11

Effect of 5-Aminolevulinic Acid (ALA) on Leaf Diurnal Photosynthetic Characteristics and Antioxidant Activity in Pear (*Pyrus Pyrifolia* Nakai)

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1. Introduction

Photosynthesis is the basis of fruit growth and development. The higher photosynthetic efficiency of tree leaves, the more photosynthate is accumulated, which is beneficial to tree growth, root development, flower bud initialization, and the ultimate guarantee of quality and yield of fruits.

5-Aminolevulinic acid (ALA) is a key precursor of all porphyrin compounds, such as chlorophyll (Chl), heme, and phytochrome (von Wettstein et al., 1995). Exogenous application of ALA at low concentrations was found to promote growth and yield of several crops and vegetables (Hotta et al., 1997a). It also improved chlorophyll content and gas exchange capacity of melon seedlings under low light and chilling conditions (Wang et al., 2004), increased CO_2 fixation in the light, and suppressed the release of CO_2 in darkness (Hotta et al., 1997a), and promoted salt tolerance of cotton plants by manipulating the Na^+ uptake (Watanabe et al., 2000). ALA-based fertilizer also enhanced the photosynthetic rate, chlorophyll content, and stomatal conductance in spinach and date palm seedlings under salinity (Nishihara et al., 2003; Youssef and Awad, 2008). However when the plant was treated with exogenous ALA at high concentrations (such as ≥1000 mg/L), it was assumed that the induced chlorophyll intermediate accumulation acted as a photosensitizer for the formation of $^1O_2$, triggering photodynamic damage in ALA-treated plants (Chakrabory et al., 1992). Thus, ALA could be used as a natural bioherbicide.

ALA has been suggested to be a new natural and environmental friendly regulator, which can be widely used in agriculture (Wang et al., 2003). However, whether it can be used in woody trees such as pear has not been reported, and the mechanisms of ALA regulation on plant growth have not yet been elucidated. In the work, we found ALA promotion on pear photosynthesis might be related with the increase of antioxidant enzyme activities, and well as H_2O_2, which might act as signaling molecules involved in the regulation process.

2. Materials and methods

2.1 Plant growth and treatment

The experiment was started in the late of May 2010 in the Horticultural Experimental Station, Nanjing Agricultural University, Jiangsu Province China. Ten years old pear trees (*Pyrus pyrifolia* Nakai. 'Akemizu') were used, which were grew in the brown yellow soil with space of 4x5m. ALA solution in concentration of 0.5 mg $\cdot L^{-1}$ was sprayed to the leaves, with the distilled water as the control. The experimental trees were arranged in complete random design, with ten tree repeats for ALA treatment or control, respectively. The measurements of the gas exchange and chlorophyll fast fluorescence parameters were conducted one week after ALA spray at an interval of 2 hours from morning to dusk. And meanwhile, the leaf samples were taken and stored in liquid nitrogen for subsequent analysis.

2.2 Gas exchange parameters

The measurements of diurnal gas exchange parameters were carried out according to the method described by Wang et al. (2004) with a portable photosynthesis system CIRAS-2 (PP Systems, UK). At each time point, from 6:00 am to 18:00 pm, the net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i) and stomata conductance (G_s) were measured simultaneously under the photon flux densities (PFD) from a built-in light source equal to the natural light intensities with the ambient temperatures. Each measurement was conducted at least 10 times, and the means were used to compare the ALA's effect.

2.3 Chlorophyll a fast fluorescence and JIP test

Chlorophyll fast fluorescence transient was measured by a Plant Efficiency Analyzer (Hansatech, UK), according to methods of Strasser et al. (1995) and Sun et al. (2009a). All the leaves were immediately exposed to a saturating light pulse (3000 mmol $\cdot m^{-2} \cdot s^{-1}$ PFD) for 1 s after dark adapted for 20min. Each transient obtained from the dark-adapted samples was analyzed according to the JIP-test (Srivastava et al., 1997; Li et al., 2005; Sun et al., 2009a).

2.4 Determination of Rubisco initial activity and its coding gene expression

Rubisco activity was determined spectrophotometrically by monitoring NADH oxidation at A340 (Lilley et al., 1974; Xia et al., 2009). Leaf samples were homogenized in a chilled mortar within the ice-cold extraction buffer solution, 40mM Tris–HCl (pH 7.6), which contained 10mM $MgCl_2$, 0.25mM EDTA, 5mM glutathione, 2% β-mercaptoethanol and 1.5% PVP (W/V). The homogenate was centrifuged at 4℃ for 15 min at 10,000g. The resulting supernatant was used for assay of the enzyme. The reaction mixture contained 100 mM Tris–HCl buffer solution, which contained 12 mM $MgCl_2$ and 0.4 mM EDTA (pH 7.8), 0.2 M $NaHCO_3$, 5 mM NADH, 50 mM ATP, 50 mM creatine phosphate, 1 U of glyceraldehyde 3-phosphodehydrogenase and 1 U of 3-phosphoglycerate kinase. The activity was estimated after the addition of enzyme extract and 0.2 mM ribulose-1, 5- bisphosphate (RuBP). Enzyme activity was expressed as mmol CO_2 fixed per min g FW.

To measure the gene expression, leaf RNA was extracted according to Louime et al. (2008). Firstly, a washing buffer, which contained 100 mM Tris-boric acid (pH 7.4), 0.35 mol/L sorbitol and 10% (w/v) PEG-6000, was added to remove the secondary material of samples in the free state. Then an extraction buffer, containing 0.25 M Tris-Boric acid (pH 7.4), 0.05 M EDTA, 2.5 M NaCl, 2% CTAB, 3% PVP, and 5% β-mercaptoethanol was used. The yield and

quality of total RNA were measured by absorbance at 230, 260, and 280 nm (A_{260}/A_{230} and A_{260}/A_{280} ratios) using UV-spectrophotometer and by running samples on a 1.5% non-denaturing agarose gel electrophoresis. Message RNAs in total RNA solution were reversed transcribed to their complementary cDNA (I strand) by oligo-dT primer using MLV reverse transcriptase Kit (Takara Bio) according to the manufacturer's recommendations.

To amplify the cDNA produced from RNA by the RT reaction, PCR was performed according to the protocol. As a template, the RT product was used. According to published pear *Actin* (GenBank: GU830958.1) and *Rubisco small subunit* sequences (GenBank: D00572.1), two pairs of oligonucleotide primers were designed for expression analysis. Gene-specific primers for *Actin* (forward: 5′-CAATGTGCCTGCCATGTATG-3′; reverse: 5′-CCAGCAGCTTCCATTCCAAT-3′) and for *Rubisco small subunit* (forward: 5′-CTTGGAATTTGAGTTGGAGAC-3′; reverse: 5′-GTAA GCGATGAAACTGATGC-3′) were used in RT-PCR. Each pair of primers cycling parameters were *Actin*: 29 cycles, Tm 51℃ and *Rubisco small subunit*: 32 cycles, Tm 59℃. PCR products were analyzed following electrophoresis on a 1% agarose gel containing ethidium bromide.

2.5 Determination of antioxidant enzymes

One hundred milligrams of leaves were homogenized in 2 ml of 50 mM phosphate buffer (pH 7.8) which contained 0.4 % polyvinyl pyrrolidone (PVP), an inhibitor of phenolic compounds in a pre-chilled mortar and pestle on ice. The homogenate was centrifuged at 10 000×g for 20 min at 4 °C and the supernatant was collected as crude enzyme extraction (Tan et al., 2008).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by monitoring the inhibition of nitro blue tetrazolium (NBT) reduction at 560 nm. The reaction mixture (3 ml) contained 195 mM methionine, 1.125 mM NBT, 3 µM EDTA, and 100 µl of enzyme extract in 50 mM PBS (pH 7.8). After addition of 20 µM riboflavin, the cuvettes were exposed to a 15-W circular "white light" tube for photoreaction 10 min. Then the reaction mixture was measured as absorbance of 1 cm cuvette at 560 nm. One unit of SOD activity was defined as the amount of enzyme per fresh mass sample causing 50 % inhibition of the photochemical reduction of NBT (Beauchamp et al., 1971).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by measuring the oxidation of ascorbate at 290 nm according to Nakano and Asada (1981). Total 3 ml of reaction solution contained 50 mM PBS (pH 7.0), 1 mM H_2O_2, and 1mM ascorbate. The reaction was started by adding 100 µl of enzyme extraction. Changes of absorbance at 290 nm were then recorded within 3 min after the start of the reaction at 1 min intervals.

