DOI: 10.1007/s11099-016-0661-4 PHOTOSYNTHETICA 55 (3): 478-490, 2017

Ozone sensitivity of four Pakchoi cultivars with different leaf colors: physiological and biochemical mechanisms

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Abstract

Ozone (O_3) is important air pollutant inducing severe losses of horticultural production. Cultivars of the same species, but with different leaf colors, may differ in their ozone sensitivity. However, it has not been clarified yet if different leaf coloration influences such a sensitivity. In this study, two purple-leafed and two green-leafed cultivars of Pakchoi were selected for ozone fumigation (240 ± 20 nmol mol^{-1}, 09:00–16:00 h). Elevated O_3 decreased chlorophyll content, increased anthocyanin (Ant) content, damaged cell membrane integrity, enhanced antioxidative enzyme activities, depressed photosynthetic rate (P_N) and stomatal conductance (g_s), inhibited maximal quantum yield (F_v/F_m) and effective quantum yield [Y_{II}] of PSII photochemistry, and caused visible injury. Purple-leafed cultivars with higher Ant contents were more tolerant than green-leafed cultivars as indicated by lower relative enhancement in malondialdehyde content and lower relative losses in P_N, g_s, F_v/F_m, and Y_{II}. The higher ability to synthesize Ant in the purple-leafed cultivars contributed to their higher photoprotective ability.

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

Introduction

Tropospheric O_3 is one of the key air pollutants in many developed and developing countries (Paoletti *et al*. 2010, Bortolin *et al*. 2014). With industrialization and vehicle exhaust, concentrations of O_3 precursors, *e.g*., NO_x and volatile organic compounds, increase quickly. As a result, global mean of surface O_3 has increased remarkably to 50 nmol mol^{-1} in current summer seasons, and is predicted to increase more in the near future (Royal Society 2008).

It has been well documented that elevated O_3 causes oxidative detrimental effects in plants (Ashmore 2005, Hayes *et al*. 2007). Ozone fluxes into plant leaves through stomata and dissolves in water solutions in the apoplast, where rapidly decomposes and forms various reactive oxygen species (ROS) (Castagna and Ranieri 2009). These ROS attack lipid and protein components of plasma membrane causing leakage of cell membranes and programmed cell death (Overmyer *et al*. 2003). It causes a visible injury in plant leaves. In a long term, the crop yield and growth of natural vegetation may be adversely affected by O_3 pollution.

Received 17 February 2016, *accepted* 23 August 2016, *published as online-first* 30 September 2016.
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Abbreviations: Ant – anthocyanins; APX – ascorbate peroxidase; CAT – catalase; Car – carotenoids; CF – charcoal-filtered air; C_i – intercellular CO_2 concentration; Chl – chlorophyll; E – transpiration rate; EC – electrical conductivity; ETR – electron transport rate; F_o – minimal fluorescence of the dark-adapted state; F_m – maximal fluorescence of the dark-adapted state; FM - fresh mass; F_v/F_m – maximal efficiency of PSII photochemistry in the dark-adapted state; g_s – stomatal conductance; JG – Jingguan; MDA – malondialdehyde; MG – Meiguan; NPQ – nonphotochemical quenching; OTCs – open top chambers; P_N – net photosynthetic rate; POD – peroxidase; q_L – coefficient of photochemical fluorescence quenching based on lake model; q_N – coefficient of nonphotochemical fluorescence quenching; q_P – coefficient of photochemical fluorescence quenching based on puddle model; ROS – reactive oxygen species; SOD – superoxide dismutase; VOCs – volatile organic compounds; Y_{II} – the effective quantum yield of PSII photochemistry; Y_{NPQ} – quantum yield of light-induced nonphotochemical fluorescence quenching; Y_{NO} – quantum yield of nonregulated heat dissipation and fluorescence emission; ZY – Ziyu; ZZ – Zizuan.
Acknowledgement: We thank Dr. Elena Paoletti at the National Research Council of Italy and Dr. Caihong Li at the Chinese Academy of Sciences for their kindly revisions of this manuscript. This study was financially supported by National Natural Science Foundation of China (31401895), Natural Science Foundation of Heilongjiang Province of China (C201221), and 'Young Talents' project and Doctoral Fund of Northeast Agricultural University of China (14Q10, 2012RCB36).

In general, plants possess some abilities to minimize oxidative damage through protection by endogenous antioxidant systems (Foyer *et al.* 1994), which, to some extent, leads to elimination of ROS and to improvement of plant tolerance (Ryang *et al*. 2009). To protect themselves, plant cells are equipped with oxygen-radical detoxifying enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) (Abassi *et al*. 1998), as well as antioxidants, such as ascorbic acid. However, responses are species-specific and/or cultivar-specific.

Anthocyanins (Ant) are products of secondary metabolism in plants. Contents of Ant vary in different cultivars and species, even in different parts of a single plant specimen. Foliar Ant might mitigate the severity of photoinhibition under excessive light. It has been reported that a loss in the photochemical quantum yield of PSII for acyanic leaves was higher than that for red leaves in *Cornus stolonifera* when exposed to saturated light (Feild *et al*. 2001). It has also been found that Ant leaves were less photoinhibited than green leaves in *Galax urceolata* under high-light stress (Hughes and Smith 2007). Moreover, Ant can scavenge hydroxyl radical, and other ROS induced by some abiotic stresses (Gould *et al.* 2002). Under O_3 pollution, ROS induced in leaves attack chloroplasts, resulting in a decrease of the saturation irradiance for photosynthesis and causing photoinhibition. Given their functions in the mitigation of photoinhibition, we wonder whether Ant can protect plants against O_3 damage *in vivo*.

Visible injury is often considered as an index of plant sensitivity to O_3 (Guidi *et al*. 2000, Bussotti *et al*. 2003). As useful nondestructive tools for stress detection (Owens 1994), gas exchange and chlorophyll (Chl) *a* fluorescence have been used to analyze species-specific sensitivities to O_3, which can in part explain the O_3 effects on plant growth (Biswas *et al*. 2008, Contran *et al*. 2009). Furthermore, changes in Chl *a* fluorescence can reflect the impact of O_3 on photoinhibition.

Many species of the genus *Brassica* are important vegetables. It has been reported that O_3 pollution can influence the physiological activities, growth, reproduction, yield, and quality of *Brassica* plants, individually or together with other ecological factors, such as elevated CO_2 or fertilizers *etc*. (Black *et al*. 2007, Wang *et al*. 2008, De Bock *et al*. 2012, Tripathi and Agrawal 2012, Vandermeiren *et al*. 2012, Singh *et al*. 2012, 2013; Rozpadek *et al*. 2015). Pakchoi is one of the most popular cruciferous vegetables in China (Cao *et al*. 2015). There are both purple-leafed cultivars and green-leafed cultivars of Pakchoi. We hypothesize that the purple-leafed cultivars with their high contents of Ant could be more tolerant to elevated O_3. We selected four cultivars of *Brassica* campestris, two with green leaves and two with purple leaves, in order to clarify the possible effects of Ant in leaves on O_3 damages to plants. We compared their different responses to elevated O_3 in terms of visible injury, gas exchanges, Chl *a* fluorescence, and biochemical parameters.

