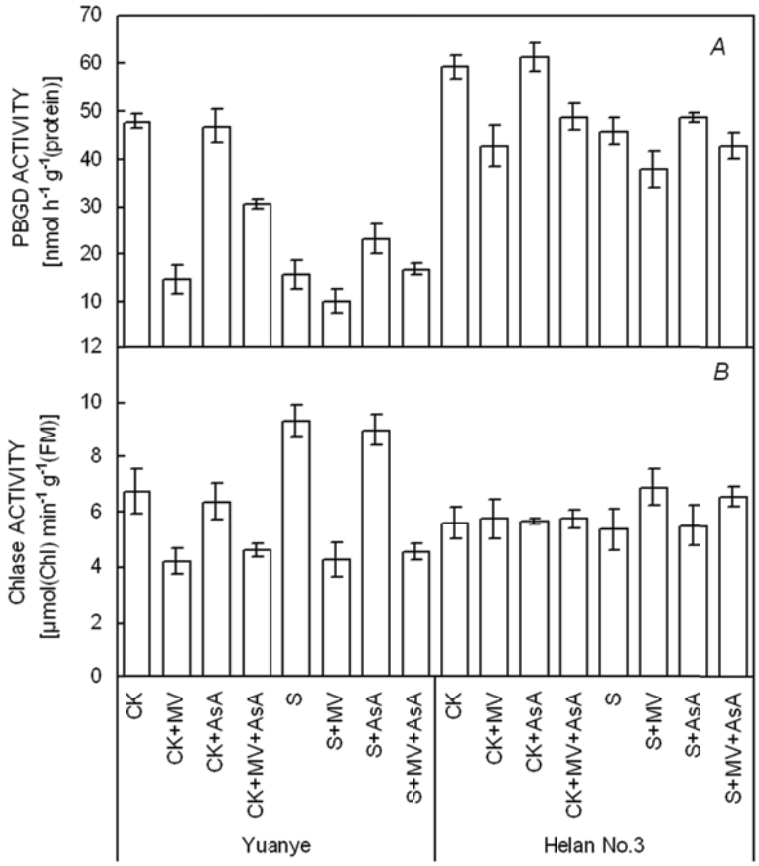

Fig. 5. Effects of methyl viologen (MV) and ascorbic acid (AsA) on the activities of porphobilinogen deaminase (PBGD) (*A*) and chlorophyllase (Chlase) (*B*) of spinach leaves. The enzyme activities were determined using the 4th or 5th leaves from the top of plants at sixth day after supplying seawater (40%). *Vertical bars* represent the means ± SD of results from five replicates. For CK, CK+MV, CK+MV+AsA, S, S+MV, S+AsA and S+MV+AsA, *see* Fig. 4.

acid peroxidase (APX; EC 1.11.1.11) and VDE in chloroplasts (Dmytro *et al.* 2003). The content of AsA in chloroplasts of Helan No. 3 spinach leaves [7.1 mg g^{-1} (Chl)] is greater than that in Yuanye spinach leaves [3.3 mg g^{-1} (Chl)] under seawater stress. The activity of de-epoxidation of xanthophyll cycle seems to be promoted by available AsA in chloroplasts of Helan No. 3 leaves (seawater-tolerant cultivar). As a result, the higher NPQ and DES are shown in Helan No. 3 stressed leaves, compared with Yuanye spinach. In the xanthophyll cycle, Z plays a role of direct quencher (Dall'Osto *et al.* 2006). Havaux and Niyogi (1999) have suggested that the xanthophyll cycle is involved specifically in the protection of the photosynthetic membranes against lipid peroxidation, and the Z-related reduction of lipid peroxidation could be the manifestation of the transitory presence of Z in the thylakoid membrane lipid phase, where it acts as a terminator of peroxy-radical chain reactions. In this study we have shown that the $O_2^{\cdot -}$ generation rate (Fig. 4*A*), H_2O_2 (Fig. 4*B*), and MDA (Fig. 4*C*) contents in stressed leaves of Helan No. 3 spinach are lower than in Yuanye spinach; this phenomenon may correlate to higher DES, which enhances the level of Z, but suppresses V. As a result, ROS in chloroplasts of Helan No. 3 spinach stressed leaves is more effectively quenched to protect photosynthetic membranes against lipid peroxidation. Thus, the higher DES seems to suppress the risk of excess light-induced generation of ROS and the lipid peroxidation in plants. As mentioned above, the correlation between NPQ and DES in leaves of both cultivars has been shown. Therefore, higher xanthophyll-dependent NPQ of Helan No. 3 leaves would suppress accumulation of ROS in chloroplasts (Fig. 4).