Catalase (CAT, EC 1.11.1.6) activity was determined according to the method of Zavaleta-Mancera et al. (2007). The total reaction mixture (3 mL) contained 50 mM PBS pH 7.0 and 100 µL of enzyme extract. The reaction was initiated by the addition of 10 mM H_2O_2. The decomposition was followed directly by the decrease in absorbance at 240 nm every 20 s for 3 min.

2.6 Determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)

Hydrogen peroxide content was estimated through the formation of a titanium-hydro peroxide complex (Agarwal et al., 2005). One hundred milligram of leaf sample was ground with liquid nitrogen and the fine powdered material was mixed with 2 ml cooled acetone. Then the mixture was centrifuged at 10,000×g for 10 min and the supernatant was collected

as hydrogen peroxide extraction. One milliliter of the extract was added by 0.1 ml 5% titanium sulfate and 0.2 ml ammonium solution to precipitate the titanium-hydro peroxide complex. Reaction mixture was centrifuged at 5,000×g for 10 min in the centrifuge and the precipitate was washed 5 times by cooled acetone. Precipitate was dissolved in 10 ml 2 M H_2SO_4 and then recentrifuged. Supernatant was read at 415 nm against reagent blank in UV-spectrophotometer. The hydrogen peroxide content was calculated by comparing with a standard curve drawn with known hydrogen peroxide concentrations.

The MDA content was measured following the method of Heath et al. (1968) with modification and expressed as nmol per g of fresh weight. Five hundred milligram of frozen powder was added to about 5 ml of 5% trichloroacetic acid (TCA) and centrifuged at 10,000×g for 5 min. Two milliliter aliquot of supernatant was added to 2 ml of 0.67% 2-thiobarbituric acid (TBA). The mixture was incubated in boiling water for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000×g for 10 min, A_{532}, A_{600} and A_{450} of the supernatant were recorded. MDA content was estimated by the formula C (μmol/L) $=6.45(A_{532}-A_{600})-0.56A_{450}$.

2.7 Statistical analysis

All data were subjected to ANOVA test and the means were compared by the Duncan's test. Comparisons with $p<0.05$ were considered significant difference. Pearson correlation analysis between parameters was performed to test for relationships between variables by SPSS. 13 software.

3. Results

3.1 Diurnal variation of air temperature and light intensity in the orchard

On the testing day, the weather was fine when the temperature and photon flux density (*PFD*) exhibited a single peak curve (Fig.1). In the morning (6:00 am), the air temperature

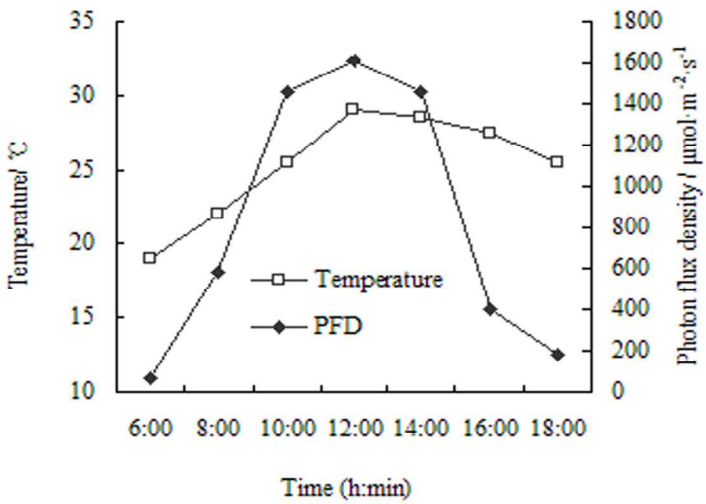

Fig. 1. Diurnal variations of air temperature and light intensity in pear orchard

was about 19℃,which linearly increased to the maximum temperature about 29℃ at noon (12:00), then decreased to 25.5℃ at dusk (6:00 pm). In the aspect of light intensity, the PFD was 65.6 μmol·m^{-2}·s^{-1} in the morning, and the maximum was 1612 μmol·m^{-2}·s^{-1} at noon.

3.2 Effect of ALA on gas exchange characteristics in pear leaves

The measurement of diurnal variations of leaf gas exchange characteristics showed that the net photosynthetic rate (P_n) possessed a twin-peaks curve (Fig. 2A) and ALA treatment significantly increased P_n of pear leaves compared with the control, especially at noon time. P_n/C_i, representing the instantaneous carboxylation efficiency, exhibited a single peak curve in diurnal variation (Fig. 2B), where ALA treatment generally promoted P_n/C_i of pear leaves, especially at noontide. Changes in stomatal conductance was similar with P_n, and ALA treatment promoted stomtal open in most of day time (Fig. 2C). However, there was no difference in the intercellular CO_2 concentrations of pear leaves between control and treatment (Fig. 2D), suggesting that 150 μmol/mol CO_2 in the experiment did not limit photosynthesis in pear leaves.

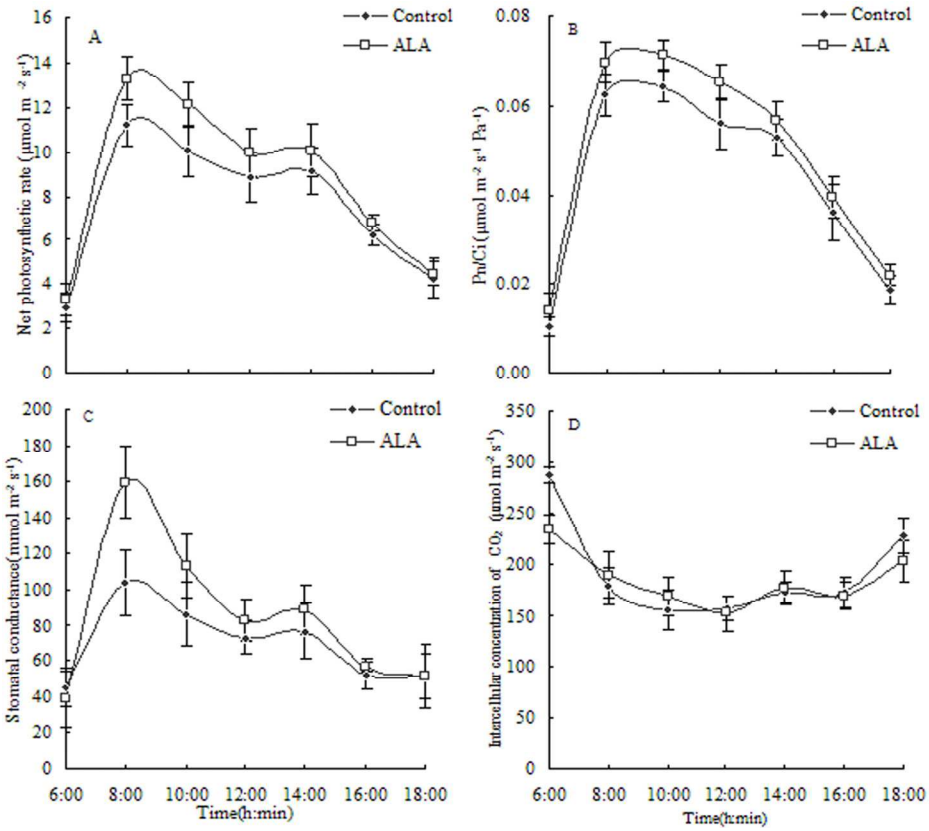

Fig. 2. Effect of ALA on diurnal variations of gas exchange parameters of pear leaves. A: Net photosynthetic rate; B: Instantaneous carboxylation efficiency; C: Stomatal conductance; D: Intercellular CO_2 concentration

