Differential effects of ozone on photosynthesis of winter wheat among cultivars depend on antioxidative enzymes rather than stomatal conductance

Zhaozhong Feng^{a,b,*}, Liang Wang^a, Håkan Pleijel^c, Jianguo Zhu^d, Kazuhiko Kobayashi^b

^a State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Shuangqing Road 18, Haidian District, Beijing 100085, China

^b Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

^c Department of Biological and Environmental Sciences, University of Gothenburg, P.O. Box 461, 40530 Gothenburg, Sweden

^d State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Sciences, Chinese Academy of Sciences, Nanjing 210008, China

HIGHLIGHTS

- 5 modern cultivars of wheat were investigated under fully open-air field conditions.
- Significant O₃ effects were only found during the mid-grain filling stage.
- The lower photosynthetic rates were mainly due to nonstomatal factors.
- Antioxidative enzymes contributed to the differential response to E-O₃ among cultivars.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:

Received 10 May 2016

Received in revised form 11 August 2016

Accepted 12 August 2016

Available online 17 August 2016

Editor: Elena PAOLETTI

Keywords:

Air pollution

Antioxidant system

Gas exchange

Ozone

Photosynthesis

Wheat

ABSTRACT

Five modern cultivars of winter wheat (*Triticum aestivum* L.): Yangmai16 (Y16), Yangmai 15 (Y15), Yangfumai 2 (Y2), Yannong 19 (Y19) and Jiaxing 002 (J2) were investigated to determine the impacts of elevated ozone concentration (E-O₃) on photosynthesis-related parameters and the antioxidant system under fully open-air field conditions in China. The plants were exposed to E-O₃ at 1.5 times the ambient ozone concentration (A-O₃) from the initiation of tillering to final harvest. Pigments, gas exchange rates, chlorophyll *a* fluorescence, antioxidants contents, antioxidative enzyme activity and lipid oxidation were measured in three replicated plots throughout flag leaf development. Results showed that significant O₃ effects on most variables were only found during the mid-grain filling stage. Across five cultivars, E-O₃ significantly accelerated leaf senescence, as indicated by increased lipid oxidation as well as faster declines in pigment amounts and photosynthetic rates. The lower photosynthetic rates were mainly due to non-stomatal factors, e.g. lower maximum carboxylation capacity and electron transport rates. There were strong interactions between O₃ and cultivar in photosynthetic pigments, light-saturated photosynthesis rate and chlorophyll *a* fluorescence with O₃-sensitive (Y19, Y2 and Y15) and O₃-tolerant (J2, Y16) cultivars being clearly differentiated in their responses to E-O₃. E-O₃ significantly influenced the antioxidative enzymes but not antioxidant contents. Significant interactions between O₃ and

* Corresponding author at: Shuangqing Road 18, Haidian District, Beijing 100085, China.

E-mail address: fzz@rcees.ac.cn (Z. Feng).

cultivar were found in antioxidative enzymes, such as SOD and CAT, but not in stomatal conductance (g_s). Therefore, it can be concluded that antioxidative enzymes rather than g_s or antioxidants are responsible for the differential responses to E- O_3 among cultivars. These findings provide important information for the development of accurate modeling O_3 effects on crops, especially with respect to the developmental stage when O_3 damage to photosynthesis becomes manifest.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Ground-level ozone (O_3) is the most important phytotoxic air pollutant at global scale with adverse impacts on terrestrial vegetation (Ashmore, 2005; Booker et al., 2009; Bytnerowicz et al., 2007; Feng and Kobayashi, 2009; Feng et al., 2014, 2015; Van Dingenen et al., 2009). A meta-analysis based on numerous chamber studies revealed that elevated O_3 concentration (average 73 ppb) reduced leaf photosynthesis and grain yield of wheat by 20% and 29%, respectively, as compared with plants grown in carbon-filtered air (Feng et al., 2008). The grain filling period is the most sensitive developmental period to O_3 , during which the decreased photosynthesis induced by elevated O_3 concentration appears to be the key driver for the yield loss (Gelang et al., 2000; Pleijel et al., 1998). Although the photosynthetic rate in saturating light (A_{sat}) is often co-limited by stomatal and biochemical factors under field conditions, many scientists argued that the central biochemical process rather than the stomatal limitation (L_s) of A_{sat} was the main cause for the O_3 -induced reduction in photosynthesis (Farage and Long, 1999; Feng et al., 2011; Fiscus et al., 1997; Morgan et al., 2004; Zheng et al., 2002). Among the non-stomatal factors, maximum carboxylation (V_{cmax}), reflecting in vivo Rubisco activity, as well as the maximum rate of RuBP regeneration (J_{max}) have been reported to play the most important role (Farage and Long, 1999; Fiscus et al., 2005; Morgan et al., 2004; Sun et al., 2014). Other important physiological responses reported to be affected by O_3 include the changes in chlorophyll *a* fluorescence variables such as reduction in quenching of photochemical efficiency (qP) and quantum yield of PSII in the light (PhiPS2) (Feng et al., 2011), as well as endogenous antioxidant metabolism (Inada et al., 2012).