Materials and methods

Plant materials and growth conditions: Four cultivars [Jingguan (JG), Meiguan (MG), Ziyu (ZY), and Zizuan (ZZ)] of Pakchoi (*Brassica campestris* L. *ssp. chinensis* L. Makino) were selected for this experiment. Leaves of two cultivars (JG and MG) are green and two (ZY and ZZ) are purple. Seeds of JG were purchased from Beijing Vegetable Research Center, Beijing Academy of Agriculture and Forestry Sciences, China. Seeds of MG were purchased from *Xiangsu Seed Company* in Zhuzhou, Hunan province, China. Seeds of ZZ and ZY were purchased from *Dongsheng Seed Company*, Beijing, China. On 1 July 2013, seeds of all cultivars were placed in pots (10 cm in diameter) filled with a mixture of vermiculite, peat, and field soil (1:3:6, v/v/v). Water was supplied every day throughout the experiment to avoid water deficiency. The plants were managed under ambient conditions until they reach a stage of 6 leaves in total on 3 August 2013.

Experimental design: The experiment was conducted in six open top chambers (OTCs, 1.8 m in diameter and 2.4 m in height) at the Hunsandake Research Station (43º56´47"N, 116º08´15"E), Institute of Botany, Chinese Academy of Sciences, China. The OTCs were made of transparent polymethylmethacrylate plates. The maximum/minimum temperature and relative humidity in the OTCs were 35/20°C and 60/45%, respectively. The PPFD inside the OTCs was averaged at 1,000 μmol $m^{-2}s^{-1}$. The O_3 treatment method was referred to Biswas *et al.* (2008), and the ventilation tube was adjusted 20 cm above the plants. On 26 July 2013, six seedlings of each species were randomly placed in each OTC. All plants received charcoal-filtered air [CF, < 5 nmol(O_3) mol^{-1}] for one week in order to adapt the environment. After one week, three chambers were ventilated with O_3, where concentration was maintained at three fold of Chinese national threshold of ozone pollution (240 ± 20 nmol mol^{-1}) (09:00–16:00 h) for 7 h, and the other three were treated with CF air on 3 August 2013. Ozone was generated by electrical discharge using charcoal-filtered ambient oxygen with an O_3 generator (*CF-KG1*, *Beijing Sumsun EP Hi-Tech.*, Beijing, China) and bubbled through distilled water before entering the elevated O_3 chambers (Zhang *et al.* 2010). Glass tube rotameters regulated the flow of injected O_3 to the tubes. Ozone concentrations in the OTCs were monitored once an hour at approximately 10 cm above the plant canopy using an O_3 analyzer (*Model 205, 2B Technologies Inc.*, Boulder, Colorado, USA).

Visible injury: Leaf injuries were assessed on the plants immediately after 7 h of O_3 exposure. The percentage of damaged area (mottled or necrotic) on all leaves was assessed by two surveyors for five plants per cultivar sampled from CF and O_3 treatments, respectively.

Gas exchange: On 4 August 2013, instant gas exchange was measured on the fourth leaf from the top from 08:30 to 11:30 h using a gas-exchange system (*Li-6400*, *Li-Cor*, USA). Six plants of each cultivar, in O_3 or CF, were randomly selected for measurements. The cuvette condition were set under light intensity of 1,500 μmol(photon) m^{-2} s^{-1} provided by the special artificial light, with air temperature of 30 ± 0.5°C. Gas exchange was measured using ambient CO_2 concentration (380 ± 10 μmol mol^{-1}). Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981).

Chl *a* fluorescence: On 4 August 2013, Chl *a* fluorescence was measured on the fourth leaf from the top using a *PAM-2500* fluorometer (*Heinz Walz*, Effeltrich, Germany). For each measurement, the middle portion of the leaf segment was dark adapted with a leaf clip (*DLC-8*, *Heinz Walz*, Effeltrich, Germany) for 30 min. The Chl *a* fluorescence parameters were measured on the adaxial leaf surface immediately after the dark adaptation. Fluorescence induction curves were automatically recorded using the slow kinetic program in the saturation pulse analysis mode. The minimal (F_o) and maximal fluorescence (F_m) in the dark-adapted state were determined with a modulated irradiation [630 nm, 0.1 μmol(photon) m^{-2} s^{-1}] and a 0.8 s saturating pulse [630 nm, 10,000 μmol(photon) m^{-2} s^{-1}], respectively. After 40 s, an actinic light [630 nm, 104 μmol(photon) m^{-2} s^{-1}] was switched on. Then a saturating pulse was imposed every 20 s until 5 min in order to determine the maximal fluorescence in the irradiation-adapted state (F_m'). The F_v/F_m was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (Schreiber 2004). The *PamWin-3* software automatically calculated four quenching coefficients (q_P, q_L, q_N, and NPQ), Y_{II}, Y_{NO}, and Y_{NPQ}, as well as the electron transport rate (ETR). All fluorescence measurements were carried out between 08:00 to 11:00 h and repeated six times.

Leaf pigment contents: Fresh leaf (FM) samples (0.2 g) were collected to determine total Chl and carotenoid (Car) contents. Chl and Car were extracted using 5 ml of acetone (80%, v/v) at 4°C for 48 h in the dark. The absorbance was measured using a UV-visible spectrophotometer (*UV-2550*, *Shimadzu*, Japan) at 470, 647, and 663 nm, using the equations of Lichtenthaler (1987). Another 0.2 g of fresh leaf samples were collected for Ant content measurement. Ant were extracted in 10 ml acidified methanol (99 CH_3OH:1 HCl, v/v) at 4°C for 24 h in the dark, clarified by centrifugation at 12,000 × *g* for 2 min, and the absorbance of supernatants was determined using a UV-visible spectrophotometer (*UV-2550*, *Shimadzu*, Japan) from optical density (OD) at 530 and 600 nm. Ant concentrations were expressed as U g^{-1}(FM), where U was calculated as $(OD_{530} - OD_{600})/0.1$ (Lin *et al.* 1998).

Antioxidative enzyme activity: Fresh leaf samples (0.5 g) were homogenized in ice-cold 50 mM phosphate extraction buffer (pH 7.8) containing 0.2 mM EDTA, and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were then used to determine enzyme activities of SOD (EC 1.15.1.1), POD (EC 1.11.1.7), and CAT (EC 1.11.1.6). Activities of SOD were determined according to the methods of Syeed *et al.* (2011). One unit of SOD activity (U) was defined as the amount of enzyme that inhibited 50% of NBT photoreduction and the SOD activity was expressed as U g^{-1}(FM). The activity of CAT was estimated according to the method reported by Díaz-Vivancos *et al.* (2008), which measures the decline of H_2O_2 at the maximum absorption at 240 nm. One unit was defined as the amount of enzyme catalyzing the decomposition of 1 μmol(H_2O_2) min^{-1} g^{-1}(FM). The activity assay of POD was based on the method described by Liu *et al.* (2014) with minor modifications. The reaction mixture contained 0.05 mM guaiacol and 2% H_2O_2 as substrate. After adding the enzyme extract, the increase in absorbance at 465 nm was monitored for 5 min. Unit of POD activity expressed the amount of enzyme oxidizing 1 μmol(guaiacol) min^{-1} g^{-1}(FM). The APX (EC 1.11.1.11) activity assay was performed according to the method of Yang *et al.* (2010) with some modifications. Fresh leaves (0.5 g) were ground with ice-cold 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM ascorbate. The homogenates were centrifuged at 15,000 × *g* for 30 min, and then the supernatants were collected for the measurement of APX activity. One unit was defined as the amount of enzyme oxidizing 1 μmol(ascorbate) min^{-1} g^{-1}(FM).