3.3 Effects of ALA treatment on chlorophyll fast fluorescence characteristics

3.3.1 Fast induction curves of chlorophyll fluorescence

Fig. 3 displays the fast fluorescence transients measured from 6:00 am to 18:00 pm in the control and ALA-treated pear leaves. There were many differences between OJIP curves at different time, however, the most important difference was found from the P-step, which was the highest at 6:00 am, then decreased to the lowest at 12:00 at noon, and recovered to higher levels in the afternoon. The valley of P level at noon might be a characteristic of photosynthetic midday nap, or photoinhibition under high light condition. From Fig. 3, it can be seen that the P was generally higher in ALA-treated leaves than that in the control, especially at noontide, which may suggest that ALA treatment was favorable to leaf photosynthesis against photoinhibition under high light stress.

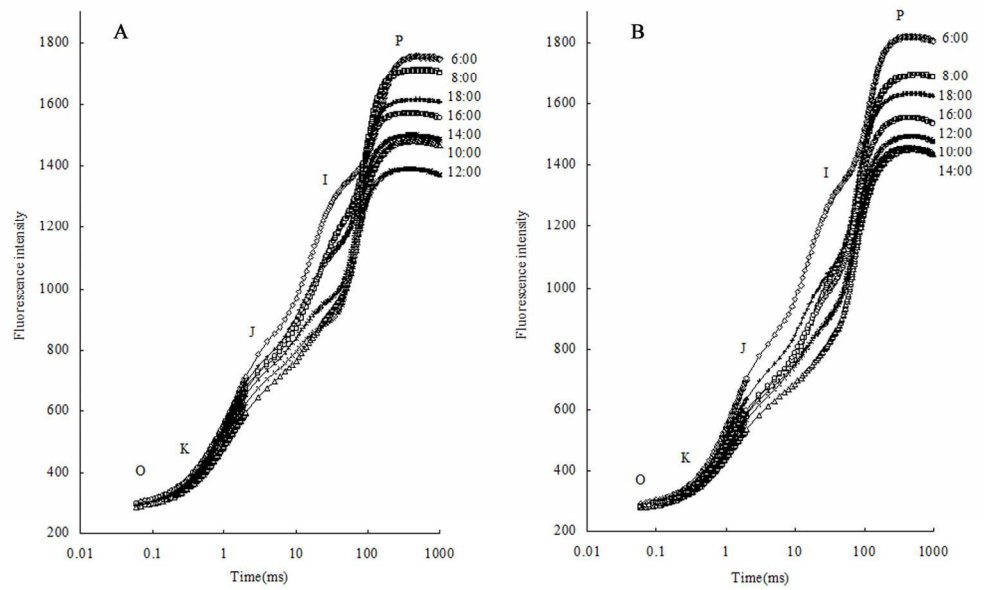

Fig. 3. Effect of ALA on diurnal variations of fast induction curves of chlorophyll a fluorescence (OJIP curve) of pear leaves. A: control, B: ALA treatment

3.3.2 Performance index on absorption basis and performance index of electron transport

The result of JIP-test showed that PI_{ABS}, the photosynthetic performance index on absorption basis, in the ALA treatment was generally higher than that of the control, although the trends of both diurnal variations were similar (Fig. 4A). Moreover, P_{ET}, the performance index of electron transport of PSII photochemical reaction was also higher in the ALA pretreatment than that of the control (Fig. 4B), suggesting that ALA treatment could improve photochemical electron transport and photosynthesis. The diurnal means of PI_{ABS} and P_{ET} were about 38% and 26% higher in the ALA treatment than that of the control, respectively.

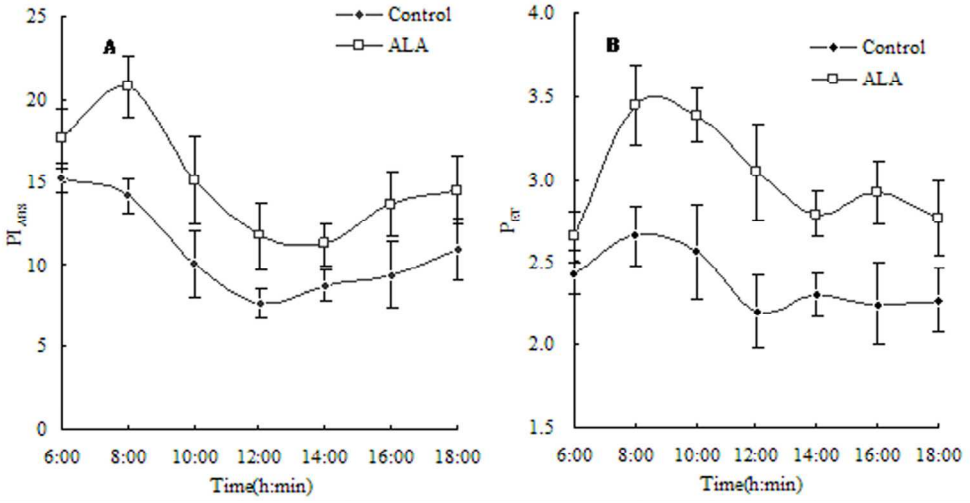

Fig. 4. Effect of ALA on diurnal variations of performance index on absorption basis (A) and performance due to electron transport probability (B) of pear leaves

3.3.3 Flux ratios of PSII photochemical reaction

Fig. 5 is the results of photosynthesis of pear leaves from the perspective of flux ratios of PSII photochemical reaction. There were differences in diurnal variation curve of φP_o, Ψ_o, φE_o and φD_o.

Fig.5A shows that ALA treatment significantly increased the maximal photochemical efficiency of PSII ($\varphi P_o \equiv F_v/F_m$) of pear leaves compared with the control. The highest of φP_o in diurnal variation curves was found at 8:00, and dropped to the lowest at 12:00, and then recovered in the afternoon, suggesting that the high light at noon time depressed the maximal photochemical efficiency of PSII, and ALA treatment was prone to alleviate the photoinhibtion.

In Fig. 5B, Ψ_o, a parameter of the PSII acceptor-side which means the possibility of a trapped exciton moves an electron into the electron transport chain beyond Q_A^-, was generally higher in ALA-treated leaves than that of the control, and the diurnal means in the former was 6.5% higher than that of the latter.

In Fig. 5C, φE_o, another parameter of quantum yield for electron transport exhibited obvious diurnal variation in the day, which was lowest at 12:00 of noon time. ALA treatment significantly improved φE_o of pear leaves, and the diurnal means of the former was 7% higher than that of the latter.

Conversely, a single peak curve was found in the aspect of energy dissipation through heat (φD_o), which reached the highest at 12:00, and ALA treatment depressed φD_o, suggesting that ALA decreased non-photochemical energy dissipation in the pear leaves (Fig.5D).