Many studies have been done to clarify the mechanism of plant defense to O_3 damage in relation to detoxification of reactive oxygen species (ROS) (Dizengremel et al., 2008; Paoletti et al., 2008), and reported that the responses of antioxidant metabolism varied by species/cultivars, depending on the concentrations of O_3 and the duration of the fumigation, e.g. acute vs. chronic ozone exposure (Baier et al., 2005; Betzelberger et al., 2010; Burke et al., 2003; Feng et al., 2011; Kollist et al., 2000; Wang et al., 2014a, 2014b). In general, acute exposure to relatively high O_3 concentrations often caused leaf lesions resulting from programmed cell death associated with plant-pathogen

interaction (Kangasjarvi et al., 2005; Overmyer et al., 2003; Rao et al., 2000) and induces an increase in antioxidant metabolism (Conklin and Barth, 2004; Kangasjarvi et al., 1994). The chronic exposure to relatively low O_3 concentrations always induces declined photosynthesis along with accelerated leaf senescence and consequently reductions in productivity and yield (Ashmore, 2005; Burkart et al., 2013; Feng and Kobayashi, 2009; Feng et al., 2008; Feng et al., 2011; Zhu et al., 2011). Recent evidence showed an increased total antioxidant capacity of soybean plants when chronically exposed to the elevated O_3 concentration (Gillespie et al., 2011, 2012). These authors also suggested that the antioxidant metabolism was primed by previous exposure to oxidative stress, indicating the importance of the endogenous antioxidant metabolism in detoxifying ROS. However, the results from soybean plants contrast with earlier studies conducted in wheat in O_3 -FACE platform with two cultivars, which generally showed a declining trend of antioxidant pools and key enzymes in the apoplast and leaf tissues under O_3 stress (Feng et al., 2010; Wang et al., 2014a, 2014b).

In the present study, therefore, we examined the photosynthetic potential and antioxidant metabolism in the leaf tissues among five wheat cultivars in order to determine whether there are different responses to O_3 among cultivars, and if so, which factors contribute to the differences. To this aim, we re-examined part of the data set presented in Feng et al. (2011) and compared them with new data obtained under the same experimental conditions.

2. Materials and methods

2.1. Experiment site

The fully open-air O_3 fumigation system (O_3 -FACE) is located in a paddy field in Xiaoji Town, Jiangsu Province, China (119° 42' E, 32° 35' N) with continuous rice/wheat or rice/rapeseed rotation for > 1000 years. The soil is Shajiang Aquic Cambisols with a sandy-loamy texture. The site belongs to the subtropical marine climatic zone with mean annual precipitation and mean annual temperature being 1100–1200 mm and 16 °C, respectively. Total annual sunshine hour is > 2000 h and frost-free period > 230 days. During the growth period from 1st March (around turning green stage) to final harvest in this study, mean daily maximum and minimum temperature were 20.6 and 10.1 °C, respectively. Mean daily maximum photosynthetic photon flux density (PPFD) was 1376 $\text{mmol m}^{-2} \text{s}^{-1}$ and accumulated precipitation was 171 mm.

Table 1

ANOVA results for the photosynthesis related parameters measured across the measurements at heading, flowering, early-grain filling, and mid-grain filling stages.

