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Influence of shading on photosynthesis and antioxidative activities of enzymes in apple trees

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Abstract

This study investigated the effects of shading on photosynthesis in 3-year-old potted apple trees. The trees were grown either in full sunlight or in a shade environment with about 50% of sunlight filtered through black plastic nets. Under the shade conditions, the net photosynthetic rate (P_N), the stomatal conductance, the transpiration rate, and the intercellular CO_2 concentration decreased before midday, which was due to low light energy but the P_N of the shaded trees increased relative to the control trees during the afternoon. In addition, the concentrations of chlorophyll (Chl) *a*, *b*, and total Chl increased in the shaded trees. In shaded apple trees, the photochemical efficiency increased. There was a lower content of reactive oxygen species and activities of antioxidant enzymes. The results showed that moderate shading can improve the total photosynthetic efficiency, which is mainly attributed to alleviation of photodamage and low accumulation of reactive oxygen species.

Additional key words: chlorophyll fluorescence; gas exchange; *Malus domestica*; photoinhibition.

Introduction

Plants need sunlight to maintain their normal productive, physiological, and biochemical functions. However, the sunlight harvested by the pigments of the photosynthetic apparatus may also induce damage to the photosynthetic system, resulting in photoinhibition and ultimately reducing plant photosynthetic activity, growth, and productivity (Guo *et al*. 2006a,b; Takahashi and Murata 2008, Takahashi *et al*. 2010). PSII has long been considered the primary target for photoinhibition in the photosynthetic apparatus. Photoinhibition occurs when the amount of absorbed photon energy, which is transferred to photosynthetic reaction centers, exceeds its utilization (Aro *et al*. 1993, Jung *et al*. 2000, Goh *et al*. 2012). As a consequence of excessive light exposure, electron transport can be inhibited, and the protein structures in PSII can be damaged (Adams *et al*. 2013). In addition, under excess light, different reactive oxygen species (ROS) are produced, such as the singlet state oxygen ($^1O_2$) and H_2O_2 (Apel and Hirt 2004, Takahashi and Murata 2006, Wang *et al*. 2012, Campbell *et al*. 2013). To alleviate this oxidative damage, plants use complex defense systems including the antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and guaiacol peroxidase (GPX, EC 1.11.17), and enzymes in the ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2); these antioxidant enzymes participate in scavenging of ROS in plants (Asada 2000, Guo *et al*. 2006, Kayihan *et al*. 2012) .

As stated earlier, normal plant growth requires optimal irradiance because excessively high or low irradiance results in photoinhibition or light deficiency, respectively; both severely limiting plant growth (Lei *et al*. 2006, Calcerrada *et al*. 2008, Deng *et al*. 2012). Plants grown in weak light are generally less likely to suffer photodamage to their photosynthetic apparatus than plants grown under strong light in short term, such as the midday depression of photosynthesis.

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Abbreviations: APX – ascorbate peroxidase; CAT – catalase; C_i – intercellular CO_2 concentration; DHAR – dehydroascorbate reductase; E – transpiration rate; F_0 – initial fluorescence; F_m – maximal fluorescence; F_v/F_m – maximum photochemical efficiency; GPX – guaiacol peroxidase; GR – glutathione reductase; g_s – stomatal conductance; MDHAR – monodehydroascorbate reductase; NPQ – nonphotochemical quenching coefficient; P_N – net photosynthetic rate; q_p – photochemical quenching coefficient; ROS – reactive oxygen species; SOD – superoxide dismutase; Φ_{PSII} – electron transport capacity of PSII photochemistry.
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The apple tree (*Malus domestica*) is a deciduous tree in the *Rosaceae* family and is one of the most widely cultivated fruit species. Although it is considered a sun plant (Guo *et al.* 2013), a midday depression of photosynthesis of apple trees is of a common occurrence (Glenn 2009). Photodamage to its photosynthetic apparatus caused by excess light during midday to the afternoon can depress its photosynthetic efficiency, which affects fruit production and quality. Therefore, apple yield and quality are largely influenced by light intensity during the growth period. Many authors have reported the phenomenon of photodamage in apple leaves exposed to strong light (Blot *et al*. 2011, Allerent *et al*. 2013, Saez *et al*. 2013). Some researchers have reported that different horticultural plants respond differently to shade. After measuring Chl content, net photosynthetic rate (P_N), and Chl fluorescence parameters, approximately 67% shade is suggested to be the optimum light irradiance conditions for *Tetrastigma hemsleyanum* cultivation (Dai *et al*. 2009). In the blueberry, shade level above approximately 60% of full sunlight must be avoided for optimal photosynthesis and growth (Kim *et al*. 2011). In the tomato plants, compared with the unshaded control, plants grown in 50% shade had similar yield and shoot fresh and dry mass and lesser photochemical stress (Masabni *et al*. 2016). In addition, sweet pepper plants cultivated under 60% shade had higher contents of Chl and yields, which suggests that the use of shading decreased the unmarketable yield (Lopez-Marin *et al*. 2012). In contrast, some researchers think that early shading reduces yield and late shading reduces fruit quality in peach plants (Georgea *et al.* 1996). After kiwifruit vines (cv. Hayward) shading, the mean fresh mass of individual fruits was significantly reduced (Snelgara and Hopkirk 1988). It has been reported that shade affected photosynthesis, growth, and yield in apple plants (Jacksona and Palmera 1977), but moderate shading and the mechanism of reduced photodamage has scarcely been reported. Therefore, our study explored possibilities to alleviate the midday depression using shading. We examined photodamage to the PSII reaction center in apple leaves under full sunlight or shade by evaluating the parameters of gas exchange, Chl *a* fluorescence, Chl content, ROS, and the activities of antioxidant enzymes.