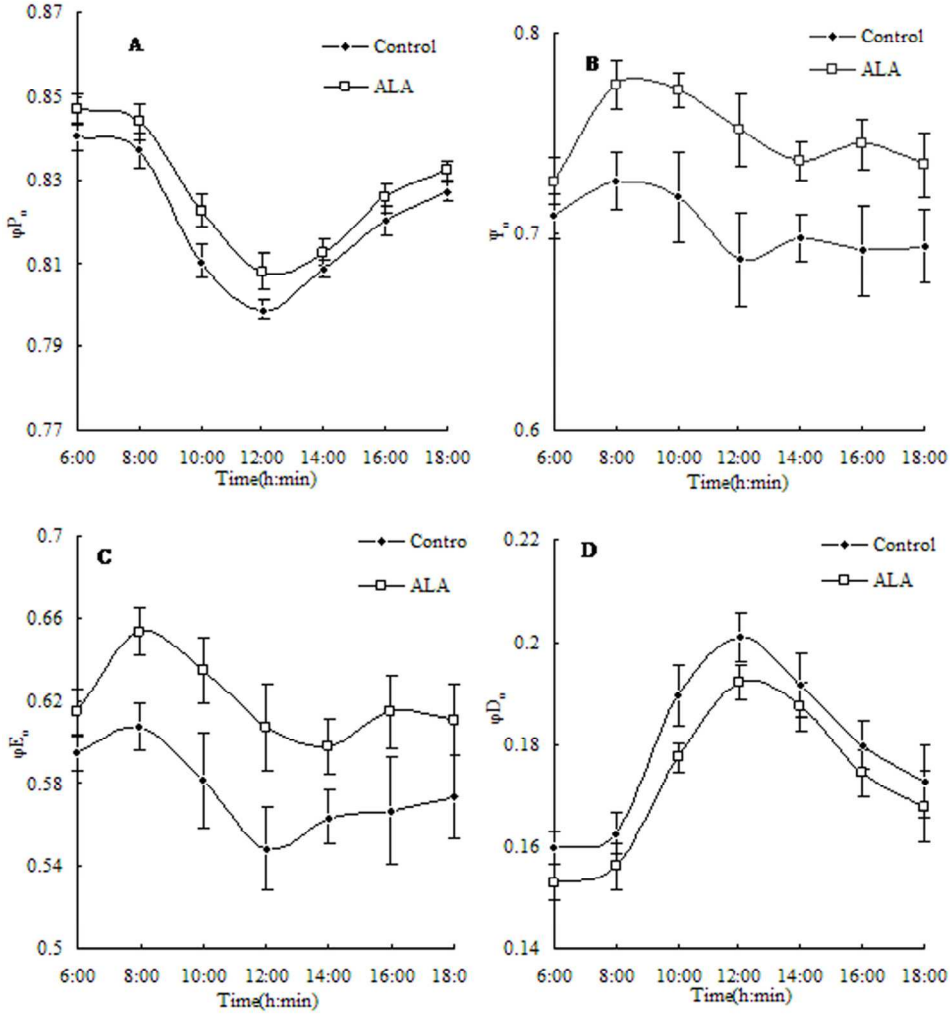

Fig. 5. Effect of ALA on diurnal variations of flux ratios of PSII photochemical reaction of pear leaves. A: Maximum quantum yield for primary photochemistry (φP_o), B: Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (Ψ_o), C: Quantum yield for electron transport (φE_o), D: Quantum yield for heat dissipation (φD_o).

3.3.4 Activity of donor side and acceptor side of PSII reaction

Amplitude of the K step (W_k) as a parameter of the PSII donor-side, expresses the inactivity of the oxygen evolving complex (OEC). The smaller of the W_k, the stronger of the OEC activity is. As in the Fig. 6A, the diurnal variation of W_k showed a plateau from 10:00 am to 16:00 pm in the control, which was obviously higher than that of ALA treatment. This suggests that ALA treatment alleviated OEC inactivity at high light environment.

M_o, an approximate slope at the origin of the fluorescence rise, represents the maximum rate Q_A reduction. From Fig.6A, M_0 of pear leaves rose gradually in the morning and kept high level in the afternoon. However, ALA treatment significantly reduced M_0, which was about 80% of the control in the diurnal mean, suggesting that ALA could decrease Q_A reduction rate in pear leaves.

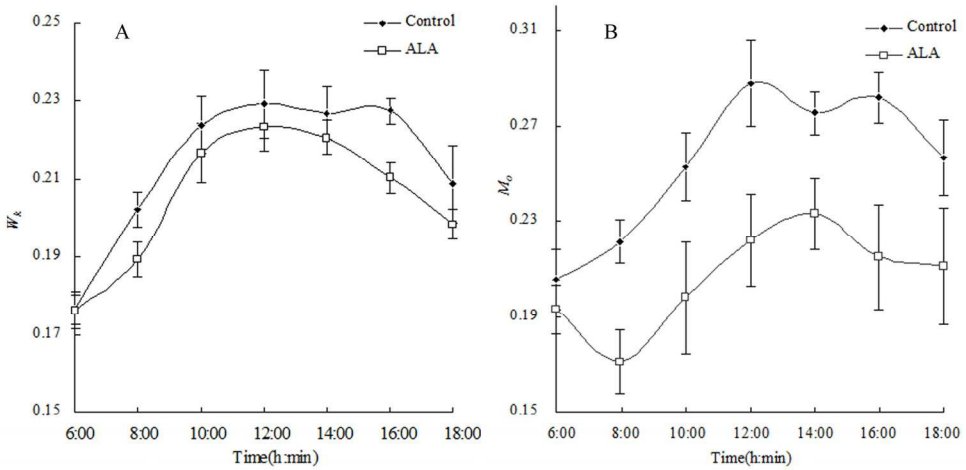

Fig. 6. Effect of ALA on diurnal variations of donor side and acceptor side parameter of PSII reaction of pear leaves. A: Amplitude of the K step (W_k); B: An approximation of the slope at the origin of the fluorescence rise (M_o)

3.4 Rubisco initial activity and RT-PCR analysis

The diurnal variation of the Rubisco initial activity of pear leaves showed a bimodal curve, where the first maximum occurred at 8:00 am, and the second at 16:00 pm. A significant valley occurred at noontide (Fig. 7A). In most cases, ALA treatment stimulated the activity, compared with the control. From the result of RT-PCR of the coding gene (Fig.7B and C), it can be seen that expression of *Rubisco small subunit* gene in pear leaves also revealed a bimodal curve, which was similar with the change of the enzyme activity in Fig. 7A. The relative expression was significantly higher in ALA-treated leaves than that of the control, especially at 8:00, which was more than 2 times. Therefore, ALA treatment improved the expression of *Rubisco small subunit* gene at transcript level.

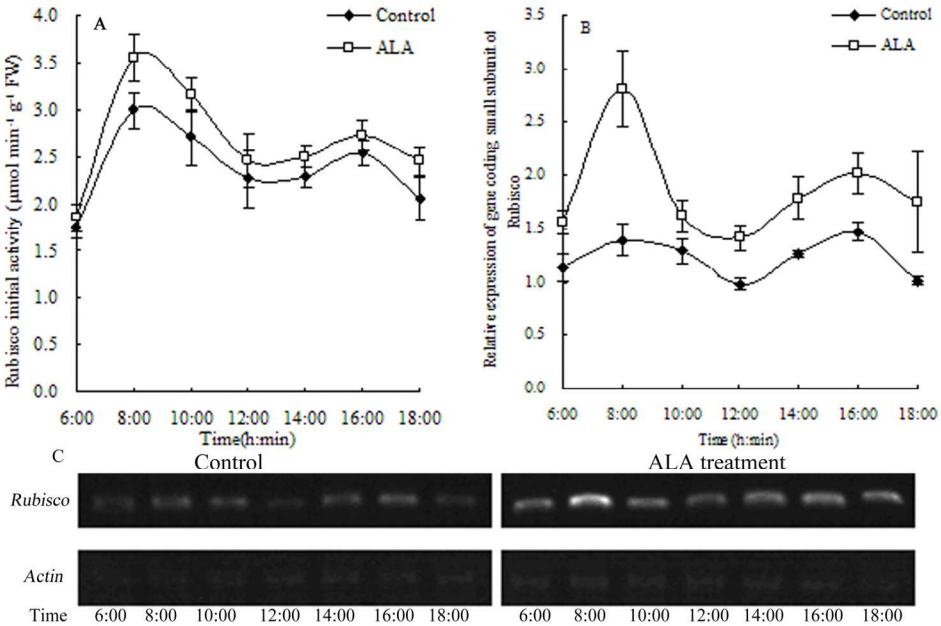

Fig. 7. Effect of ALA on diurnal variations of ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) initial activity (A) and relative expression of *Rubisco small subunit gene* (B) of pear leaves. C is electrophorogram of the gene expression detected by RT-PCR.