Variables	O_3	Cultivar (C)	Date (D)	$O_3 \times C$	$O_3 \times D$	$O_3 \times C \times D$
A_{sat}	0.293	0.017	<0.001	0.546	<0.001	0.001
Ci	0.720	0.041	<0.001	0.369	<0.001	<0.001
g_s	0.395	0.061	<0.001	0.383	0.001	0.171
V_{cmax}	0.986	0.001	<0.001	0.601	<0.001	<0.001
J_{max}	0.478	<0.001	<0.001	0.661	<0.001	0.003
L_s	0.609	0.005	<0.001	0.089	<0.001	0.041
V_{cmax}/J_{max}	0.616	0.012	0.001	0.456	<0.001	<0.001
Fv'/Fm'	0.128	0.054	<0.001	0.916	<0.001	0.001
PhiPS2	0.260	0.003	<0.001	0.226	<0.001	0.003
PhiCO ₂	0.129	0.901	<0.001	0.624	<0.001	<0.001
qP	0.651	0.002	<0.001	0.144	<0.001	0.003
Chl	0.695	0.154	<0.001	0.803	<0.001	<0.001
Car	0.852	0.447	<0.001	0.993	<0.001	0.007

Table 2

ANOVA results for the antioxidant-related parameters across the measurements at flowering and mid-grain filling stages.

Variables	O_3	Cultivar (C)	Date (D)	$O_3 \times C$	$O_3 \times D$	$O_3 \times C \times D$
APX	0.351	0.010	0.001	0.335	0.035	0.319
POD	0.471	<0.001	0.001	0.891	0.839	0.497
CAT	0.917	0.177	<0.001	0.471	0.005	<0.001
SOD	0.978	<0.001	<0.001	0.666	0.084	0.049
GSH	0.981	0.013	0.006	0.261	0.697	0.248
Phenolics	0.684	0.002	<0.001	0.379	0.375	0.032
MDA	0.537	0.552	<0.001	0.931	<0.001	0.038
AsA	0.100	<0.001	<0.001	0.537	<0.001	0.430

2.2. Plant material

Five modern cultivars i.e. Yannong 19 (Y19), Yangmai 16 (Y16), Yangmai 15 (Y15) and Yangfumi 2 (Y2), and Jiaying 002 (J2) were selected for the comparison in the 2007/2008 growing season. The cultivation followed the standard local practices (Zhu et al., 2011). All wheat seeds were sown on 13 November 2007. The N fertilizer was applied as urea and diammonium phosphate with the total amount being 210 kg N ha⁻¹, of which 60% was applied at planting, 10% at early tillering and 30% at elongation stage. The P and K fertilizers were applied at the total amount of 90 kg P₂O₅ ha⁻¹ and 90 kg K₂O ha⁻¹, respectively, of which 60% was applied at planting and 40% at elongation stage. No irrigation was applied during the experiment due to subtropical climate with sufficient rainfall.

2.3. Fumigation treatment

Two O₃ treatments were ambient (A-O₃) and elevated (E-O₃) O₃ concentration ([O₃]), and the targeted concentration in E-O₃ was 1.5 times ambient [O₃]. Wheat plants were grown in three replicate rings (240 m²) for each treatment. O₃ addition started on 5 March 2008, when wheat plants were at tillering stage, and continued until the grain maturity. The daily fumigation period was from 9:00 h to sunset, when ambient [O₃] was below 20 ppb. More details of the design and the performance of the O₃-FACE are available elsewhere (Feng et al., 2011; Tang et al., 2011). During the study, M7 (9:00–16:00) in A-O₃ averaged 52 ppb with a maximum of 110 ppb. The observed maximum ambient 1 h mean [O₃] was 140 ppb in one afternoon, suggesting a serious O₃ pollution in this region of China (Feng et al., 2011).

2.4. Plant sampling

Plant samples for biochemical assay of antioxidant system were taken twice during flowering and mid-grain filling stages. For each ring, six plants of each cultivar were randomly selected and divided into three groups on each sampling occasion. Fully expanded flag leaves of each plant were punched quickly and then immersed in liquid N₂. The leaf samples were stored at -80 °C until further biochemical analysis. The measurements of gas exchange and chlorophyll *a* fluorescence were carried out at four developmental stages: heading, flowering, early- and mid- grain fillings.