Materials and methods

Plant materials and study site: Three-year-old dwarf 'Red Fuji' apple (*Malus domestica* Borkh./M.26/*Malus robusta* Rehd.) trees were grown in soil (volume ratio of field topsoil to organic matter was 2:1, pH 6.5) in plastic pots (24 cm diameter, 27 cm tall) in an orchard located in the Northwest A&F University (34°20'N, 108°24'E).

Shading treatments: Eighteen plants were grown under shade or control conditions. The control temperatures were approximately 40°C maximum and 15°C minimum. The apple trees received full sunlight of 1,800 μmol(photon) m^{-2} s^{1} in the field. The shade conditions were achieved using black mesh of about 50% transmittance placed 2.0 m above the ground. The mesh width was 3.0 m, and the length was 4.0 m. The experiments were conducted in September 2012. Trees in shade and full sunlight were arranged in a randomized split block design. The trees were watered until drainage on a daily basis. Six plants were randomly selected for parameter analysis.

Gas-exchange parameters: A portable photosynthesis system (*LI-6400T*, *Li-Cor Inc*., USA) with a *6400-02B* light source was used to estimate the net photosynthetic rate [P_N, μmol(CO_2) m^{-2} s^{-1}], the stomatal conductance [g_s, mmol(H_2O) m^{-2} s^{-1}], the transpiration rate [E, mmol(H_2O) m^{-2} s^{-1}], and the intercellular CO_2 concentration [C_i, μmol(CO_2) mol^{-1}] at 1,000 μmol(photon) m^{-2} s^{-1}. The CO_2 concentration was maintained at 400 μmol mol^{-1} using a CO_2 injection system. The parameters were measured when the system reached equilibrium.

Chl content: Fresh mature leaves from the middle of apple trees were selected to evaluate the Chl content. Twenty leaf discs from fresh leaves were obtained using a punch (0.4 cm in diameter) and then incubated in 5 ml of 80% acetone in the dark for 24 h at 4°C (Zai *et al*. 2012). After incubation, the extract was analyzed at 645 and 663 nm using a spectrophotometer (*UV-2800*, *UNICO*, China).

Chl fluorescence parameters: *In vivo* Chl fluorescence was measured using a pulse amplitude modulation fluorometer (*PAM-2500*, *Walz*, Effeltrich, Germany) connected to a computer with data recorder software (*PAMWIN 3.0*). Before the measurement, the leaves were dark-adapted for 20 min with a leaf-clip holder (*DLC-8*). When all PSII reaction centers were open, the minimal fluorescence (F_0) in the dark-adapted state was determined with extremely low modulated light [<0.1 μmol(photon) m^{-2} s^{-1}] to avoid inducing any significant variable fluorescence. When all PSII reaction centers were closed, the maximal fluorescence (F_m) was measured with a saturating pulse [>8,000 μmol(photon) m^{-2} s^{-1}] on dark-adapted leaves. After that, the leaf was illuminated with white actinic light [1,160 μmol(photon) m^{-2} s^{-1}]. The steady-state value of fluorescence (F_s) was determined, and the maximal fluorescence in the light-adapted state (F_m') was measured with a second saturating pulse [>8,000 μmol(photon) m^{-2} s^{-1}]. Simultaneously the actinic light was turned off and the far-red light was turned on. With far-red light, the minimal fluorescence in the light-adapted state (F_0') was recorded. The maximum quantum yield of PSII (F_v/F_m), photochemical quenching coefficient (q_p), and actual photochemical efficiency of PSII (Φ_{PSII}) were calculated according to Genty *et al*. (1989): $F_v/F_m = (F_m - F_0)/F_m$, $q_p = (F_m' - F_s)/(F_m' - F_0')$, and $\Phi_{PSII} = (F_m' - F_s)/F_m'$. Nonphotochemical quenching (NPQ) was calculated as $F_m/F_m' - 1$.

Estimation of H_2O_2: The marked leaves were cut at the petiole and immediately dipped into water containing 1.0 mg(3,3-diaminobenzidine, DAB) mL^{-1} (pH 3.8) (Thordal-Christensen *et al*. 1997) and maintained at 25°C in 30 μmol(photon) m^{-2} s^{-1} conditions for 3 h to take up the chromagen. Subsequently, treated leaves were divided

into two groups, a group of the leaves were illuminated for 4 h at light of 1,200 μmol(photon) m^{-2} s^{-1}, and another group of the leaves were illuminated for 4 h at light of 600 μmol(photon) m^2 s^1, throughout keeping the petioles immersed in the DAB solutions. DAB formed a deep brown polymerization product upon reaction with H_2O_2, and the content reflected the distribution of H_2O_2 in leaves. When the treated leaves were boiled in 95% ethanol until their green color disappeared, the deep brown polymerization product was displayed.

Activities of antioxidant enzymes: Treated leaves were cut and immediately frozen in liquid nitrogen and then preserved at –80°C. The frozen leaves (0.5 g) were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 3 mM 2-mercaptoethanol, and 2% (w/v) polyvinylpolypyrrolidone with a chilled pestle. The homogenate was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant was used for the enzyme assays.