Electrical conductivity (EC): Leaf samples (0.2 g) were cut into uniformly sized discs and placed in test tubes containing 20 ml of double distilled water in two sets. One set was kept at 25°C for 24 h and the other set was maintained at 100°C in boiling water bath for 20 min. The electrical conductivities C1 and C2 of the sets were measured by a conductivity meter (*Delta 326*, *Mettler-Toledo*, Switzerland). The relative conductivity index was calculated as (C1/C2) × 100%.

Malondialdehyde (MDA) content: The MDA content assay was performed according to the method of Hao *et al.* (2004). Fresh leaf samples (0.2 g) were ground and then homogenized in trichloroacetic acid (10%). The homogenates were centrifuged at 4,000 × *g* for 10 min. The supernatants (1 ml) were mixed with 5 ml of thiobarbituric acid (0.6%) and then maintained in boiling water bath for

15 min. After cooling, the mixture was centrifuged at 4,000 × *g* for 10 min. The absorbances of supernatants were measured using a UV-visible spectrophotometer (*UV-2550*, *Shimadzu*, Japan) at 450, 532, and 600 nm. The MDA content was calculated as:

$$MDA = [6.45 (OD_{532} - OD_{600}) - 0.56 OD_{450}]/0.2.$$

Statistical analysis: All data analyses were performed using *SPSS version 12* (*SPSS*, Chicago, IL, USA). Data were checked for normal distribution by the *Kolmogorov-Smirnov*'s *d* test, if $P>0.05$ means that distribution is normal. Means of each parameter were analyzed using factorial analysis of variance (*ANOVA*) to compare values. Interactions between treatment and cultivar were identified using two-way *ANOVA*. *Pearson*'s correlation among relative changes in gas exchange, Chl *a* fluorescence, and other physiological parameters in the four cultivars exposed to elevated O_3 were analyzed.

Results

Visible injuries on fully expanded leaves of all cultivars of Pakchoi were observed under O_3 fumigation, whereas plants in CF air showed no visible injury. Permanent wilting of the first and second leaves from the bottom was noted in all O_3-exposed cultivars. No visible injury was found on the youngest leaves (leaf 6) in all cultivars except JG. There was a significant variation among the cultivars in O_3 injury developed on the fully expanded leaf (Leaf 3) (45–97%) after 7h of O_3 fumigation. The least visible injuries were observed in ZZ, followed by ZY, whereas both the green-leafed cultivars (JG and MG) showed the highest damage (Table 1).

Leaf pigment contents: Elevated O_3 significantly decreased the concentration of total Chl and Car and increased that of Ant. There were significant interactions between treatments and cultivars concerning these pigments (Fig. 1). The total Chl concentrations in the green-leafed cultivars (JG and MG) were significantly higher than those in the purple-leafed cultivars (ZY and ZZ) under CF air (Fig. 1). Under O_3 fumigation, the largest relative loss of total Chl was observed in MG, followed by ZY and JG, and the lowest was found in ZZ (Fig. 1*A*). In CF air, the Car content was the highest in JG, followed by MG and ZY, and the lowest was found in ZZ. Compared with CF, the Car content of ZY showed the largest relative reduction, followed by MG and JG, and the lowest was found in ZZ under elevated O_3 concentration (Fig. 1*B*). There were significant differences between cultivars in Ant contents under CF air. The Ant concentration of the purple-leafed cultivars (ZY and ZZ) was much higher than those of the green-leafed cultivars (JG and MG). After fumigation, the Ant content of the purpled-leafed cultivars significantly increased while that of the green-leafed cultivars remained almost stable (Fig. 1*C*).

Table 1. Visible symptoms of O_3 damage in different leaves of four cultivars of Pakchoi exposed to elevated O_3 or charcoal-filtered air (CF). Leaves of each sampled plant were named from the oldest (Leaf 1) to the youngest (Leaf 6). Overall, the green-leafed cultivars (Jingguan and Meiguan) showed higher level of visible symptoms of O_3 injury than the purple-leafed cultivars (Ziyu and Zizuan). Results are shown as means ± SD (n = 6).

Treatment	Visible symptoms of O_3 damage [%]					
	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Leaf 6
Jingguan						
CF	0	0	0	0	0	0
O_3	100 ± 2	100 ± 2	97±3	93 ± 7	55 ± 4	21 ± 5
Meiguan						
CF	0	0	0	0	0	0
O_3	100 ± 0	100 ± 2	95±8	66 ± 2	40 ± 2	0
Ziyu						
CF	0	0	0	0	0	0
O_3	100 ± 1	82 ± 3	55±2	43 ±5	18 ± 1	0
Zizuan						
CF	0	0	0	0	0	0
O_3	92 ± 2	73 ± 9	45±3	33 ± 1	17 ± 3	0

Membrane integrity: The EC and MDA contents of all cultivars enhanced significantly by O_3 fumigation (Fig. 2) and significant interactions between treatments and cultivars were found (Fig. 2). A higher relative rise in MDA was observed in the green-leafed cultivars (JG and MG) compared to that in the purple-leafed cultivars (ZY and ZZ) (Fig. 2*B*). The highest relative increase of EC was observed in ZZ, followed by MG and JG, and the lowest value was found in ZY (Fig. 2*A*).

Antioxidative enzyme activity: Four antioxidative enzymes (SOD, POD, CAT, and APX) showed significantly higher activities under O_3 exposure compared with CF control (Fig. 3) and significant interactions between treatments and cultivars were found (Fig. 3). In CF air, the SOD activity of MG was much lower than those of other cultivars. Under O_3 fumigation, SOD activities of JG, ZY, and ZZ were much higher than that of MG. The highest relative increase of SOD activity was found in ZY, followed by MG and ZZ, and the lowest was observed in JG (Fig. 3*A*). In CF air, POD activities of MG was the highest, followed by ZY and JG, while that of ZZ was the lowest one. Under O_3 exposure, POD activities of MG and ZY were higher than those of JG and ZZ. The highest relative increase of POD activity was found in ZY, followed by MG. Under O_3 fumigation, JG and ZZ exhibited insignificantly different POD activities compared with CF (Fig. 3*B*). CAT activities of MG were the highest, while that of the ZZ was the lowest in CF air.