Very low concentrations of ROS, *i.e.* when $^1O_2$ does not induce necrotic oxidative damage, rather it acts as a signalling molecule leading to programmed cell death (Wagner *et al.* 2004, Danon *et al.* 2005, Tanaka and Tanaka 2006). However, generation of excess amounts of $^1O_2$ could lead to severe necrosis and plant death within hours.

Upon illumination, Chl biosynthesis intermediates *i.e.*, Pchl or Proto IX produce $^1O_2$ in plants and cause oxidative damage (Rebeiz *et al.* 1984, 1988; Tripathy and Chakraborty 1991, Chakraborty and Tripathy 1992, Lermontova and Grimm 2000, Tripathy *et al.* 2007). The

site of generation of $^1O_2$ is mostly in the thylakoids. This is because Chl biosynthesis intermediates are partially hydrophobic, and consequently are loosely attached to the thylakoid membranes (Mohapatra and Tripathy 2002, 2007). Although they are associated with the thylakoid membranes, these tetrapyrroles do not form pigment-protein complexes and hence are not connected to the reaction center. Although some of the carotenoids are present in the lipid bilayer, a lot more are located in the pigment-protein complexes and they are spatially too far from Chl biosynthesis intermediates to quench their triplet states (Havaux *et al.* 2007, Mozzo *et al.* 2008). Synthesis of Chl biosynthetic intermediates are highly regulated and are not overproduced in plants. However, impairment of Chl biosynthesis, *i.e*. PBGD in salinity-stressed plants, could deregulate accumulation of Chl biosynthesis intermediates that could produce $^1O_2$ by type II photosensitization reaction causing oxidative damage to plants.

In addition, H_2O_2 closely participates in the senescence of plants, which is accompanied by the collapse of chloroplasts (Brennan and O'Neill 1995). As shown in Fig. 4*B*, compared with that of Helan No. 3, in chloroplasts of Yuanye spinach leaves, seawater stress induces more accumulation of H_2O_2, which appears to initiate a senescence process, and chloroplasts collapse. Furthermore, Chlase seems to show an apparent latent behavior, and the enzyme activity can continue to be enhanced during the process of senescence in barley (Sabater and Rodríguez 1978) and spinach plants (Yamauchi and Watada 1991). The phenomenon is attributed to combination/affinity between substance and enzyme, and the post-translational modification of Chlase seems to be a key point in Chl-decomposition process (Harpaz-Saad *et al.* 2007). It is concluded that H_2O_2-induced collapse of chloroplasts of Yuanye spinach leaves results in combination/affinity between substance and enzyme to activate the Chlase (Fig. 5*B*) and the subsequent decomposition of Chls. However, invariant activity of Chlase in Helan No. 3 spinach leaves is probably attributed to lower accumulation of H_2O_2 in chloroplasts under seawater stress. As mentioned above, higher xanthophyll-dependent NPQ seems to suppress the risk of excess light-induced generation of ROS (*e.g*. H_2O_2) in plants. Therefore, higher activity of xanthophyll cycle in Helan No. 3 spinach leaves seems to suppress the decomposition of Chl through reducing accumulation of ROS in chloroplasts under seawater stress.

In summary, seawater stress enhanced photoinhibition, which aggravated accumulation of ROS in chloroplasts of spinach leaves, thus decreasing PBGD activity, inhibiting conversion of PBG into Uro III. As a result, Chl content in leaves of two cultivars with seawater is decreased. The higher activity of xanthophyll cycle in seawater tolerant spinach plays a role in maintaining Chl metabolic processes, probably by decreasing the levels of ROS, when the plants are cultured in the nutrients solution with seawater.

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