2.5. Biochemical assay of antioxidant system in leaf tissues

Frozen leaf tissues were ground into powder in liquid N₂ with a mortar and a pestle for the biochemical assay. Ascorbic acid (AsA) contents were measured by monitoring the changes of absorbance at 265 nm (an extinction coefficient of 14 mM⁻¹ cm⁻¹) after addition of ascorbate oxidase (Luwe and Heber, 1995). Glutathione (GSH) content was measured with a 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB)-glutathione disulfide reductase recycling assay following the method of Griffith (1980). The content of total phenols was determined by a Folin-Ciocalteu assay according to the method of Singleton and Rossi (1965) using gallic acid as a standard. Malondialdehyde (MDA) content was assessed for estimation of lipid peroxidation by 2-thiobarbituric acid-reactive metabolite (TBA) according to the method of Heath and Packer (1968) and calculated by the equation of C_{MDA} (mmol L⁻¹) = 6.45 * (A₅₃₂ - A₆₀₀) - 0.56 * A₄₅₀ in order to rule out the disturbances from nonspecific (A₆₀₀) and sugar (A₄₅₀) absorbance. All measurements were conducted by microplate reader (SPECTRA MAX 190, MD Com. USA).

For antioxidant enzymes: two leaflets were extracted using 2 ml of 50 mM sodium phosphate buffer (pH 7.0, containing 1% vinyl pyrrolidone). The samples were centrifuged at 15,000 r min⁻¹ for 15 min and the supernatant was collected at 4 °C. The activities of antioxidant enzymes of superoxide dismutase (SOD), peroxidases (POD) and

ascorbate peroxidase (APX) were determined following the description by Wang et al. (2014a). The SOD activity was determined by estimating its ability of inhibiting the photochemical reduction of nitro blue tetrazolium (NBT) and the absorbance was read at 560 nm. The amount of enzyme that inhibited the 50% NBT reduction was defined as one unit of SOD activity. The POD activity was measured by monitoring the changes of the absorbance at 470 nm (an extinction coefficient of 26.6 mM⁻¹ cm⁻¹) after the enzyme extract was mixed with a 50 mM phosphate buffer (pH 6.0) containing 1 mM guaiacol and 0.5 mM H₂O₂. The APX activity was assayed by monitoring the change of the absorbance at 290 nm (an extinction coefficient of 2.8 mM⁻¹ cm⁻¹) after the enzyme extract was mixed a 50 mM potassium phosphate buffer (pH 7.0) containing 250 mM ascorbate and 1 mM H₂O₂. The hydrogen peroxidase (CAT) activity was determined based on the description by McKee et al. (1997). The changes of the absorbance at 240 nm (an extinction coefficient of 39.4 mM⁻¹ cm⁻¹) was monitored after the enzyme extract was mixed with a 30 mM KH₂PO₄/K₂HPO₄ (pH 7.0) containing 10 mM H₂O₂. All measurements were conducted by using the spectrophotometric method.

Fig. 1. Light-saturated photosynthesis rate (A_{sat}), stomatal conductance (g_s) and intercellular CO₂ concentration (C_i) of winter wheat in the measurement at the mid-grain filling stage under ambient [O₃] (A-O₃) and elevated [O₃] (E-O₃) (mean ± SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the four measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

2.6. Gas exchange, chlorophyll *a* fluorescence and leaf pigment content

An in vitro measurement of the gas exchange and fluorescence was carried out following the method of a preceding study (Morgan et al., 2004), as described previously (Feng et al., 2011). Three fully expanded flag leaves of each cultivar per ring were randomly selected. The stems attached with flag leaves were cut under water at predawn and immediately transported to laboratory in low light ($\text{PPFD} < 20 \mu\text{mol m}^{-2} \text{s}^{-1}$). The measurement was then initiated after light acclimation at $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ outside the gas exchange chamber. The LI-6400 photosynthesis system mounted with a 6400–40 leaf chamber fluorometer (LICOR, Lincoln, NE, USA) was employed to measure the gas exchange and fluorescence. The net CO_2 assimilation rate vs. intercellular CO_2 concentration (A-Ci) and A vs. photosynthetic photon flux density (A-PPFD) curves were determined in two of the fully expanded flag leaves for each cultivar per subplot. V_{cmax} , J_{max} and L_s were determined according to the method of Farquhar and Sharkey (1982) from the A-Ci curve, as described previously by Long and Bernacchi (2003). The apparent quantum efficiency (AQY) was estimated from the initial slope of A-PPFD curve fitted with a linear function (Morgan et al., 2004) and A_{sat} was from the entire A-PPFD curve fitted with a nonrectangular empirical function (Long and Drake, 1991). The measurement of modulated chlorophyll fluorescence was conducted simultaneously with steady-state (F_s) and maximal (F_m') fluorescence being automatically logged along with the measurement of A-PPFD curve. The actual photochemical efficiency of PSII in the saturated light (F_v'/F_m'), quenching of photochemical efficiency of PSII (qP), and the quantum yield of non-cyclic electron transport (ΦPSII) were provided by the software in the instrument.