The CAT activity was estimated using the method of Aebi (1984). The 1.5 ml of reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 (30%), and 40 μl of enzyme extract. The reaction was initiated by adding H_2O_2. Changes in the reaction solution absorbance at 240 nm (*UV-2600*, *SHIMADZU*, Japan) were read every 30 s. One CAT unit was defined as the amount of enzyme necessary to decompose 1 mM(H_2O_2) min^{-1} under the above-mentioned assay conditions. The specific CAT activity was expressed as U g^{-1}(FM) min^{-1}.

The SOD activity was determined by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) and estimated by the method of Dhindsa *et al*. (1981). The 1.0 ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 6.5 mM methionine, 50 μM NBT, 100 μM EDTA, 10 μM riboflavin, and 20 μl of enzyme extract. This reaction was started by the addition of riboflavin, and the glass test tubes were shaken and placed under fluorescent lamps [150 μmol(photon) m^{-2} s^{-1}]. The reaction proceeded for 5 min before being stopped by switching off the light. The absorbance was read at 560 nm. The mixture that lacked enzyme was used to zero the absorbance at 560 nm (*UV-2600*, *SHIMADZU*, Japan). Blanks or controls were analyzed in the same manner but without illumination or enzyme, respectively. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under assay conditions. The specific SOD activity was expressed as U g^{-1}(FM).

The GPX activity was measured according to the method of Egley *et al.* (1983). The reaction mixture (2.0 ml) consisted of 0.05 M potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, 10 mM guaiacol, and 5 μl of enzyme extract. The reaction was started by the addition H_2O_2. Reaction time was 5 min. The initial rate of guaiacol oxidation was estimated by the increase in absorbance measured at 470 nm. The specific GPX activity was expressed as U g^{-1}(FM) min^{-1}.

The APX activity was determined by monitoring the decrease in absorbance at 290 nm according to the method of Nakano and Asada (1981). The 1 ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.5 mM AsA, 1 mM H_2O_2, and 20 μl of enzyme extract. The reaction was initiated by adding H_2O_2. One unit of APX activity was defined as the amount of the enzyme causing a change in 1.0 μmol(AsA oxid.) per min. The specific APX activity [U g^{-1}(FM) min^{-1}] = $(\Delta A_{290} \times V)/(2.8 \times M \times V \times t)$, where ΔA_{290} was the change of A_{290} during 30 s; V was total volume of crude enzyme solution; 2.8 mM^{-1} cm^{-1} was an extinction coefficient; M was mass of fresh materials; t was reaction time, 0.5 min.

The GR activity was determined at 25°C by measuring the rate of NADPH oxidation as a decrease in absorbance at 340 nm according to the method of Halliwell and Foyer (1978). The 1 ml of reaction mixture contained 0.05 M potassium phosphate buffer (pH 7.5), 1 mM EDTA, 0.1 mM NADPH, 0.25 mM oxidized GSH, and 20 μl of enzyme extract. The reaction was initiated by adding NADPH. One unit of GR activity was defined as the amount of the enzyme causing a change in 1.0 μmol(NADPH oxid.) per min. The specific GR activity [U g^{-1}(FM) min^{-1}] = $(\Delta A_{340} \times V)/(M \times V_s \times 6.22 \times t)$, where ΔA_{340} was the change of A_{340} during every min; V was total volume of crude enzyme solution; V_s was volume of crude enzyme used in the determination; M was mass of fresh materials; 6.22 mM^{-1} cm^{-1} was an extinction coefficient; t was reaction time, 3.5 min.

The DHAR activity was assayed according to the method of Nakano and Asada (1981). The 1.0 ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 2.5 mM reduced GSH, 0.1 mM EDTA, 0.2 mM dehydroascorbate (DHA), and 20 μl of enzyme extract. The DHAR activity was estimated by the increase in absorbance measured at 265 nm. The reaction was initiated by adding DHA. One unit of DHAR activity was defined as the amount of the enzyme causing a change in 1.0 μmol(AsA formation) per min. The specific DHAR activity [U g^{-1}(FM) min^{-1}] = $(\Delta A_{265} \times V)/(M \times V_s \times 5.42 \times t)$, where ΔA_{265} was the change of A_{265} during every minute; V was total volume of crude enzyme solution; V_s was volume of crude enzyme used in the determination; M was mass of fresh materials; 5.42 mM^{-1} cm^{-1} was an extinction coefficient; t was reaction time, 3.0 min.

The MDHAR activity was determined at 340 nm in 1.0 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.6), 2.5 mM AsA, 0.1 mM EDTA, 0.1 mM NADH, 0.5 units of AsA oxidase (EC 1.10.3.3), and 20 μl of enzyme extract according to the method of Nakano and Asada (1981). The reaction was initiated by adding AsA oxidase. One unit of MDHAR activity was defined as the amount of the enzyme causing a change in 1.0 μmol(NADH oxid.) per min. The specific MDHAR activity was expressed as U g^{-1}(FM) min^{-1}.

Statistical analysis: The obtained data were tested for significance by using analysis of variance (*ANOVA*) test. Means were compared by least significant differences (LSD) test at $P<0.05$ levels. All statistical tests were carried out using *SPSS* software (*Version 19, SPSS Inc.*, IL, USA).