3.5 Effect of ALA treatment on the H_2O_2 and MDA content

There is a difference in the H_2O_2 and MDA content in pear leaves between ALA treatment and control (Fig. 8). The H_2O_2 content in ALA treated-leaves maintained at a relatively

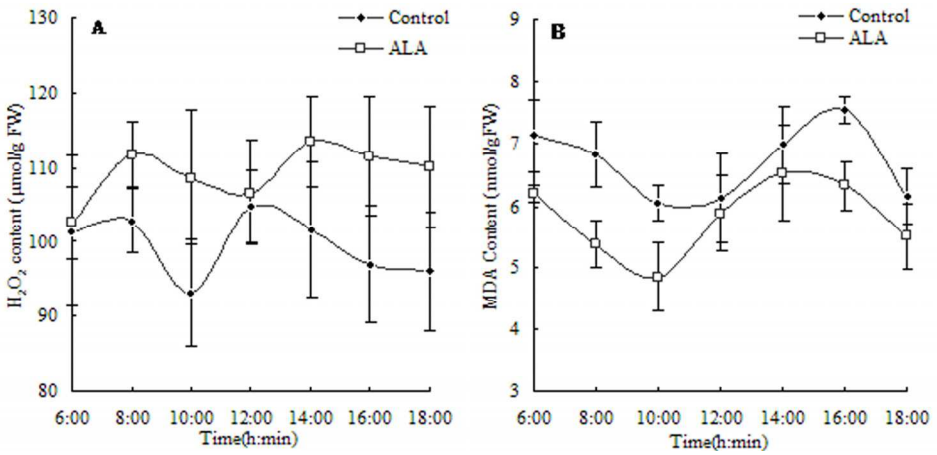

Fig. 8. Effect of ALA on diurnal variations of H_2O_2 and MDA content of pear leaves. A: H_2O_2 content; B: MDA content

stable higher level in one day than that of the control. The diurnal mean of the former was about 10% higher that of the latter, suggesting that ALA treatment could increase the H_2O_2 content of pear leaves (Fig. 8A).

As for the MDA content of pear leaves, it revealed a wave daily variation (Fig.8B). The lowest in the diurnal variation curve was at about 10:00 am and the highest at 16:00 pm. ALA pretreatment decreased the content of MDA, and the significant differences were found in the morning and afternoon. At noon time, the MDA content in the ALA-treated leaves was also slightly higher than that of the control, however, the difference was not significant at P=0.05 level.

3.6 Activities of antioxidant enzymes

The diurnal variation of SOD, APX and CAT activities in response to ALA treatment are shown in Fig. 9. In SOD, the diurnal variation was a two-peak curve, where the first big peak was recorded at 8:00 am and the second small one at 16:00 pm (Fig. 9A). Compared with the control, SOD activities in ALA-treated leaves were generally increased, especially in the early morning and evenfall.

Different with SOD, no peak could be found in the diurnal variation of APX activities of pear leaves (Fig. 9B). Instead, it was lowest at noontide, but kept higher levels in the morning or afternoon. ALA treatment significantly stimulated the enzyme activity in all day time, and the diurnal mean in the ALA-treated was 37% higher than that of the control.

The diurnal variation of CAT activities in pear leaves was similar with a sine curve, which exhibited a peak at 8:00 am and valley at noontide, and then recovered to earlier levels (Fig. 9C). At any time, the activities in ALA-treated leaves were generally higher than that of the control, suggesting that ALA improved the CAT activity in pear leaves.

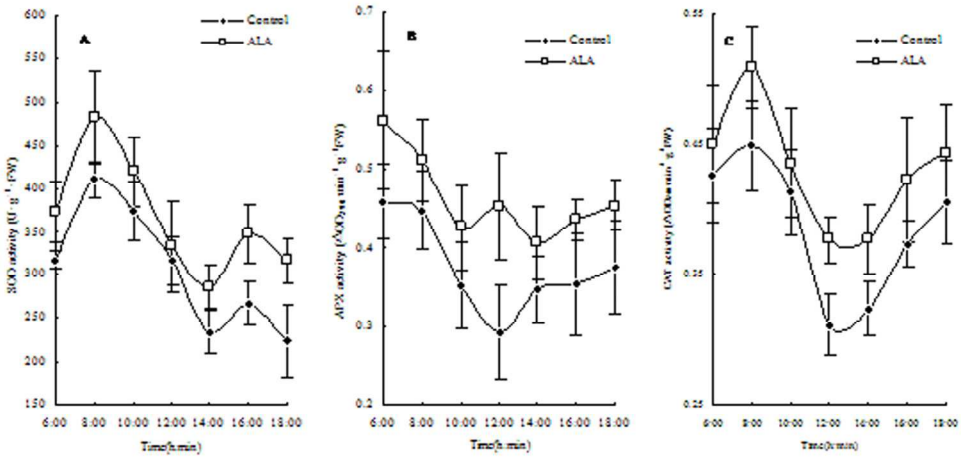

Fig. 9. Effect of ALA on diurnal variations of antioxidant enzymes activities of pear leaf. A: SOD; B: APX; C: CAT

3.7 Analysis of correlation between environmental factors, photosynthetic parameters and antioxidant activities

The results of Pearson correlation analysis by SPSS 13.0 showed that there were a lot of high correlations between environmental factors, gas exchange, chlorophyll fast fluorescence, antioxidant activity and Rubisco initial activity in pear leaves, significant at either the 0.05 level or the 0.01 level (Table 1).

Firstly, the light intensity (*PFD*) was positively correlated with air temperature, P_n, P_n/C_i, W_k and φD_0, but negatively correlated with C_i, φP_0 and CAT activity, which means that high light intensity led to increase of temperature, net photosynthetic rate and instantaneous carboxylation efficiency, and meanwhile, OEC was possibly inactivated and the absorbed energy dissipation through heat was increased. Additionally, the intercellular CO_2 concentration was prone to decrease, and the maximal photochemical efficiency and CAT activity were also inhibited under high light stress.

Secondly, air temperature was positively correlated with W_k, M_0, and φD_0, but negatively with Ci, PI_{ABS}, φP_0, APX and CAT activity. This may mean that high temperature led to inactivate both donor and acceptor sides of PSII reaction center in pear leaves. Therefore, photosynthetic performance index on absorption basis and the maximal photochemical efficiency decreased under high temperature. Additionally, the activities of two H_2O_2 eliminating enzymes APX and CAT decreased as temperature increased.

Among the gas exchange characteristics, P_n was closely related with P_n/C_i and Gs, but negatively with C_i, which means that under the experimental condition, C_i was beyond a limited factor for photosynthesis. P_n was also positively with P_{ET}, SOD and Rubisco initial activity. Therefore, the electron transport activity of PSII reaction center, $O_2^{\cdot-}$ scavenging enzyme SOD activity and the enzyme for CO_2 fixation were key for net photosynthetic rate.

Among the fluorescence indexes, PI_{ABS} was positively correlated with P_{ET}, Ψ_0, φP_0 and φE_0. Meanwhile, PI_{ABS} was also highly correlated with the activities of SOD, APX and CAT. Additionally, it was correlated with the relative expression of *Rubisco small unit* coded gene (P=0.005).

The initial activity of Rubisco was significantly correlated with P_n, P_n/C_i, P_{ET}, Ψ_0, φE_0, expression of the coding gene, and SOD activity, which means that $O_2^{\cdot-}$ scavenging enzyme activity and photochemical electron transfer activity were closely related with the CO_2 fixation enzyme activity. Furthermore, the expression of gene coding *Rubisco small unit* was highly correlated with PI_{ABS}, P_{ET}, Ψ_0, φE_0, H_2O_2 content and three antioxidant enzyme activity.