After gas exchange and chlorophyll *a* fluorescence measurements, leaf photosynthetic pigment content was determined spectrophotometrically following the protocol of Lichtenthaler (1987) with the extracts in 95% ethanol in the dark at 4°C for 72 h.

2.7. Statistics

The experiment was laid out in a split plot design with the main plot being allocated to the $[\text{O}_3]$ treatment and the subplot being cultivar.

Statistical analysis was conducted based on subplot means for all variables in JMP software (SAS Institute, USA). Analysis of variance (ANOVA) for each variable was performed in a mixed linear model with main plot as a random effect and $[\text{O}_3]$, cultivar and sampling date as fixed effects. Tukey-Kramer HSD test was used for the comparison of variables between A- O_3 and E- O_3 . All the data were presented as mean $\pm$ standard deviation of three rings replicates with significance level set at $P \leq 0.05$.

3. Result

Across all measurements, E- O_3 had no significant effects on all investigated variables (Tables 1 & 2). However, significant interaction between O_3 and measurement dates was found in most variables excluding POD, SOD, GSH and phenolics, indicating that effects of E- O_3 depends on growing stages. Similarly, the responses to E- O_3 among cultivars also depended on growing stages, as indicated by no significant interaction between O_3 and cultivar but significant interaction of O_3 , cultivar and measurement dates for most variables (Tables 1 & 2). Before the last measurement date, no statistically significant difference was detected between wheat plants exposed to A- O_3 and E- O_3 for any of the physiological variables except the total chlorophyll and carotenoids content (data not shown). Therefore, we present only the results from the final measurement, i.e. mid grain filling stage.

Figs. 1–3 show the photosynthetic responses of each cultivar to E- O_3 . Except g_s , the interactions between cultivar and O_3 were statistically significant and most of the photosynthetic parameters showed the varietal differences. Averaged across the five cultivars, A_{sat} was reduced by 46% for plants grown at E- O_3 , and the E- O_3 -induced loss reached statistically significant levels in Y19 (58%), Y2 (78%) and Y15 (62%), but not in J2 (21%) or Y16 (16%) (Fig. 1). In parallel with the decrease in A_{sat} by E- O_3 , g_s showed a similar declining trend in Y19, Y2 and Y15, while Ci and L_s generally increased at E- O_3 for all cultivars (Fig. 1–2). E- O_3 significantly reduced V_{cmax} and J_{max} with a larger decrease in V_{cmax} , especially in Y19, Y2 and Y15 (Fig. 2). The same was the case for parameters related to fluorescence such as F_v'/F_m' , qP , ΦPSII and AQY (Fig. 3), which showed E- O_3 -induced reductions being significant in Y19, Y2 and Y15 only.

Fig. 2. The maximum carboxylation (V_{cmax}), the maximum rate of RuBP regeneration (J_{max}), stomatal limitation (L_s) and the ratio of V_{cmax} and J_{max} ($V_{\text{cmax}}/J_{\text{max}}$) of winter wheat in the measurement at the mid-grain filling stage under ambient $[\text{O}_3]$ (A- O_3) and elevated $[\text{O}_3]$ (E- O_3) (mean $\pm$ SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the four measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

Fig. 3. The actual photochemical efficiency of PSII in the saturated light (F_v/F_m'), quenching of photochemical efficiency of PSII (q_p), and the quantum yield of non-cyclic electron transport (Φ_{PSII}) and apparent quantum efficiency (Φ_{CO_2}) of winter wheat in the measurement at the mid-grain filling stage under ambient $[O_3]$ (A- O_3) and elevated $[O_3]$ (E- O_3) (mean $\pm$ SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the four measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

Leaf senescence was significantly accelerated in Y19, Y2 and Y15 at E- O_3 , as indicated by significant difference compared with at A- O_3 in chlorophyll, carotenoids and MDA contents (Figs. 4–5). E- O_3 did not significantly influence the antioxidant pools including GSH and Phenolics at the last measurement date, but a significant decrease in AsA in Y19 was found (Fig. 5). Among the antioxidant enzymes, APX and POD did

not respond to E- O_3 for any of the five cultivars, whereas CAT (Y19 and Y2) as well as SOD (Y2) showed reductions by E- O_3 (Fig. 6).