Results

Growth environment: The light intensity and air temperature in the orchard during the trial are shown in Fig. 1. The daily average light intensity ranged from 800 to 1,000 μmol(photon) m^{-2} s^{-1} under full sunlight except on the 20th day, when the average light intensity and temperature were extremely high (Fig. 1*A*,*C*). After shading, the daily average light intensity decreased of about 50% and the air temperature decreased. In addition, the recordings of diurnal variation of light intensity and air temperature on the 25th day were designated as reference values (Fig. 1*B*,*D*).

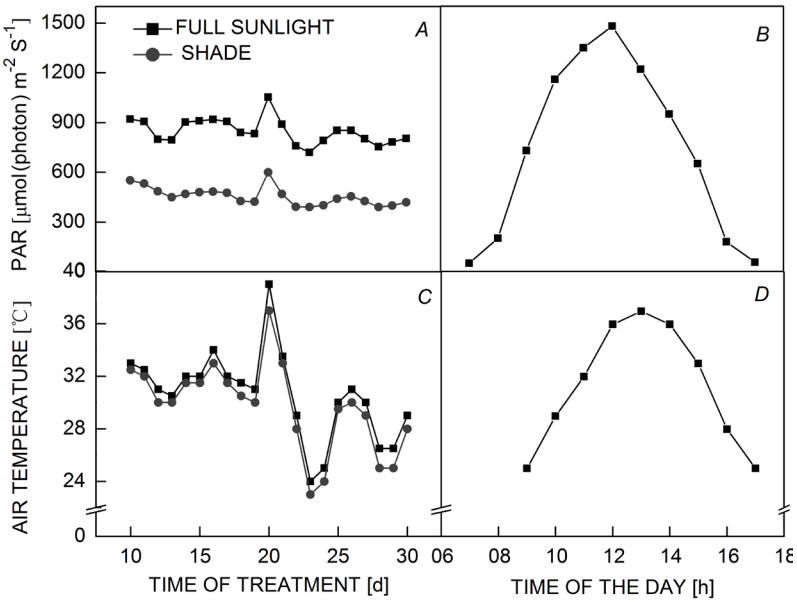

Fig. 1. Light intensity and temperature in orchard recorded during the experiment. (*A*) Diurnal average light intensity of full sunlight (FS) and shade (S) environment. (*B*) Records of light intensity at 25th day. (*C*) Diurnal average air temperature of FS and S environment. (*D*) Records of air temperature at 25th day.

Gas exchange: The changes in gas-exchange parameters of apple leaves exposed to full sunlight and shaded are shown in Table 1. The P_N values of leaves under the shade treatment were 17–42% lower than those exposed to full sunlight before midday but were higher by 46.5–69.5% in the afternoon. Additionally, before midday, the shade leaves had lower values of g_s, E, and C_i but higher values occurred in the afternoon. According to the curve-fitting equations (Fig. 2), we calculated that the fixation of CO_2 in leaves under the shade treatment was 12.1% higher than in leaves exposed to full sunlight for a whole day. The P_N of leaves under full sunlight significantly changed in the afternoon, with almost a 50% reduction, and were significantly lower than those under shade conditions. The values of g_s, E, and C_i of leaves under full sunlight decreased during the afternoon, and were lower than those under shade. The E values were markedly higher on the 20th day due to the temperature peak on this day; this phenomenon could be a self-protection for the apple trees.

The Chl content in leaves is an important physiological index representing the light-harvesting ability for photosynthesis in plants. After shade treatment, the Chl *a* and Chl *b* contents were significantly higher, and the Chl *a*/*b* ratio was lower than those of leaves grown under full sunlight (Table 2).

Chl fluorescence: To explore the variation of PSII activity, the parameters of Chl fluorescence of leaves was measured. The parameters F_0, F_v/F_m, q_p, and Φ_{PSII} are a measure of the capacity of the primary photochemistry of PSII, and they are good indicators of the effect of environmental stress in photosynthesis (Baker and Rosenqvist 2004, Henriques 2009, Zai *et al*. 2012). The F_0 of leaves under shade were significantly higher than those exposed to full sunlight in the morning but were markedly lower in the afternoon. In addition, F_0 values changed between 0.39 and 0.40 in shaded leaves but from 0.42 to 0.45 in leaves exposed to full sunlight during the afternoon (Table 3), thus F_0 values in leaves exposed to full sunlight were higher than shade leaves in the afternoon. Moreover, leaves in the shade had a significantly lower F_m before midday but higher F_m in the afternoon. The F_v/F_m values of leaves exposed to full sunlight were significantly higher than those under shade conditions before midday, but the opposite occurred during the afternoon. These two treatments showed a significant difference in q_p (reflects the balance between the excitation rate of the PSII antennae and the electron flow through the photosynthetic electron transport chain) and Φ_{PSII} (reflects the fraction of absorbed light used in photochemistry), and the leaves exposed to full sunlight had higher values in the morning but lower values during the afternoon. This mechanism inhibited photosynthetic electron transfer from the reaction center of PSII to the Q_A, Q_B, and PQ pools, thus leading to a significant reduction in P_N. The NPQ values (reflect the thermal dissipation of part of the light absorbed by the PSII antenna pigments) showed opposite changes for the two treatments.

Evaluation of H_2O_2: In the presence of peroxidase, DAB formed a deep brown polymerization product upon reaction

Table 1. The net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) in apple trees grown in full sunlight (FS) and shade (S) environment (means ± SE, n = 6) (records of 25th day). AM – measuring time was 9:00–10:00 h, PM – measuring time was 14:00–15:00 h. *Different letters* indicate significant differences by the *Duncan*'s multiple range test at p=0.05.