H_2O_2, a famous reaction oxygen species, was correlated with P_{ET}, Ψ_0, φE_0, the activities of SOD, APX and CAT, the Rubisco initial activity and the relative expression of *Rubisco small unit* coded gene, which might imply the ROS was a active factor for photosynthesis of pear leaves under the experimental condition. MDA, a lipid peroxidation product, was the only parameter, negatively correlated with P_{ET}, Ψ_0, φE_0, and SOD activity, which might be an adverse factor for photosynthesis.

Among the antioxidant enzymes, APX and CAT activities were negatively correlated with *PFD* and (or) temperature, although the coefficient between APX and *PFD* was -0.518, slight higher than the 0.05 level, which means the enzymes was affected by environmental factors. Nevertheless, SOD activity was not affected by *PFD* or temperature. SOD was significantly correlated with P_n, PI_{ABS}, P_{ET}, φ_0, φE_0, APX, CAT, Rubisco initial activity, and the relative expression of *Rubisco small unit* coded gene, but negatively with M_0 and MDA content,

	Temperature	P_n	P_n/C_i	G_S	C_i	PI_{ABS}	P_{ET}	W_k	Ψ_0	M_0	φP_0	φD_0	φE_o	Rubisco	Gene expression	H_2O_2	MDA	SOD	APX	CAT
PFD	0.660**	0.692**	0.791**	0.425	-0.743**	-0.507	0.130	0.754**	0.107	0.359	-0.832**	0.831**	-0.221	0.267	-0.158	0.110	-0.228	0.008	-0.518	-0.563**
Temperature		0.306	0.424	0.016	-0.759**	-0.745**	-0.131	0.921**	-0.152	0.635*	-0.882**	0.885**	-0.451	0.122	-0.143	0.134	-0.013	-0.468	-0.706**	-0.722**
P_n			0.979**	0.918**	-0.707**	0.043	0.558*	0.430	0.522	-0.096	-0.312	0.310	0.315	0.835**	0.401	0.320	-0.378	0.540*	-0.130	0.056
P_n/C_i				0.831**	-0.804**	-0.10	0.490	0.556**	0.457	0.018	-0.462	0.460	0.205	0.772**	0.289	0.258	-0.358	0.470	-0.233	-0.077
G_S					-0.428	0.359	0.677**	0.105	0.633*	-0.352	0.013	-0.014	0.528	0.870	0.593	0.379	-0.475	0.683	0.112	0.354
C_i						0.440	-0.199	-0.824**	-0.178	-0.364	0.695**	-0.694**	0.110	-0.570*	-0.130	-0.150	0.213	-0.124	0.460	0.367
PI_{ABS}							0.699**	-0.826**	0.707**	-0.952**	0.864**	-0.864**	0.901**	0.336	0.698**	0.436	-0.426	0.730**	0.910**	0.915**
P_{ET}								-0.217	0.995**	-0.804**	0.299	-0.297	0.924**	0.708**	0.768**	0.675**	-0.725**	0.758**	0.594*	0.640**
W_k									-0.247	0.747**	-0.921**	0.922**	-0.544*	0.191	-0.288	-0.133	0.071	-0.383	-0.824**	-0.738**
Ψ_0										-0.828**	0.320	-0.318	0.936**	0.673**	0.760**	0.685**	-0.701**	0.750**	0.632*	0.654**
M_0											-0.745**	0.744**	-0.952**	-0.331	-0.663**	-0.542*	0.517	-0.722**	-0.910**	-0.867**
φP_0												-1.00**	0.633*	0.050	0.427	0.113	-0.062	0.443	0.821**	0.857**
φD_0													-0.632*	-0.050	-0.426	-0.112	0.062	-0.447	-0.821**	-0.857**
φE_o														0.575*	0.785**	0.604*	-0.599*	0.782**	0.820**	0.854**
Rubisco															0.688**	0.396	-0.473	0.692**	0.091	0.438
Gene expressiom																0.671**	-0.399	0.612*	0.556*	0.646**
H_2O_2																	-0.441	0.352	0.395	0.238
MDA																		-0.548*	-0.281	-0.418
SOD																			0.560*	0.726**
APX																				0.793**

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 1. Correlations between environmental factors, gas exchange, chlorophyll fast fluorescence, antioxidant activity and Rubisco initial activity in pear leaves

suggesting that SOD was not only important in prevention of lipid peroxidation, but also in prevention PSII reaction center close, and therefore promotion of photochemical electron transfer and photosynthetic dark reaction.

Additionally, the correlations of APX and CAT activities were similar with the SOD in the most parameters. This means that three antioxidant enzymes synergized in eliminating reaction oxygen species to prevent peroxidation of lipid in plant cells. However, the correlations of MDA with APX and CAT were not significant (P>0.05), implying the role of APX and CAT activity was not enough to impact lipid peroxidation, i.e, the H_2O_2 level was not adverse in the experimental condition.

4. Discussion

Previous reports have demonstrated that ALA treatment can improve the net photosynthetic rate in spinach (Nishihara et al., 2003), melon (Wang et al., 2004), pakchoi (Wang et al., 2004b), radish (Hotta et al., 1997b; Wang et al., 2005b), strawberry (Liu et al., 2006) and watermelon (Sun et al., 2009a), under normal or stress conditions. The result of this work confirmed that exogenous ALA at a concentration of 0.5 mg/L could increase the net photosynthetic rate of pear leaves (Fig. 2A), which were related with the increase of P_n/Ci (Fig. 2B) and Gs (Fig.2C). This means that ALA treatment did not only promote stomatal opening, but also affect the non-stomatal factors related with photosynthesis (Farquhar and Sharkey, 1982). In other studies, exogenous ALA promotion on stomatal opening has been reported in melon (Wang et al., 2004a) or watermelon (Kang et al., 2006). A transgenic tobacco, which could over-produce endogenous ALA, also possessed higher stomatal conductivity (Zhang et al., 2010). Therefore, ALA inducing stomatal conductance might be a universal phenomena. However, the mechanism need further to be elucidated.

The shape of the OJIP transient has been found to be sensitive to stress such as excess light, temperature and drought (Appenroth et al., 2001; Thach et al., 2007). In our data (Fig. 3), the P-step in pear leaves was significantly lower at noon than that in the morning or at dusk, suggesting the environmental factors at noon impaired chlorophyll fast fluorescence yield. Wang et al. (2005a) suggested that the optimal temperature for photosynthesis of pear leaves was about 27℃. In this work, the highest temperature was 29℃ (Fig. 1), which was near to the theoretic optimal temperature, and might not be the key inhibitory factor for pear photosynthesis. Instead, the typical midday nap characteristic of pear leaves was possible to be result of the high light intensity at noontide (Fig. 2, Fig.3). ALA treatment increased the fluorescence yield, especially at noontide, suggesting that ALA could promote resistance of pear leaves against high light stress. Sun et al. (2008) also found that ALA treatment could alleviate photoinhibition of watermelon seedlings switched to high light from shaded condition. Our results here were similar with the previous observation. Because ALA treatment could also improve leaf photosynthesis of plants grown under low light condition (Wang et al., 2004; Sun and Wang, 2007), it can be deduced that ALA might stabilize photosynthetic capacity against light stresses. In fact, this effect of ALA is important because plants are not always grown under optimal light intensity condition, and low light or high light often affects plant photosynthesis. ALA improvement on photosynthesis under light stresses can increase photosynthate accumulation in many crops.

The mechanisms of ALA improvement on photosynthesis by non-stomata have been mentioned in many aspects. Firstly, ALA increased the chlorophyll content, since it is the key biosynthetic precursor of all tetrapyrrole compounds (von Wettstein et al., 1995; Jahn

and Heinz, 2009), which has been suggested to contribute to increase of photosynthesis (Tanaka et al., 1993). However, in the most cases, the chlorophyll content was not a limiting factor for leaf photosynthesis.