4. Discussion

It has been reported that modern wheat cultivars are more sensitive to O_3 than old accessions due to higher g_s and lower levels of antioxidant capacity (Pleijel et al., 2006), and that they are subjected to larger oxidative damages to proteins and cellular membranes (Biswas et al., 2008). In this study, five modern cultivars showed variable reduction of A_{sat} at mid-grain filling stage with a wide range from 16% to 78% when exposed to an elevation of average 8 h- $[O_3]$ (9:00–17:00) from 44.4 ppb to 56.4 ppb (Feng et al., 2011), which suggests the presence of intraspecific variation in the response to elevated $[O_3]$. However, the varietal difference in response to E- O_3 was observed only at the latest developmental stage (mid-grain filling), implying accumulative O_3 effects. It is consistent with previous chamber studies of wheat plants (Feng et al., 2008), as well as with SoyFACE experiments using ten soybean cultivars (Betzberger et al., 2010). At the final measurement stage, O_3 -induced reduction of A_{sat} was significant in Y19, Y2 and Y15 but not in J2 and Y16 (Fig. 1), indicating that J2 and Y16 were the least sensitive cultivar followed by Y15, while Y2 and Y19 were the most sensitive ones to O_3 . This is consistent with the O_3 -induced grain yield losses in the same cultivars: 13.1% in J2 as averaged across the years 2008–2010 (unpublished data), and 15.3% in Y15, 17.7% in Y16, 24.2% in Y2 and 20.1% in Y19 on average across the years 2007–2009 (Zhu et al., 2011).

The sensitivity of plants to O_3 has been linked to O_3 flux controlled by stomata (Dizengremel et al., 2009; Fiscus et al., 2005) and antioxidant capacity determined by antioxidant pools and enzymes (Biswas et al., 2008; Feng et al., 2010; Inada et al., 2012; Li et al., 2016; Wang et al., 2014a, 2014b). However, there was no significant interaction between cultivar and O_3 in g_s across the four measurements (Table 1, Fig.

Fig. 4. The chlorophyll and carotenoid contents of winter wheat in the measurement at the mid-grain filling stage under ambient $[O_3]$ (A- O_3) and elevated $[O_3]$ (E- O_3) (mean $\pm$ SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the four measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

Fig. 5. Ascorbic acid (AsA), Glutathione (GSH), phenolics and lipid peroxidation (MDA) contents of winter wheat in the measurement at the mid-grain filling stage under ambient $[O_3]$ (A- O_3) and elevated $[O_3]$ (E- O_3) (mean $\pm$ SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the two measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

1), suggesting that g_s does not contribute to the significant difference among cultivars in response to E- O_3 . Similar results were also found in the study of Y16 and Y2 sensitivity to O_3 in terms of photosynthesis (Feng et al., 2011). Therefore, O_3 -induced reduction in A_{sat} would be largely attributed to non-stomatal factors, i.e. impaired physiological activity of mesophyll cells (Akhtar et al., 2010; Biswas et al., 2008; Farage and Long, 1999). Further evidence was the increased C_i (Fig. 1) and decreased Rubisco activity as indicated by V_{cmax} (Fig. 2). For instance, the larger losses in V_{cmax} and J_{max} at the final measurement stage for Y19, Y2 and Y15 (Fig. 2) suggested that both the Rubisco activity and RuBP

(1,5-bisphosphate) regeneration contributed to the reduction of A_{sat} . The larger decline in V_{cmax} compared to J_{max} (V_{cmax}/J_{max}) of Y19 underlined the paramount importance of the Rubisco linked photochemical reactions among the non-stomatal factors. This view is supported by previous wheat studies that the greater reduction in A_{sat} in E- O_3 was related to a reduction of Rubisco activity (Farage and Long, 1999; Inada et al., 2012; McKee et al., 1995). The effects of E- O_3 on photoinhibition was significant for Y19, Y2 and Y15 at the final measurement date as represented by reduced AQY (Fig. 3), leading to decrease in qP and Φ_{PS2} (Fig. 3) (Morgan et al., 2004). This may be