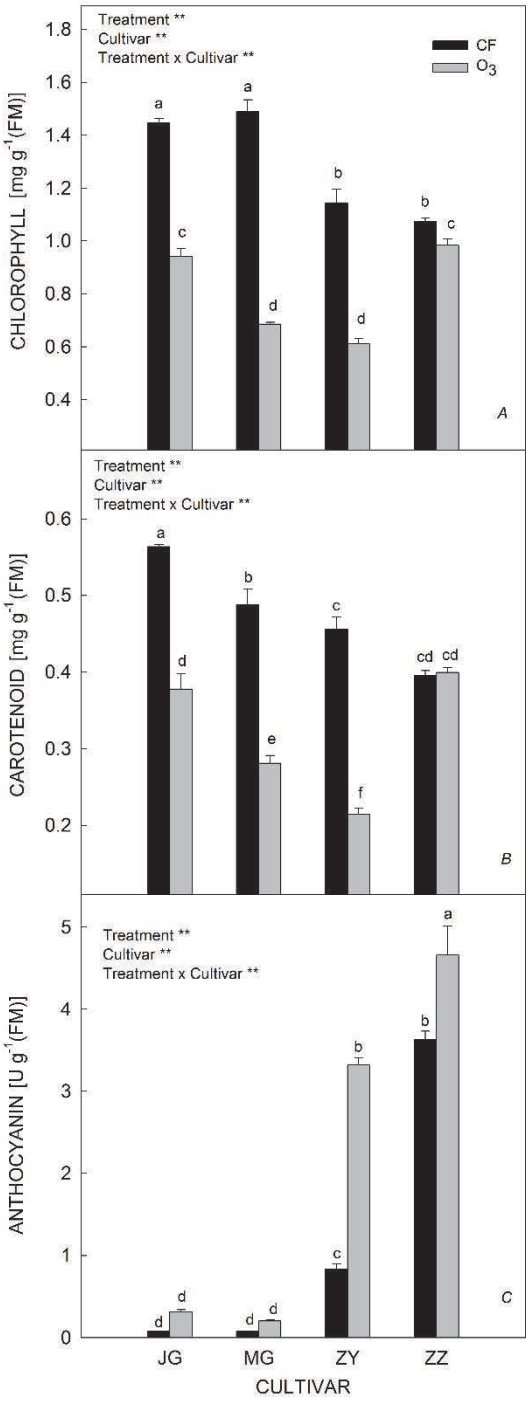

Fig. 1. Total chlorophyll (*A*), carotenoid (*B*), and anthocyanin content (*C*) of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 4 pooled foliar samples, *Duncan*'s test). Results of a two-way *ANOVA* are shown in *the inset*, where ** stands for $P \leq 0.01$.

Higher relative increases of CAT activity were observed in the purple-leafed cultivars (ZY and ZZ) compared to those in the green-leafed cultivars (JG and MG) (Fig. 3*C*). The highest APX was observed in ZZ, followed by MG and ZY, and the lowest was in JG in CF air (Fig. 3*D*). Under elevated O_3, the highest APX was observed in JG, followed by ZY and MG, and the lowest was found in ZZ. The highest relative increase of APX activity was found in JG, followed by MG and ZY, while the APX activity of ZZ was not significantly different between treatments.

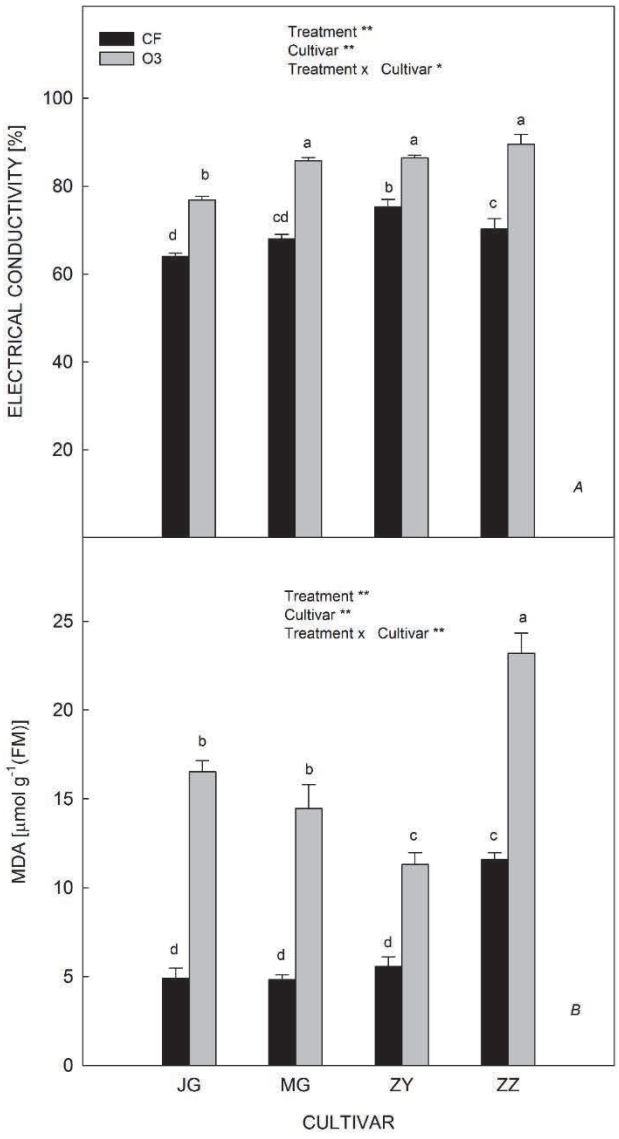

Fig. 2. Electrical conductivity (*A*) and malondialdehyde (MDA) content (*B*) of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 4 pooled foliar samples, *Duncan*'s test). Results of a two-way *ANOVA* are shown in *the inset*, where ** stands for $P \leq 0.01$ and * for $P \leq 0.05$.

Gas exchange: Ozone fumigation significantly decreased P_N, E, and g_s, and increased C_i; however, there were

Table 2. Correlation among leaf pigment contents, relative changes in membrane integrity parameters, antioxidative enzyme activities, chlorophyll *a* fluorescence parameters and gas exchange parameters in four cultivars of pakchoi exposed to elevated O_3. Relative changes are expressed as percent differences between O_3-exposed (O_3) plants and charcoal-filtered air (CF) plants, (CF-O_3)/CF, before *Pearson*'s correlation test. Asterisks denote significant correlation, * <0.05, ** <0.01. VJ, ChlR, CarR and AntR stand for visible injury, relative change of chlorophyll, carotenoid and anthocyanin, respectively; ChlB, CarB and AntB stand for chlorophyll, carotenoid and anthocyanin content in CF, respectively; ChlA, CarA, AntA stand for chlorophyll, carotenoid, and anthocyanin content after O_3 treatment, respectively; CarC, AntC stand for the differences of carotenoid and anthocyanin between CF and O_3 treatment (CF-O_3), respectively. MDA and CAT – relative change of malondialdehyde and catalase, respectively; F_v/F_m, Y_{II}, Y_{NO}, Y_{NPQ}, NPQ, q_N, q_P, q_L, and ETR – relative change of maximal efficiency of PSII photochemistry in the dark-adapted state, the effective quantum yield of PSII photochemistry, quantum yield of nonregulated heat dissipation and fluorescence emission, quantum yield of light-induced nonphotochemical fluorescence quenching, nonphotochemical quenching, coefficient of nonphotochemical fluorescence quenching, coefficient of photochemical fluorescence quenching based on puddle model, coefficient of photochemical fluorescence quenching based on lake model, and electron transport rate, respectively; P_N, g_s and E – relative change of net photosynthetic rate, stomatal conductance and transpiration rate, respectively.