Treatment	Time of treatment [d]	P_N [μmol(CO_2) m^{-2} s^{-1}]	g_s [mmol(H_2O) m^{-2} s^{-1}]	E [mmol(H_2O) m^{-2} s^{-1}]	C_i [μmol(CO_2) mol^{-1}]
FS-AM	0	10.12 ± 0.4^{ab}	146 ± 3^{a}	2.56 ± 0.41^{cd}	266 ± 13^{a}
	10	10.51 ± 1.42^{ab}	147 ± 22^{a}	2.60 ± 0.32^{cd}	273 ± 9^{a}
	15	11.53 ± 1.01^{a}	146 ± 11^{a}	3.00 ± 0.10^{c}	252 ± 9^{ab}
	20	11.59 ± 1.18^{a}	182 ± 12^{a}	4.90 ± 0.18^{a}	250 ± 14^{ab}
	25	11.13 ± 1.38^{a}	177 ± 17^{a}	3.90 ± 0.22^{b}	264 ± 9^{a}
S-AM	0	10.12 ± 0.32^{ab}	152 ± 14^{a}	2.94 ± 0.12^{c}	267 ± 18^{a}
	10	8.68 ± 0.50^{abc}	82 ± 7^{b}	1.68 ± 0.12^{e}	224 ± 9^{c}
	15	7.40 ± 0.73^{bc}	71 ± 10^{b}	1.70 ± 0.23^{e}	222 ± 7^{c}
	20	6.52 ± 0.91^{c}	76 ± 13^{b}	2.40 ± 0.30^{cde}	225 ± 10^{c}
	25	6.39 ± 1.37^{c}	78 ± 16^{b}	2.00 ± 0.36^{de}	246 ± 16^{bc}
FS-PM	0	5.50 ± 0.21^{c}	62 ± 6^{c}	1.60 ± 0.21^{d}	227 ± 7^{b}
	10	5.81 ± 0.63^{c}	58 ± 8^{c}	1.53 ± 0.22^{d}	220 ± 12^{ab}
	15	6.29 ± 0.89^{bc}	70 ± 9^{c}	1.52 ± 0.20^{d}	245 ± 9^{ab}
	20	5.50 ± 0.80^{c}	65 ± 10^{c}	2.11 ± 0.20^{cd}	225 ± 9^{b}
	25	5.39 ± 1.02^{c}	7 9 ± 10b^{c}	1.97 ± 0.21^{d}	260 ± 9^{ab}
S-PM	0	5 .50 ± 0.51^{c}	58 ± 8^{c}	1.50 ± 0.20^{d}	231 ± 8^{b}
	10	8.51 ± 0.52^{ab}	107 ± 11^{ab}	2.80 ± 0.13^{b}	242 ± 20^{ab}
	15	9.20 ± 0.39^{a}	127 ± 9^{a}	2.71 ± 0.14^{bc}	261 ± 4^{a}
	20	9.32 ± 0.80^{a}	136 ± 10^{a}	3.75 ± 0.30^{a}	243 ± 4^{ab}
	25	8.91 ± 1.21^{a}	139 ± 16^{a}	2.92 ± 0.32^{b}	260 ± 10^{a}

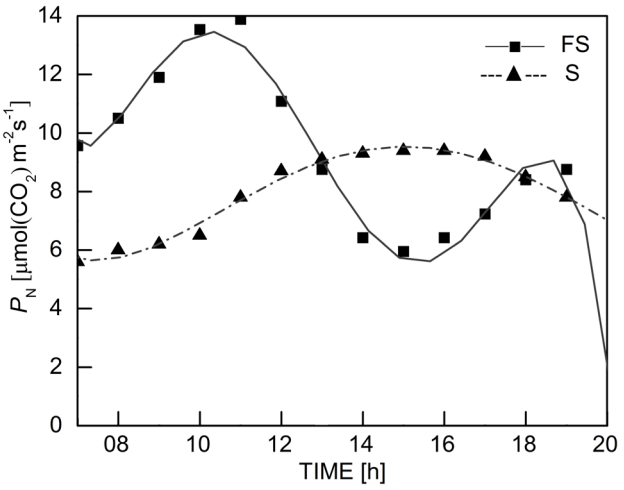

Fig. 2. Diurnal variation curve of net photosynthetic rate (P_N) in apple trees grown in full sunlight (FS) and shade (S) environment (records of P_N at 25th day).

with H_2O_2, and the content reflected the distribution of H_2O_2 in leaves. As shown in Fig. 3, the leaf grown under shade conditions accumulated H_2O_2 around the petiole and veins; however, another leaf exposed to full sunlight exhibited a strong brown color throughout the leaf.

Activities of antioxidant enzymes: Activities of SOD and CAT were enhanced significantly in apple leaves exposed to full sunlight conditions. Simultaneously, full sunlight also enhanced significantly activities of ascorbate-glutathione cycle enzymes, *i.e*., GPX, MDHAR, DHAR, APX, and GR (Table 4). The results showed that strong light intensity accelerated the accumulation of H_2O_2 and increased the activities of antioxidant enzymes in apple leaves.