ALA has been suggested to increase the activity of OEC at the donor side of PSII reaction center under stress condition. Sun et al. (2009b) found that W_k, which represented the inhibition of OEC activity, was lower in ALA-treated leaves than that in the control of watermelon seedlings under chilling stress. Zhang et al. (2010) observed that W_k in the transgenic tobacco with capacity to over- produce endogenous ALA was also lower than that of the wild type. In the work, W_k in ALA-treated pear leaves was always lower than that of the control (Fig. 6A). Thus, ALA promotion in OEC activity might be a general effect.

ALA might improve the photochemical efficiency of PSII reaction center. Whether the dark-adapted or light-adapted maximal photochemical efficiency, it has been reported that ALA had significant effect (Sun et al., 2009a; Wang et al., 2010). Recently, the similar effect was confirmed in the transgenic tobacco (Zhang et al., 2010). In this work, we observed that φP_0 of pear leaves treated by ALA was higher than that of the control (Fig. 5A), suggesting the maximal photochemical efficiency was improved.

ALA might also improve the activity of acceptor side of PSII reaction center. Two important fluorescence parameters M_o and ψ_0, where the M_0 represents the proximate rate of Q_A completely being reduced, and ψ_0 was the probability of a trapped exciton moves an electron into the electron transport chain beyond Q_A^-, often responded to ALA treatment. In most situations, ALA decreased M_0 but stimulated ψ_0, which was beneficial to electron transfer through Q_A^- electron acceptor of PSII reaction center (Strasser et al., 1995; Li et al., 2005). The results in the work approved the previous observations that Q_A was retardant to be completely reduced (Fig. 6B) and the electron was easily transferred to the downstream electron acceptors beyond Q_A^- in the chain after ALA treatment (Fig. 5B).

Liu et al. (2010) suggested that ALA treatment alleviated the decrease of Rubisco activity of cucumber under suboptimal temperature and light intensity stress. In this work, we found the diurnal variation of Rubisco initial activity in pear leaves (Fig. 7A), which was improved by ALA treatment and highly correlated with P_n (r=0.835, $P<10^{-5}$). It was the first time to observe that ALA could up-regulate transcription of gene coding *Rubisco small unit* in pear leaves (Fig. 7). The level of transcript in ALA-treated leaves at 8:00 am was more than 2 times as high as that of the control, which means that ALA treatment did not only affect light reaction of photosynthesis, but also dark reaction, even the expression of the key enzyme. The effect of ALA has been not mentioned before. However, the mechanism of ALA regulation on gene transcription needs to be elucidated further.

In aspect of antioxidant enzymes, it has been suggested that ALA treatment stimulated SOD activity around PSI reaction center, which can scavenge ROS aroused from photosynthetic electron transport in electron transfer chain to improve photochemical electron transfer rate (Sun et al., 2009a, b). Diethyldithiocarbamate (DDC), an inhibitor of Cu-Zn-SOD, could eliminate ALA' effect on photochemical efficiency (Liu et al., 2006; Sun et al., 2009a), which suggested the important role of SOD on ALA promotion. In this work, ALA treatment also induced SOD activity in pear leaves (Fig. 9A), which was positively correlated with many photosynthesis and chlorophyll fluorescence parameters but negatively with M_0 and the MDA content (Table 1), suggesting that it might play an important role on the acceptor side activity of PSII reaction center and preventing lipid peroxidation of photosynthetic apparatus during daytime. On the other hand, Jung et al. (2008) found higher levels of SOD

activity in transgenic rice than in the wild type. The same was true in transgenic tobacco and *Arabidopsis* (Wang et al., unpublished). Thus, enhancement of SOD activity was accompanied with increase of endogenous or exogenous ALA levels. However, the mechanism of SOD activity induced by ALA has no been known.

That ALA induced the increase of enzymes eliminating H_2O_2, such as APX, CAT and POD, has been suggested (Nishihara et al., 2003; Liu et al., 2006). Since ALA is the essential biosynthetic precursor of tetrapyrrole compounds including heme, and the latter is a necessary component for the activity of all three enzymes (Tsiftsoglou et al., 2006), it is reasonable to deduce that ALA induces heme accumulation, which is beneficial for H_2O_2 eliminating enzyme activity. In this work, it was also observed that ALA induced increase of activities of APX and CAT in pear leaves (Fig. 9). However, ALA treatment also increased the content of H_2O_2 in pear leaves, which might be at a safe level, because MDA content with ALA treatment was significantly lower than the control (Fig. 8). In Table 1, H_2O_2 level was correlated with P_{ET}, Ψ_0, φE_0, the activities of SOD, APX, CAT and Rubisco initial activity, as well as transcript of gene coding *Rubisco small unit*. This means that H_2O_2 might be an active signal molecule rather than an adverse ROS, involved in regulation of antioxidant enzyme activity and physiological or molecular processes. H_2O_2 has been suggested as a cellular signal, and has wide-ranging effects in many biological processes (Finkel and Holbrook, 2000). It can also regulate gene expression in plants (Neill et al., 2002; Apel and Hirt, 2004). However, whether ALA promotion on plant photosynthesis is dependent on H_2O_2 signal need further study.

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6. References

Agarwal S, Sairam RK, Srivastava GC, Tyagi A, Meena RC. Role of ABA, salicylic acid, calcium and hydrogen peroxide on antioxidant enzymes induction in wheat seedlings. Plant Sci, 2005, 169:559-570

Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Ann Rev Plant Biol, 2004, 55: 373-399

Appenroth KJ, Stockel J, Srivastava A, Strasser RJ. Multiple effects of chromate on the photosynthetic apparatus of *Sprirodela polyrhiza* as probed by OJIP chlorophyll a fluorescence measurements. Environ Pollu, 2001, 115:49-64

Beauchamp CO, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem, 1971, 44:276-287

Chakrabory N, Tripathy BC. Involvement of singlet oxygen in 5-aminolevulinic acid induced photodynamic damage of cucumber chloroplast. Plant Physiol, 1992, 98: 7-11

Farquhar GD, Sharkey TD. Stomatal conductance and photosynthesis. Ann Rev Plant Physiol, 1982, 33: 317- 345

Finkel T, Holbrook NJ. Oxidants, oxidative stress and biology of ageing. Nature, 2000, 408:239-247

Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968, 125: 189-198

Hotta Y, Tanaka T, Takaoka H, Takeuchi Y, Konnai M. New physiological effects of 5-aminolevulinic acid in plants: the increase of photosynthesis, chlorophyll content, and plant growth. Biosci Biotech Biochem, 1997b, 61:2025-2028

Hotta Y, Tanaka T, Takaoka H, Takeuchi Y, Konnai M. Promotive effects of 5-aminolevulinic acid on the yield of several crops. Plant Growth Regul, 1997a, 22:109-114

Jahn D, Heinz DW. Biosynthesis of 5-aminolevulinic acid. In: Tetrapyrroles: Birth, Lift and Death, ed by Warren M, Smith AG. Landes Bioscience and Springer Science+Business Media. 2009, 29-42

Jung S, Back K, Yang K, Kuk YI, Chon SU. Defence response produced during photodynamic damage in transgenic rice overexpressing 5-aminolevulinic acid synthase. Photosynthetica, 2008, 46: 3-9

Kang L, Cheng Y, Wang LJ. Effects of 5-aminoluveulinic acid (ALA) on the photosyntehsis and anti-oxidative enzyme activity of leaves of greenhouse watermelon in summer and winter. Acta Bot Boreal-Occident Sin, 2006, 26: 2297-2301

Li PM, Gao HY, Strasser RJ. Application of the fast chlorophyll fluorescence induction dynamics analysis in photosynthesis study. J Plant Physiol Mol Biol, 2005, 31: 559-566