	VJ	ChlB	ChlA	CarR	CarA	CarC	AntB	AntA	AntC	MDA	CAT	F_v/F_m	Y_{II}	Y_{NO}	Y_{NPQ}	NPQ	q_N	q_P	q_L	ETR	P_N	g_s	E
VJ	1.00																						
ChlB	0.99**	1.00																					
ChlA	-0.06	-0.11	1.00																				
CarR	0.43	0.43	-0.86	1.00																			
CarA	0.03	-0.01	0.99*	-0.86	1.00																		
CarC	0.54	0.53	-0.77	0.99*	-0.77	1.00																	
AntB	-0.83	-0.82	0.52	-0.85	0.48	-0.92	1.00																
AntA	-0.99**	-0.99**	0.17	-0.81	0.08	-0.62	0.88	1.00															
AntC	0.74	0.75	0.47	-0.27	0.59	-0.15	-0.25	-0.68	1.00														
MDA	-0.97*	-0.94	-0.16	-0.26	-0.23	-0.40	0.73	0.94	-0.79	1.00													
CAT	0.78	0.77	-0.58	0.89	-0.55	0.95	-1.00**	-0.84	0.16	-0.67	1.00												
F_v/F_m	0.95*	0.98*	-0.25	0.49	-0.14	0.57	-0.82	-0.97*	0.70	-0.86	0.77	1.00											
Y_{II}	0.93	0.96*	-0.03	0.25	0.10	0.34	-0.66	-0.92	0.86	-0.88	0.60	0.97*	1.00										
Y_{NO}	-0.96*	-0.98*	-0.06	-0.23	-0.17	-0.34	0.68	0.94	-0.88	0.94	-0.61	-0.95*	-0.99*	1.00									
Y_{NPQ}	0.98*	0.95	0.08	0.35	0.15	0.48	-0.79	-0.96*	0.73	-0.99**	0.74	0.87	0.86	-0.92	1.00								
NPQ	0.99*	0.98	0.08	0.29	0.17	0.41	-0.74	-0.97*	0.82	-0.99*	0.68	0.93	0.94	-0.98*	0.98*	1.00							
q_N	0.96*	0.94	0.22	0.17	0.31	0.31	-0.66	-0.92	0.86	-0.99**	0.59	0.86	0.91	-0.96*	0.98*	0.99*	1.00						
q_P	-0.08	-0.09	0.90	-0.94	0.95	-0.88	0.62	0.19	0.59	-0.08	-0.69	-0.16	0.10	-0.13	-0.02	0.06	0.18	1.00					
q_L	0.10	0.07	0.95*	-0.85	0.99*	-0.76	0.45	0.01	0.68	-0.28	-0.52	-0.04	0.20	-0.27	0.19	0.24	0.36	0.97*	1.00				
ETR	0.93	0.97*	-0.08	0.29	0.05	0.38	-0.68	-0.93	0.84	-0.87	0.62	0.98*	1.00**	-0.98*	0.85	0.94	0.89	0.05	0.16	1.00			
P_N	0.66	0.65	0.63	-0.40	0.73	-0.28	-0.13	-0.58	0.98*	-0.76	0.04	0.57	0.75	-0.80	0.69	0.76	0.82	0.69	0.80	0.72	1.00		
g_s	0.85	0.85	0.40	-0.11	0.51	0.02	-0.42	-0.79	0.98*	-0.90	0.33	0.78	0.90	-0.94	0.86	0.92	0.95	0.45	0.59	0.88	0.96*	1.00	
E	0.97*	0.97*	0.08	0.22	0.19	0.33	-0.67	-0.94	0.88	-0.95	0.60	0.94	0.98*	-1.00**	0.93	0.98*	0.97*	0.14	0.28	0.98*	0.80	0.94	1.00

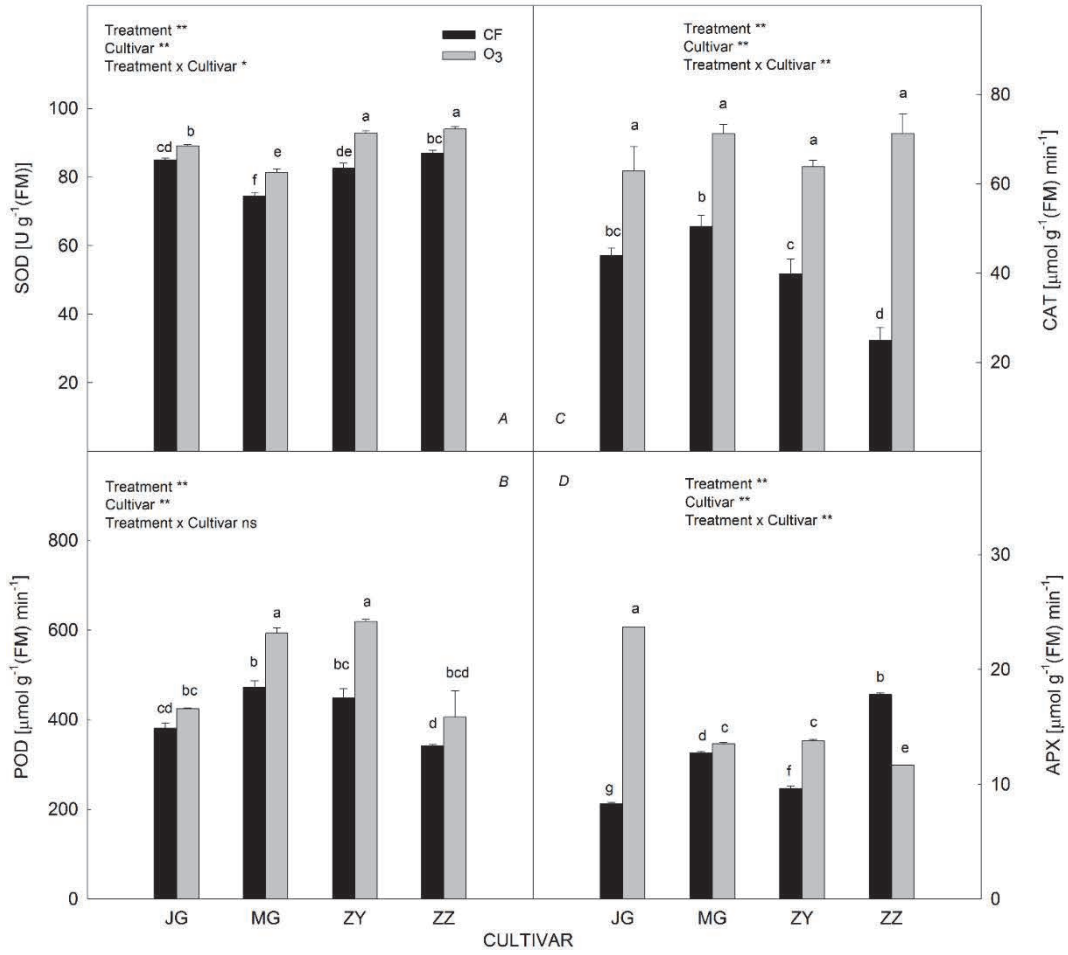

Fig. 3. Superoxide dismutase (SOD, *A*), peroxidase (POD, *B*), catalase (CAT, *C*), and ascorbate peroxidase (APX, *D*) activities of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 4 pooled foliar samples, *Duncan*'s test). Results of a two-way *ANOVA* are shown in the inset, where ** stands for $P \leq 0.01$, * for $P \leq 0.05$, and ns for $P > 0.05$.

significant interactions between treatments and cultivars (Fig. 4). The highest P_N were observed in JG and MG, followed by ZZ, and the lowest was in ZY in CF air, while ZY had the highest P_N under elevated O_3. Under O_3 fumigation, P_N, E, and g_s of all cultivars decreased notably compared with CF (Fig. 4). Higher relative reduction in P_N, g_s, and E were observed in the green-leafed cultivars (JG and MG) than those in the purple-leafed cultivars (ZY and ZZ) (Fig. 4).

Chl *a* fluorescence: Ozone exposure significantly decreased F_v/F_m, ETR, NPQ, q_N, Y_{II}, Y_{NPQ}, Y_{NO}, but the effects on q_P, and q_L was not significant (Figs. 5,6), although there were significant interactions between treatments and cultivars (Figs. 5,6). The highest F_v/F_m was observed in JG and MG, followed by ZZ, and the lowest was in ZY in CF air. F_v/F_m of all cultivars decreased notably under O_3 fumigation compared with CF (Fig. 5*A*). Higher relative loss in F_v/F_m were observed in the green-leafed cultivars (JG and MG) than that in the purple-leafed cultivars (ZY and ZZ) (Fig. 5*A*). Elevated O_3 significantly decreased the ETR of the green-leafed cultivars, while did not influence that of the purple-leafed cultivars (Fig. 5*B*). Under O_3 fumigation, Y_{II} of the green-leafed cultivars (JG and MG) was lowered significantly, however, that of the purple-leaf cultivars was kept at a stable level (Fig. 5*C*). All cultivars except JG showed no significant difference in Y_{NPQ} values both in CF air and O_3 (Fig. 5*D*). We noted that Y_{NO} significantly increased under O_3 exposure in JG, MG, and ZZ, but remained the same in ZY (Fig. 5*E*). The NPQ of all cultivars was significantly depressed by O_3 (Fig. 6*A*). Both JG and MG exhibited lower q_N under O_3 exposure compared with CF, however, the q_N of both ZZ and ZY was not significantly different (Fig. 6*B*). Ozone pollution did not change the q_P or q_L in all cultivars (Fig. 6*C, D*).