Discussion

Under the conditions of our study, the PAR and air temperature were reduced (Fig. 1), g_s, E, and C_i were simultaneously lowered; lower P_N was observed in shaded apple trees compared to apple trees exposed to full sun before midday (Table 1). Stomata usually open in response to an increase in PAR, and thus, a lower light intensity reduces stomatal opening and leads to decreased g_s, and stomatal closure leads to a subsequent decline in E. A decrease in g_s may restrict CO_2 fixation (Pires *et al*. 2011) and cause a lower C_i (Yan *et al*. 2013), with a consequent decrease in P_N in apple trees during the morning. The lower values of P_N suggest that the light energy was suboptimal during the morning and did not induce photoinhibition. Therefore, we advise not to shade before 11:00 a.m. This result can provide theoretical basis in apple culture for an automated facility. During the experiment, the integrated daily P_N was approximately 12% higher in shaded apple trees than that in trees under full sun (Fig. 2), due to increased g_s and decreased photoinhibition. Higher g_s and P_N under reduced radiation have also been reported for *Macadamia integrifolia* and *Litchi chinensis* (Lloyd *et al*. 1995).

Table 2. Content of total chlorophyll (Chl), Chl *a*, Chl *b*, and Chl *a*/*b* ratio in leaves of apple trees grown in full sunlight (FS) and shade (S) environment (means ± SE, $n = 6$) (records of 25th day). *Different letters* indicate significant differences by the *Duncan*'s multiple range test at p=0.05.

Treatment	Time of treatment [d]	Chl (*a*+*b*) [mg cm^{-2}]	Chl *a* [mg cm^{-2}]	Chl *b* [mg cm^{-2}]	Chl *a*/*b*
FS	0	0.031 ± 0.001^{h}	0.026 ± 0.001^{g}	0.005 ± 0.001^{g}	5.14 ± 0.07^{a}
	10	0.034 ± 0.002^{g}	0.029 ± 0.002^{f}	0.006 ± 0.002^{f}	5.13 ± 0.10^{a}
	16	0.037 ± 0.002^{f}	0.031 ± 0.001^{e}	0.006 ± 0.001^{ef}	5.12 ± 0.02^{a}
	21	0.039 ± 0.001^{e}	0.033 ± 0.002^{d}	0.006 ± 0.003^{e}	5.10 ± 0.01^{a}
	26	0.042 ± 0.002^{d}	0.035 ± 0.003^{c}	0.007 ± 0.001^{d}	5.10 ± 0.02^{a}
S	0	0.031 ± 0.001^{h}	0.026 ± 0.001^{g}	0.005 ± 0.000^{g}	5.14 ± 0.05^{a}
	10	0.037 ± 0.002^{f}	0.031 ± 0.002^{e}	0.006 ± 0.001^{ef}	4.81 ± 0.04^{b}
	16	0.044 ± 0.003^{c}	0.036 ± 0.002^{b}	0.008 ± 0.003^{c}	4.30 ± 0.03^{c}
	21	0.049 ± 0.002^{b}	0.039 ± 0.002^{a}	0.010 ± 0.001^{b}	3.98 ± 0.02^{d}
	26	0.050 ± 0.002^{a}	0.040 ± 0.003^{a}	0.011 ± 0.001^{a}	3.73 ± 0.02^{e}

Table 3. Changes of the chlorophyll fluorescence parameters in leaves of apple trees grown in full sunlight (FS) and shade (S) environment (means ± SE, $n = 4$) (records of 25th day). AM – measuring time was 9:00–10:00 h, PM – measuring time was 14:00–15:00 h. *Different letters* indicate significant differences by the *Duncan*'s multiple range test at p=0.05.