Lilley RM, Walker DA. An improved spectrophotometric assay for ribulose-bisphosphate carboxylase. Biochim Biophys Acta, 1974, 358: 226-229

Liu WQ, Kang L, Wang LJ. Effect of 5-aminolevulinic acid (ALA) on photosynthesis and its relationship with antioxidant enzymes of strawberry leaves. Acta Bot Boreal Occident Sin, 2006, 26:57-62

Liu YM, Ai XZ, Yu XC. Effects of ALA on photosynthesis of cucumber seedlings under suboptimal temperature and light intensity. Acta Hort Sin, 2010, 37: 65-71

Louime C, Vasanthaiah HKN, Jittayasothorn Y, Jiang L, Basha SM, Thipyapong P, Boonkerd N. A simple and efficient protocol for high quality RNA extraction and cloning of chalcone synthase partial cds from muscadine grape cultivars (*Vitis Rotundifolia* Michx.). Euro J Sci Res, 2008, 22:232-240

Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Photochem Photobiol, 1981, 37: 679-690

Neill S, Desikan R, Hancock J. Hydrogen peroxide signaling. Curr Opin Plant Biol, 2002, 5: 388-395

Nishihara E, Kondo K, Parvez MM, Takahashi K, Watanabe K, Tanaka K. Role of 5-aminolevulinic acid (ALA) on active oxygen-scavenging system in NaCl treated spinach (*Spinacia oleracea*). Plant Physiol, 2003, 160: 1085- 1091

Srivastava A, Guissé B, Greppin H, Strasser RJ. Regulation of antenna structure and electron transport in PSII of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll a fluorescence transient: OKJIP. Biochim Biophys Acta, 1997, 1320: 95-106

Strasser RJ, Srivastava A, Govindjee. Polyphasic chlorophyll-alpha fluorescence transient in plants and cyanobacteria. Photochem Photobiol, 1995, 61: 32-42

Sun YP, Wang LJ. Effects of 5-aminolevulinic acid (ALA) on chlorophyll fluorecence dynamics of watermelon seedlings under shade condition. Acta Hort Sin, 2007, 34: 901-908

Sun YP, Wei ZY, Zhang ZP, Wang LJ. Protection of 5-aminolevulinic acid (ALA) on high light photoinhibition of watermelon grown under shade condition. Acta Bot Boreal-occident Sin, 2008, 28: 1384-1390

Sun YP, Zhang ZP, Wang LJ. Promotion of 5-aminolevulinic acid treatment on leaf photosynthesis is related with increase of antioxidant enzyme activity in watermelon seedlings grown under shade condition. Photosynthetica, 2009a, 47(3):347-354

Sun YP, Zhang ZP, Xu CX, Shen CM, Gao C, Wang LJ. Effect of ALA on fast chlorophyll fluorescence induction dynamics of watermelon leaves under chilling stress. Acta Hort Sin, 2009b, 36: 671-678

Tan W, Liu J, Dai T, Jing Q, Cao W, Jiang D. Alterations in photosynthesis and antioxidant enzyme activity in winter wheat subjected to post-anthesis water-logging. Photosynthetica, 2008, 46:21-27

Tanaka Y, Tanaka A, Tsuji H. Effects of 5-aminolevulinic acid on the accumulation of chlorophyll b and apoproteins of the light-harvesting chlorophyll a/b-protein complex of photosystem II. Plant Cell Physiol, 1993, 34: 465–472

Thach LB, Shapcott A, Schmidt S, Critchley C. The OJIP fast fluorescence rise characterizes *Graptophyllum* species and their stress responses. Photosynth Res, 2007, 94:423-436

Tsiftsoglou AS, Tsamadou AI, Papadopoulou LC. Heme as key regulator of major mammalian cellular functions: Molecular, cellular, and pharmacological aspects. Pharmacol Therapeut, 2006, 111: 327-345.

Von Wettstein DV, Gough S, Kannangara CG. Chlorophyll biosynthesis. Plant Cell, 1995, 7: 1039-1057

Wang LJ, Jiang WB, Huang BJ. Promotion of 5-aminolevulinic acid on photosynthesis of melon (*Cucumis melo*) seedling under low light and chilling stress conditions. Physiol Plant, 2004a, 121:258-264

Wang LJ, Jiang WB, Gao GL, Han HZ, Kuang YL, Liang SQ. Studies on leaf photosynthesis of young pear trees with various cultivars. Acta Hort Sin, 2005a, 32: 571-577

Wang LJ, Jiang WB, Zhang Z, Yao QH, Matsui H, Ohara H. Biosynthesis and physiological activities of 5-aminolevulinic acid (ALA) and its potential application in agriculture. Plant Physiol Commun, 2003, 39:185-192

Wang LJ, Liu WQ, Sun GR, Wang JB, Jiang WB, Liu H, Li ZQ, Zhuang M. Effect of 5-aminolevulinic acid on photosynthesis and chlorophyll fluorescence of radish seedlings. Acta Bot Boreal-Occident Sin, 2005b, 25: 488- 496

Wang LJ, Shi W, Liu H, Liu WQ, Jiang WB, Hou X L. Effects of exogenous 5-aminolevulinic acid treatment on leaf photosynthesis of pak-choi. J Nanjing Agri Univer, 2004b, 47:1084-1091

Wang LJ, Sun YP, Zhang ZP, Kang L. Effects of 5-aminolevulinic acid (ALA) on photosynthesis and chlorophyll fluorescence of watermelon seedlings grown under low light and low temperature conditions. Acta Hort, 2010, 856: 159-166

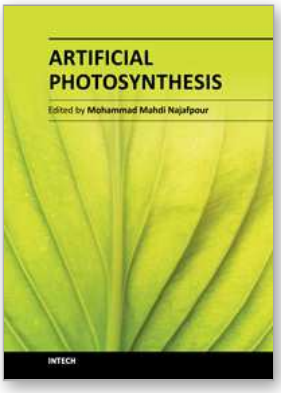
Watanabe K, Tanaka T, Hotta Y, Kuramochi H, Takeuchi Y. Improving salt tolerance of cotton seedlings with 5-aminolevulinic acid. Plant Growth Regul, 2000, 32:99-103

Xia XJ, Huang LF, Zhou YH, Mao WH, Shi K, Wu JX, Asami A, Chen ZX, Yu JQ. Brassinosteroids promote photosynthesis and growth by enhancing activation of Rubisco and expression of photosynthetic genes in *Cucumis sativus*. Planta, 2009, 230:1185-1196

Youssef T, Awad MA. Mechanisms of enhancing photosynthetic gas exchange in date palm seedlings (*Phoenix dactylifera* L.) under salinity stress by a 5-aminolevulinic acid-based fertilizer. J Plant Growth Regul, 2008, 27:1-9

Zavaleta-Mancera HA, López-Delgado H, Loza-Tavera H, Mora-Herrera M, Trevilla-García C, Vargas-Suárez M, Ougham H. Cytokinin promotes catalase and ascorbate peroxidase activities and preserves the chloroplast integrity during dark-senescence. J Plant Physiol, 2007, 164: 1572-1582

Zhang ZP, Yao QH, Wang LJ. Expression of yeast *Hem1* controlled by *Arabidopsis HemA1* promoter enhances leaf photosynthesis in transgenic tobacco. Mol Biol Rep, 2011, 38: 4369-4379



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Photosynthesis is one of the most important reactions on Earth, and it is a scientific field that is intrinsically interdisciplinary, with many research groups examining it. We could learn many strategies from photosynthesis and can apply these strategies in artificial photosynthesis. Artificial photosynthesis is a research field that attempts to replicate the natural process of photosynthesis. The goal of artificial photosynthesis is to use the energy of the sun to make different useful material or high-energy chemicals for energy production. This book is aimed at providing fundamental and applied aspects of artificial photosynthesis. In each section, important topics in the subject are discussed and reviewed by experts.

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