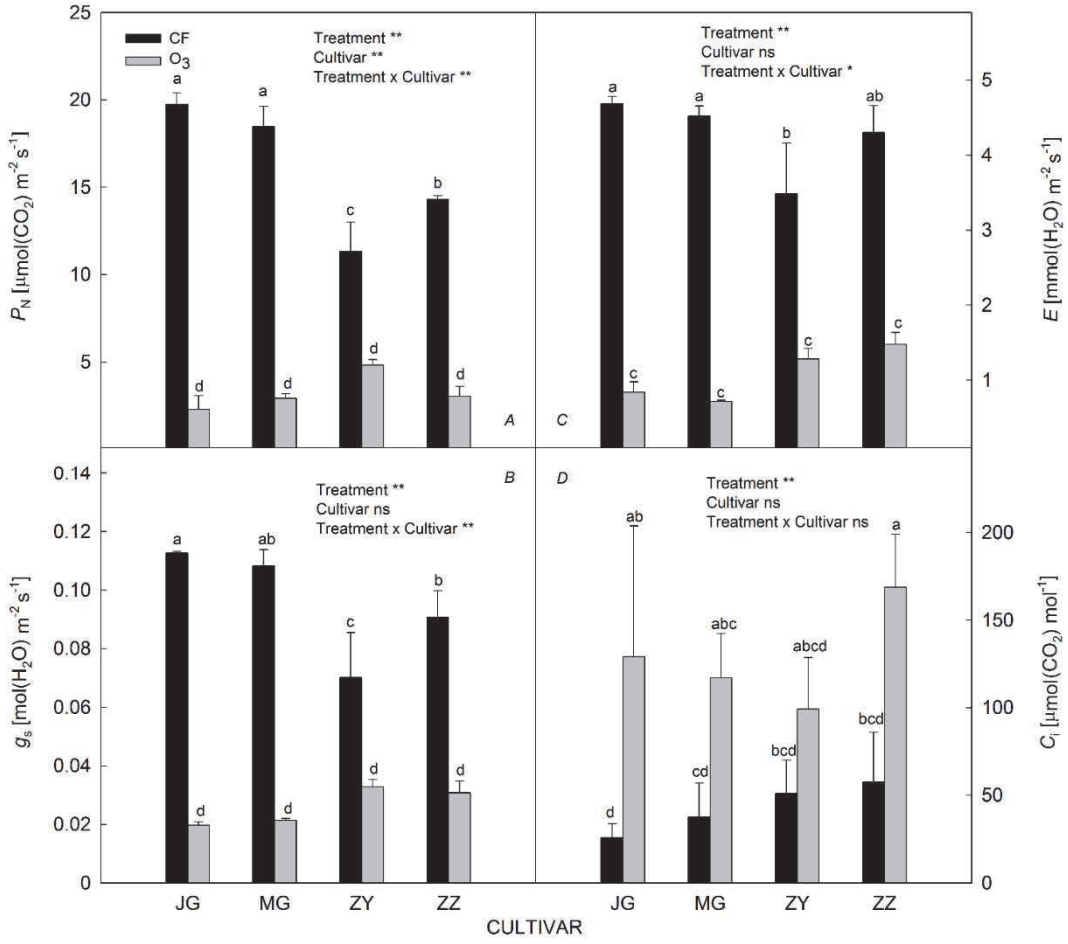

Fig. 4. Light-saturated net photosynthesis (P_N, *A*), stomatal conductance (g_s, *B*), transpiration rate (*E*, *C*), and intercellular CO_2 concentration (C_i, *D*) of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 6 plants, *Duncan*'s test). Results of a two-way *ANOVA* are shown in the inset, where ** stands for $P \leq 0.01$, * for $P \leq 0.05$,and ns for $P > 0.05$.

Correlations among gas-exchange parameters, Chl *a* fluorescence parameters, and antioxidative enzyme activities affected by O_3: After O_3 fumigation, visible leaf injury of all four cultivars positively correlated with the total leaf Chl contents before O_3 exposure, however, negatively with Ant contents after O_3 treatment. Visible leaf injury of the cultivars positively correlated with relative losses in F_v/F_m, Y_{NPQ}, NPQ, q_N, and *E*, and the relative increase in Y_{NO}. Total leaf Chl contents of four cultivars in CF air positively correlated with relative reductions in F_v/F_m, Y_{II}, ETR, and *E*, and with O_3-induced increases in Y_{NO} (Table 2). Total leaf Chl contents of four cultivars in CF air negatively correlated with leaf Ant contents after O_3 fumigation. Under O_3 exposure, total leaf Chl contents of four cultivars positively correlated with their Car contents. Leaf Ant contents before O_3 fumigation positively correlated with relative increase of CAT activity under O_3 exposure compared with CF. Leaf Ant contents under O_3 exposure adversely correlated with relative decrease of F_v/F_m, relative increase of Y_{NPQ}, and relative elevation of NPQ. Increase of leaf Ant contents from CF air to O_3 exposure adversely correlated with relative losses in P_N and g_s. Relative change in MDA exhibited significant negative relationships with Y_{NPQ} and q_N. Under O_3 fumigation, relative decrease in P_N had a significant positive correlation with relative loss of g_s. Relative decline in F_v/F_m had a significant positive correlation with those in Y_{II} and ETR, and with O_3-induced increase in Y_{NO}. The decrease of Y_{II} also positively correlated with O_3-induced increase in Y_{NO} (Table 2). Moreover, relative loss in Y_{II} correlated with ETR positively. O_3-induced increase in Y_{NO} positively correlated with relative reductions in NPQ, q_N, and ETR. Relative decrease of Y_{NPQ} exhibited a significant positive relationship with that of NPQ and q_N.