Treatment	Time of treatment [d]	F_0	F_m	F_v/F_m	q_P	NPQ	Φ_{PSII}
FS-AM	0	0.38 ± 0.01^{c}	1.93 ± 0.03^{b}	0.80 ± 0.01^{ab}	0.26 ± 0.02^{a}	1.55 ± 0.05^{a}	0.14 ± 0.01^{a}
	10	0.38 ± 0.02^{c}	1.90 ± 0.07^{b}	0.80 ± 0.01^{ab}	0.27 ± 0.02^{a}	1.56 ± 0.12^{a}	0.15 ± 0.01^{a}
	16	0.39 ± 0.01^{c}	2.16 ± 0.05^{a}	0.81 ± 0.00^{a}	0.25 ± 0.03^{ab}	1.68 ± 0.08^{a}	0.13 ± 0.01^{ab}
	21	0.39 ± 0.01^{c}	2.15 ± 0.03^{a}	0.81 ± 0.00^{a}	0.23 ± 0.02^{ab}	1.54 ± 0.09^{a}	0.12 ± 0.01^{ab}
	26	0.40 ± 0.01^{c}	2.13 ± 0.02^{a}	0.80 ± 0.01^{ab}	0.23 ± 0.01^{ab}	1.49 ± 0.09^{a}	0.13 ± 0.01^{ab}
S-AM	0	0.38 ± 0.01^{c}	1.95 ± 0.04^{b}	0.80 ± 0.01^{ab}	0.26 ± 0.03^{a}	1.55 ± 0.06^{a}	0.14 ± 0.02^{a}
	10	0.42 ± 0.00^{bc}	1.86 ± 0.14^{bc}	0.78 ± 0.01^{bc}	0.24 ± 0.05^{ab}	1.54 ± 0.06^{a}	0.11 ± 0.03^{abc}
	16	0.43 ± 0.01^{bc}	1.81 ± 0.03^{bc}	0.77 ± 0.01^{cd}	0.21 ± 0.03^{ab}	1.55 ± 0.10^{a}	0.10 ± 0.01^{bc}
	21	0.45 ± 0.03^{ab}	1.79 ± 0.11^{bc}	0.75 ± 0.01^{d}	0.18 ± 0.02^{b}	1.52 ± 0.03^{a}	0.08 ± 0.01^{c}
	26	0.48 ± 0.03^{a}	1.65 ± 0.07^{c}	0.71 ± 0.01^{e}	0.18 ± 0.01^{b}	1.39 ± 0.08^{a}	0.08 ± 0.01^{c}
FS-PM	0	0.43 ± 0.01^{a}	1.75 ± 0.05^{b}	0.76 ± 0.01^{b}	0.21 ± 0.02^{bc}	1.67 ± 0.05^{ab}	0.09 ± 0.01^{c}
	10	0.42 ± 0.02^{a}	1.76 ± 0.05^{b}	0.76 ± 0.01^{b}	0.22 ± 0.02^{bc}	1.63 ± 0.06^{ab}	0.10 ± 0.01^{c}
	16	0.43 ± 0.02^{a}	1.75 ± 0.05^{b}	0.75 ± 0.01^{b}	0.20 ± 0.04^{bc}	1.74 ± 0.08^{ab}	0.09 ± 0.02^{c}
	21	0.43 ± 0.02^{a}	1.73 ± 0.10^{b}	0.75 ± 0.01^{b}	0.19 ± 0.02^{c}	1.68 ± 0.10^{ab}	0.08 ± 0.01^{c}
	26	0.45 ± 0.03^{a}	1.71 ± 0.13^{b}	0.74 ± 0.01^{b}	0.19 ± 0.02^{c}	1.90 ± 0.09^{a}	0.08 ± 0.01^{c}
S-PM	0	0.43 ± 0.01^{a}	1.75 ± 0.05^{b}	0.75 ± 0.01^{b}	0.21 ± 0.02^{bc}	1.68 ± 0.05^{ab}	0.09 ± 0.01^{c}
	10	0.39 ± 0.00^{b}	2.00 ± 0.05^{a}	0.80 ± 0.01^{a}	0.31 ± 0.04^{a}	1.54 ± 0.05^{b}	0.17 ± 0.02^{a}
	16	0.39 ± 0.01^{b}	2.01 ± 0.02^{a}	0.80 ± 0.01^{a}	0.27 ± 0.01^{ab}	1.61 ± 0.10^{b}	0.14 ± 0.01^{ab}
	21	0.40 ± 0.01^{b}	2.01 ± 0.05^{a}	0.80 ± 0.01^{a}	0.21 ± 0.02^{bc}	1.60 ± 0.12^{b}	0.11 ± 0.01^{bc}
	26	0.40 ± 0.02^{b}	2.00 ± 0.03^{a}	0.79 ± 0.01^{a}	0.21 ± 0.02^{bc}	1.63 ± 0.06^{ab}	0.11 ± 0.01^{bc}

A decrease in photosynthetic ability of leaves exposed to strong light has previously been reported (Hunter *et al*. 2012, Miyata *et al*. 2012). Similar trends occurred in the present study, with a lower photosynthetic activity of apple leaves grown under full sunlight than those grown under shade conditions. Some research reports indicate that high temperature and strong irradiance induced photoinhibition or photodamage of photosynthetic apparatus (Adams *et al*. 2013). Changes in chloroplast membrane lipids and proteins that result in structural modifications of the thylakoid membrane can also impair photosynthesis (Carpentier 1999). In present experiment, the P_N values decreased by about 50% during the afternoon (Table 1), indicating a reduced ability to assimilate CO_2 could be attributed to photodamage caused by strong light stress.

In green plants, the PSI is a large protein complex, which works as an oxidoreductant in oxygenic photosynthesis. The LHCI complex consists of four different Lhca polypeptides (Lhca1–4). Each Lhca protein binds in total about 10 Chl *a* and Chl *b* molecules. Chl *b* mainly occurs in the light-harvesting Chl *b* protein complex I (LHCI), and shade leaves are relatively rich in Chl *b* (Zhang *et al*. 2007,

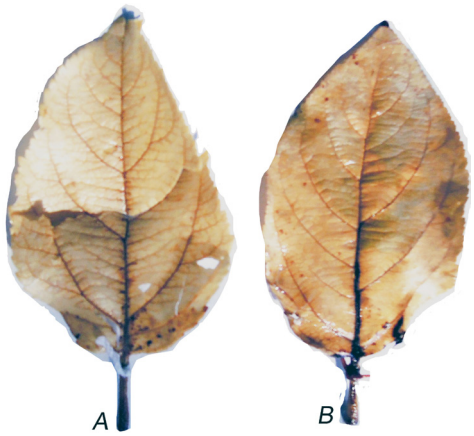

Fig. 3. Accumulation of H_2O_2 in leaf of apple trees grown in full sunlight and shade environment (leaves at 25th day). (*A*) Leaf from apple tree grown in shade environment. (*B*) Leaf from apple tree grown in full sunlight.

Table 4. Effect of full sunlight (FS) and shade (S) conditions on antioxidant enzyme activities in leaves of apple trees (means ± SE, n = 4) (records of 25th day). * – indicates that the effect of growth conditions is significant at P<0.05.