Fig. 6. The activities of superoxide dismutase (SOD), peroxidases (POD), ascorbate peroxidase (APX) and hydrogen peroxidase (CAT) of winter wheat in the measurement at the mid-grain filling stage under ambient $[O_3]$ (A- O_3) and elevated $[O_3]$ (E- O_3) (mean $\pm$ SD, $n = 3$). The letters on top of the bars are based on the Tukey test across the two measurements. Bars without a same letter are significantly different from each other at $P < 0.05$.

caused by the lower efficiency of excitation energy captured by open PSII reaction center as indicated by the decrease in F_v/F_m' (Fig. 3). The reduced quantum yield of PSII electron transport appears to be another non-stomatal factor responsible for the genetic difference among the cultivars. Therefore, the larger decline of g_s and increase of L_s in Y19, Y2 and Y15 were the result rather than the cause of the O_3 -induced A_{sat} , which is also supported by previous chamber studies (Fiscus et al., 1997; McKee et al., 1995).

Among all antioxidant variables tested in this study, APX, POD, SOD, GSH, Phenolics and AsA showed significant difference among cultivars ($P < 0.05$). Ozone-induced reduction of A_{sat} in Y19, Y2 and Y15 also resulted from a larger damage to the integrity of chloroplast membrane since greater chlorophyll degradation and lipid peroxidation were observed as indicated by decreased total chlorophyll (Fig. 4) and increased MDA (Fig. 5), respectively. The reduction in A_{sat} , chlorophyll degradation and lipid peroxidation in turn may lead to accelerated leaf senescence (Fangmeier et al., 1994; Mulholland et al., 1997; Pell et al., 1994), which has been widely reported for many species including wheat as a typical physiological symptom induced by elevated O_3 (Feng et al., 2011; Finnan et al., 1998; Miller et al., 1999; Pell et al., 1994; Ribas et al., 2005). Among foliar pigments, carotenoids play an important role in photosynthetic reaction centre by both participating the process of energy-transfer and providing a mechanism for plant photoprotection against photooxidation (Davies, 2004). Therefore, the O_3 -induced decline in carotenoids similar to that in total chlorophyll at the final measurement underlines the relations between wheat O_3 sensitivity and the antioxidant capacity determined by different antioxidant pools including AsA, carotenoids, and enzymes of APX, SOD, CAT and POD. The antioxidants of the apoplast have been reported as the first line of defense against O_3 damage (Baier et al., 2005), and the antioxidant metabolism in the leaf tissues acts as a mechanism replenishing antioxidants oxidized by O_3 in the apoplast. The insignificant responses of all the tested antioxidant pools and enzymes to E- O_3 for J2, Y16 and/or Y15 in leaf tissues indicated the antioxidant systems may prevent oxidative damage better than Y19 and Y2. Larger O_3 -induced loss of AsA in Y19, SOD in Y2 and CAT in both Y2 and Y19 resulted in the increasing membrane lipid peroxidation, and thus greater loss in A_{sat} and yield. These results were supported by the previous wheat FACE studies that Y16 exhibited higher photosynthetic rates (Feng et al., 2011) and apoplastic ascorbate contents (AsA) than Y2 (Feng et al., 2010), and lower levels of ROS and higher apoplastic antioxidant enzyme activities than Y19 (Wang et al., 2014a).

5. Conclusions

This study investigated the ecophysiological responses to elevated O_3 among five varieties of winter wheat under fully open air field conditions. The significant O_3 effects on most variables were only found during the mid-grain filling stage. Among the tested cultivars, two distinct groups with respect to O_3 sensitivity could be discerned: sensitive cultivars (Y15, Y2 and Y19) and tolerant cultivars (J2 and Y16). The lower photosynthetic rates induced by E- O_3 were mainly due to non-stomatal factors, e.g. lower maximum carboxylation capacity and electron transport rates. Significant interactions between O_3 and cultivar were found in antioxidative enzymes, such as SOD and CAT, but not in stomatal conductance and antioxidant contents, suggesting that antioxidative enzymes significantly contributed to the differential response to E- O_3 among cultivars. Current results implied that in addition to strongly needed reductions in emissions of O_3 precursors, breeding plant varieties higher in antioxidative enzymes can become on part the strategy to avoid O_3 -induced yield loss in crops. Also, the obtained results provide important information for the development of accurate modeling O_3 effects on crops, especially with respect to the developmental stage when O_3 damage to photosynthesis becomes manifest.