Discussion

Leaf appearance is highly related to the quality and market value of leafy vegetables. When O_3 concentration increases in the air, visible leaf injuries often appear (Feng *et al*. 2014, Kefauver *et al.* 2014). Visible symptom

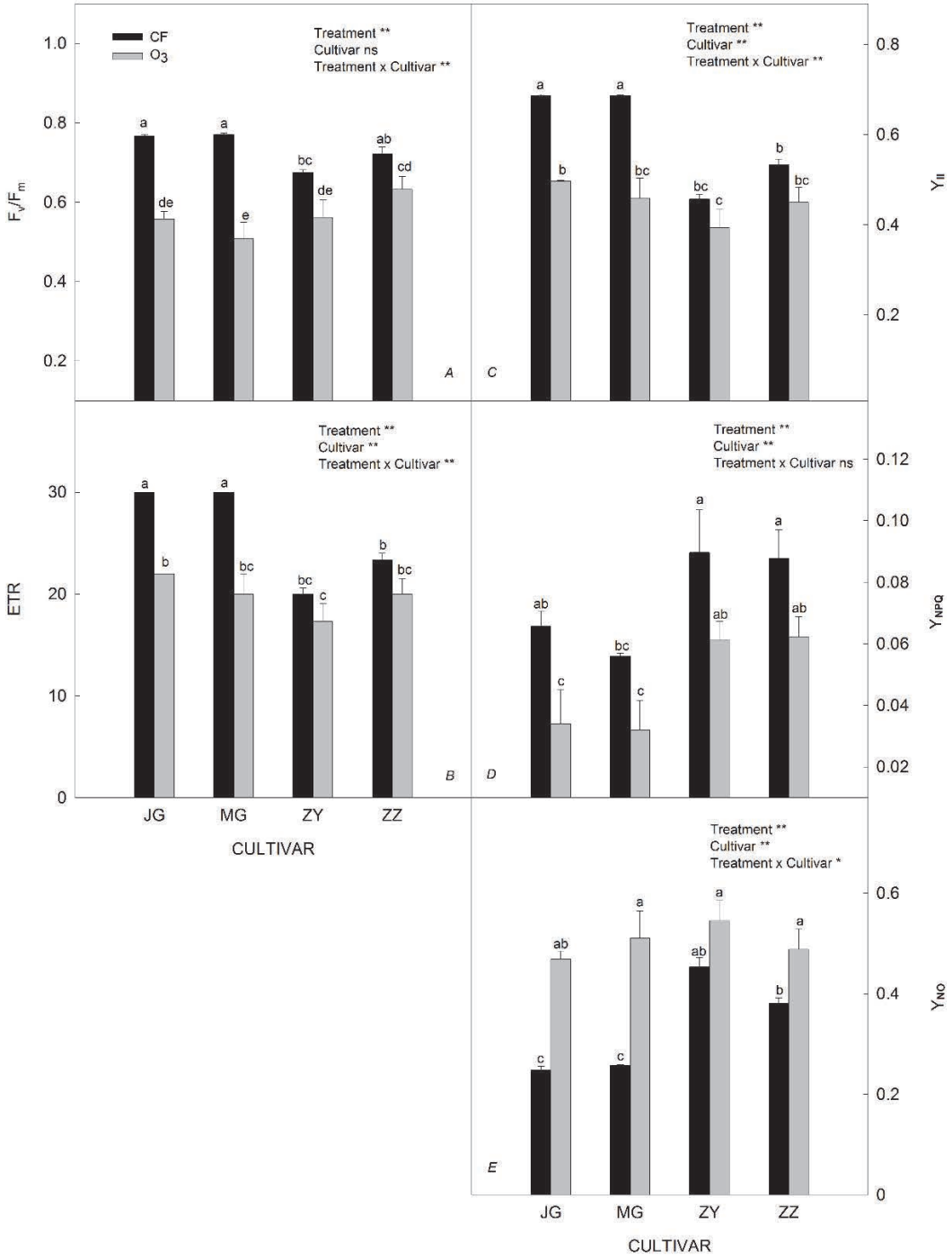

Fig. 5. The maximal photochemical quantum yield of photosystem II (F_v/F_m, *A*), electron transport rate (ETR, *B*), effective quantum yield of photosystem II [Y_{II}, *C*], quantum yield of light-induced nonphotochemical fluorescence quenching [Y_{NPQ}, *D*], and quantum yield of nonregulated heat dissipation and fluorescence emission [Y_{NO}, *E*] of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 6 plants, *Duncan*'s test). Results of a two-way *ANOVA* are shown in *the inset*, where ** stands for $P \leq 0.01$, * for $P \leq 0.05$, and ns for $P > 0.05$.

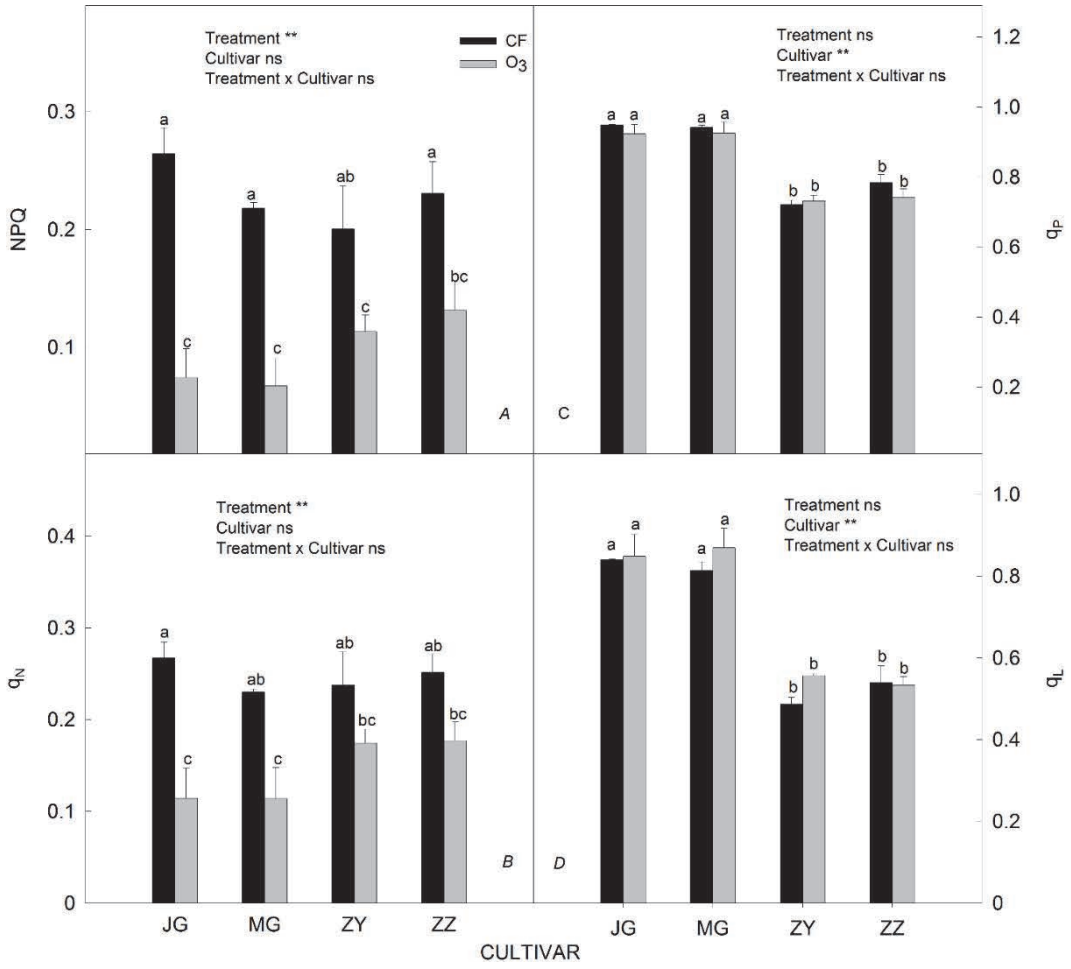

Fig. 6. The nonphotochemical quenching (NPQ, *A*), coefficient of nonphotochemical fluorescence quenching (q_N, *B*), coefficient of photochemical fluorescence quenching based on puddle model (q_P, *C*), and coefficient of photochemical fluorescence quenching based on lake model (q_L, *D*) of four cultivars ('Jingguan', JG; 'Meiguan', MG; 'Ziyu', ZY; 'Zizuan', ZZ) under charcoal-filtered air (CF) and acute O_3 fumigation (O_3) after 7-h exposure. Data are means + SE. *Different letters* show significant differences among bars (n = 6 plants, *Duncan*'s test). Results of a two-way *ANOVA* are shown in *the inset*, where ** stands for $P \leq 0.01$ and ns for $P > 0.05$.

is an important index for O_3 sensitivity. Ozone caused more visible damage in the green-leafed cultivars than in purple-leafed cultivars which indicated that green-leafed cultivars were more sensitive. This result is in accordance with the findings of Calatayud and Barreno (2004), in which lettuce variety 'Valladolid' with green leaves was more susceptible to O_3 damage than the variety 'Morella' with red leaves. For a single plant, visible symptoms emerged first in the oldest leaves, while the youngest leaves exhibited little sign of injury. It is consistent with other findings (Bender *et al.* 1994, Thwe *et al*. 2013).