Treatment	FS	S
SOD [U g^{-1}(FM)]	9.74 ± 0.11*	4.77 ± 0.09
CAT [U g^{-1}(FM)]	14.82 ± 1.22*	10.02 ± 0.19
GPX [U g^{-1}(FM) min^{-1}]	14.67 ± 1.27*	9.31 ± 0.15
APX [U g^{-1}(FM) min^{-1}]	9.88 ± 0.17*	6.12 ± 0.09
GR [U g^{-1}(FM) min^{-1}]	3.03 ± 0.10*	2.28 ± 0.06
DHAR [U g^{-1}(FM) min^{-1}]	3.13 ± 0.05*	2.67 ± 0.03
MDHAR [U g^{-1}(FM) min^{-1}]	7.81 ± 0.11*	5.20 ± 0.03

Sakuraba *et al*. 2010). In addition, Chl *b* exists exclusively in the peripheral antenna, whereas the core antenna has only Chl *a* (Green and Durnford 1996, Barber *et al*. 2000, Satoh *et al*. 2001). A higher contribution of outer antenna Chl (Chl *b*) to the total Chl content leads to lower Chl *a*/*b* ratio and enhances the efficiency of photocapture under limited light supply (Baig *et al*. 2005). In addition, the Chl *a*/*b* ratio also depends on the PSII to PSI ratio. We found that the contents of Chl *a* and Chl *b* were higher in apple leaves grown in the shade environment, leading to higher total Chl content, and lower Chl *a*/*b* ratio (Table 2); hence, a relatively high value for Chl *b*/*a*. The lower Chl *a*/*b* ratio may indicate a larger PSII outer antenna enabling a higher absorption of light energy under shaded conditions; a larger antenna does, however, not mean that the photosynthetic efficiency increases. Hence, moderately shading did not decrease the total photosynthetic capacity.

Compared to leaves in full sunlight, the leaves in the shade had a higher F_0 and lower F_m, F_v/F_m, q_p, and Φ_{PSII} during the morning, which were attributed to the limited light energy that led to lower photosynthetic capacities, although these values were significantly higher during the afternoon (Table 3). Under full sunlight during the afternoon, the higher F_0 values might be caused by photodamage to the photosynthetic apparatus due to strong light stress. In addition, very low values of F_m and F_v/F_m are indicative of the accumulation of inactive PSII reaction centers and should be ascribed to photodamage induced by strong light stress (Kalaji *et al*. 2011). The lower values of q_p and Φ_{PSII} in the apple trees were due to reduced electron transport capacity in PSII during the afternoon. Furthermore, Calvin-Benson cycle activity and stomatal opening cannot be ruled out. A similar result occurred in wheat and transgenic *Arabidopsis* plants (Araus *et al*. 1998, Sakuraba *et al*. 2010).

Under strong light, inhibition of the activity of PSII (photoinhibition) occurs due to an imbalance between the rate of photodamage to PSII and the rate of the repair of damaged PSII. Reactive oxygen species (ROS) produced under high irradiance directly inactivate the photochemical reaction center of PSII (Murata *et al*. 2007). Antioxidant enzymes are widely accepted to protect PSII against photodamage, however, it has been proposed that, rather than protecting PSII from photodamage, they stimulate protein synthesis, with resultant repair of PSII and mitigation of photoinhibition (Murata *et al*. 2012). In general, the production of active oxygen is low in normal plant cells because of well-developed defense systems against ROS. SOD constitutes the first line of cellular defense against ROS, by scavenging the primary product of oxygen reduction, superoxide anion (O_2^-). SODs rapidly convert O_2^- and water to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Asada 2006), thereby protecting the plant against oxidative damage. In addition, increase in CAT activity suggests an upregulation of the plant protective mechanisms against oxidative stress through scavenging H_2O_2 by converting it into O_2 and H_2O in peroxisomes (Dat *et al*. 2000). In the present investigation, in the afternoon, SOD activities declined to 4.77 [U g^{-1}(FM)] from 9.74 [U g^{-1}(FM)] in the shaded apple trees, in addition, CAT activities declined to 10.02 [U g^{-1}(FM)] from 14.82 [U g^{-1}(FM)] (Table 4), suggesting reduced accumulation of H_2O_2 in shaded leaves. To prove the decline in accumulation of H_2O_2 in shaded leaves, the visual DAB staining method was performed (Fig. 3). The essential antioxidative enzymes of the AsA-GSH cycle (GPX, APX, GR, MDHAR, and DHAR) maintain the cellular redox balance under stressful conditions by scavenging H_2O_2 in chloroplasts, cytosol, vacuoles, and apoplastic spaces (Mitter 2004, Liu *et al*. 2012). We found the enzymes GPX, APX, GR, MDHAR, and DHAR activities also lowered in the shaded apple trees, suggesting a chloroplast-based detoxification of ROS *via* the Mehler reaction and the NADPH-dependent reduction of the disulfide bond of oxidized GSH.

Therefore, these results suggest that the shaded apple leaves maintained higher photosynthetic ability to assimilate CO_2, to transport electrons and utilize energy more efficiently, which was attributed to the lesser photodamage of the photosynthetic apparatus due to a lower amount of ROS in the afternoon.

Conclusions: In our study, moderate shade could alleviate

the damage of strong radiation on apple trees during the afternoon and synchronously improve photosynthesis in leaves and increase the total carbon assimilation. In addition to Chl, enhanced photosynthesis was attributed to an increase in photochemical efficiency and electron transport chain activity under shade conditions. On the basis of Chl fluorescence and ROS analyses, the increased photochemical efficiency was due to an increased energy conservation capacity. This was caused by the decrease in photodamage of photosynthetic apparatus due to a lower amount of H_2O_2 under strong light conditions.

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