Percentage of visible injury in the fully expanded functional leaf (leaf 4) showed a significant positive correlation with the total leaf Chl concentration in CF air, but a negative correlation with the Ant content after O_3 exposure. These results demonstrated that cultivars with high Chl contents before O_3 treatment could be harmed seriously by O_3. In contrast, the cultivars with higher Ant concentration under O_3 fumigation had higher tolerance to elevated O_3.

Membrane integrity is an important aspect which represents the degree of injury of cell membrane. Ozone treatment significantly increased EC and MDA contents, which demonstrated that the cell membrane was oxidized by the O_3 pollution and ion leakage occurred. Percentage of visible injury significantly correlated with the relative increase of the MDA content which indicated that the less visible symptoms in the purple-leafed cultivars could be partly attributed to the lesser increase in MDA content.

In this experiment, O_3 activated the antioxidative defense system in leaves of all cultivars. Activities of SOD, POD, CAT, and APX increased significantly under the O_3 treatment. This result showed that these enzymes could be triggered to take part in cleaning up the ROS produced by the elevated O_3. Although research was often focused on changes of antioxidant enzymes in response to O_3 pollution, results obtained were often confusing (Li *et al*. 2011, Inada *et al.* 2012).

SOD is crucial for scavenging $O_2^{\cdot-}$ radicals in various cell compartments (Takahashi and Asada, 1983). When exposed to O_3, the relative increase of SOD activity in ZY was the highest, while that in JG was the lowest, suggesting that SOD might play a more important role in ZY for its O_3 resistance by detoxifying free radicals. The degrading of $O_2^{\cdot-}$ results in production of H_2O_2 which can be scavenged by POD, CAT, or APX (Li *et al*. 2009). Ozone fumigation enhanced the POD activities in Pakchoi, which meant that POD probably played an important role in scavenging H_2O_2. The O_3 treatment induced the increase of CAT in all cultivars, which is similar to previous observations (Li *et al*. 2011). The elevation of CAT activities in the green-leafed cultivars were lower than those in the purple-leafed cultivars, which suggested that CAT played more important role in the purple-leafed cultivars and helped increase their ability to tolerate O_3-induced stress. The activity of APX, which is regulated by H_2O_2, was elevated in all cultivars except ZZ after O_3 fumigation. It was in contrary to a report referring that APX activity decreased in nonheading Chinese cabbage under copper stress (Li *et al*. 2009).

Leaf gas exchange is a complex process related to plant growth and biomass accumulation. It has been found that photosynthesis is very sensitive to high concentrations of O_3 (Xu *et al*. 2009, Zhang *et al*. 2010). In the present study, elevated O_3 induced significant reductions in P_N of all four cultivars, but C_i was not significantly affected, suggesting that the inhibition of P_N was caused by limitations of mesophyll processes rather than stomatal limitation (Weber *et al.* 1993). These findings are in agreement with other reports (Biswas *et al.* 2008, Zhang *et al*. 2010). P_N is an important characteristic related to the yield of crops or vegetables. The loss of P_N significantly differed between cultivars. The relative decrease of P_N showed a significant negative correlation with the difference in the Ant content between CF air and O_3 treatment. During the fumigation period, the relative lower reduction in P_N occurred probably due to a greater increase in the Ant content in the purple-leafed cultivars than that in the green-leafed cultivars. Relative change of P_N between CF air and elevated O_3 exhibited a positive relationship with that of g_s. The change of g_s might be the result of inhibited P_N due to the nonstomatal limitation (Zhang *et al*. 2010). There was no significant relationship between the relative loss of P_N and visible injury. Many researchers have found that leaf injury symptoms do not always correlate with the decrease of growth and the loss of biomass or yield (Pleijel and Danielsson 1997, Booker *et al.* 2009). In the present study, the weak relationship between visible injury and loss of P_N might indirectly confirm those conclusions mentioned above. Meanwhile the declines in NPQ and q_N in the green-leafed cultivars were much deeper which meant the heat dissipation of excessive energy decreased, and possible photodamage increased.

Under O_3 fumigation, F_v/F_m of nonheading Chinese cabbage significantly decreased, which showed that photoinhibition occurred. The relative lowering of F_v/F_m was positively correlated with visible injury and with the total Chl content in CF air which meant that the green-leafed cultivars experienced stronger photoinhibition under O_3 pollution. After O_3 exposure, the relative decline of F_v/F_m was negatively correlated with the Ant content, which indicated that the biosynthesis of Ant in leaves could prevent photoinhibition in photosynthetic organs. This result was similar to other studies on plant responses to environmental stress, *e.g*., high light (Gould *et al*. 2002, Gould 2004). Significant decrease of Y_{II} was noted under O_3 fumigation. The Y_{II} decline in the purple-leafed cultivars was lower than that in the green-leafed cultivars. It was found that Y_{NO} was significantly elevated under O_3 exposure. The relative increase of Y_{NO} had a positive relationship with visible leaf injury and the total Chl content in CF air. This result demonstrated that the green-leafed cultivars with high Chl experienced the photoinhibition and the severe inhibition led to the photodamage. Regulated thermal energy dissipation involving ΔpH- and zeaxanthin-dependent photoprotective mechanisms was indicated by the Y_d, NPQ, and q_N. Under elevated O_3, Y_{NPQ}, NPQ, and q_N decreased significantly. The relative reductions in Y_{NPQ}, NPQ, and q_N were lesser in the purple-leafed cultivars than that in the in green-leafed cultivars, which indicated that the purple-leafed cultivars could keep relatively high thermal energy dissipation.

In conclusion, acute treatment with high O_3 concentration decreased the Chl and Car contents, increased the Ant content, damaged the cell membrane integrity, enhanced the antioxidative enzyme activities, such as SOD, POD, CAT, and APX, depressed the P_N, g_s, and E, inhibited the F_v/F_m, Y_{II}, ETR, Y_{NPQ}, NPQ, and q_N, elevated the Y_{NO}, and caused visible injury. The purple-leafed cultivars with the high Ant content displayed a greater tolerance to O_3 pollution than the green-leafed cultivars thanks to the lower relative rise in the MDA content and Y_{NO}, higher CAT activity, lower reductions in P_N, g_s, E, F_v/F_m, Y_{II}, ETR, NPQ, and q_N. The ability to produce greater amounts of Ant in the purple-leafed cultivars probably contributed to their greater photoprotective and antioxidant abilities. In ZZ, antocyanins could react as an antioxidant, together with CAT, and, in addition, SOD, POD, and CAT could further remove the reactive oxygen species, together with Ant, in ZY. Ozone tolerance is controlled by expressions of many genes, such as oxidative stress-responsive genes (Whaley *et al.* 2015). Further investigation is needed to clarify the role of anthocyanins in ozone resistence.

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