Acknowledgements

This study has been funded by the Hundred Talents Program, Chinese Academy of Sciences, Fellowship of Japan Society for the Promotion of Science (P09120), the International S & T Cooperation Program of China (2009DFA31110). We are grateful to Prof. G. Liu and Dr. H. Y. Tang for their technical support in the free-air ozone release system.

References

- Akhtar, N., Yamaguchi, M., Inada, H., Hoshino, D., Kondo, T., Izuta, T., 2010. Effects of ozone on growth, yield and leaf gas exchange rates of two Bangladeshi cultivars of wheat (*Triticum aestivum* L.). *Environ. Pollut.* 158, 1763–1767.
- Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. *Plant Cell Environ.* 28, 949–964.
- Baier, M., Kandlbinder, A., Gollack, D., Dietz, K.J., 2005. Oxidative stress and ozone: perception, signalling and response. *Plant Cell Environ.* 28, 1012–1020.
- Betzberger, A.M., Gillespie, K.M., McGrath, J.M., Koester, R.P., Nelson, R.L., Ainsworth, E.A., 2010. Effects of chronic elevated ozone concentration on antioxidant capacity, photosynthesis and seed yield of 10 soybean cultivars. *Plant Cell Environ.* 33, 1569–1581.
- Biswas, D.K., Xu, H., Li, Y.G., Sun, J.Z., Wang, X.Z., Han, X.G., Jiang, G.M., 2008. Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. *Glob. Chang. Biol.* 14, 46–59.
- Booker, F., Munfiring, R., McGrath, M., Burke, K., Decoteau, D., Fiscus, E., Manning, W., Krupa, S., Chappelka, A., Grantz, D., 2009. The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. *J. Integr. Plant Biol.* 51, 337–351.
- Burkart, S., Bender, J., Tarkotta, B., Faust, S., Castagna, A., Ranieri, A., Weigel, H.J., 2013. Effects of ozone on leaf senescence, photochemical efficiency and grain yield in two winter wheat cultivars. *J. Agron. Crop Sci.* 199, 275–285.
- Burkey, K.O., Eason, G., Fiscus, E.L., 2003. Factors that affect leaf extracellular ascorbic acid content and redox status. *Physiol. Plant.* 117, 51–57.
- Bytnerowicz, A., Omasa, K., Paoletti, E., 2007. Integrated effects of air pollution and climate change on forests: a northern hemisphere perspective. *Environ. Pollut.* 147, 438–445.
- Conklin, P.L., Barth, C., 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant Cell Environ.* 27, 959–970.
- Davies, K., 2004. Plant pigments and their manipulation. *Annu. Plant Rev.* 14, 57–91.
- Dizengremel, P., Le Thiec, D., Bagard, M., Jolivet, Y., 2008. Ozone risk assessment for plants: central role of metabolism-dependent changes in reducing power. *Environ. Pollut.* 156, 11–15.
- Dizengremel, P., Le Thiec, D., Hasenfratz-Sauder, M.P., Vaultier, M.N., Bagard, M., Jolivet, Y., 2009. Metabolic-dependent changes in plant cell redox power after ozone exposure. *Plant Biol.* 11, 35–42.
- Fangmeier, A., Brunschön, S., Jäger, H.J., 1994. Time course of oxidant stress biomarkers in flag leaves of wheat exposed to ozone and drought stress. *New Phytol.* 126, 63–69.
- Farage, P.K., Long, S.P., 1999. The effects of O_3 fumigation during leaf development on photosynthesis of wheat and pea: an in vivo analysis. *Photosynth. Res.* 59, 1–7.
- Farquhar, G.D., Sharkey, T.D., 1982. Stomatal conductance and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 33, 317–345.
- Feng, Z.Z., Kobayashi, K., 2009. Assessing the impacts of current and future concentrations of surface ozone on crop yield with meta-analysis. *Atmos. Environ.* 43, 1510–1519.
- Feng, Z.Z., Kobayashi, K., Ainsworth, E.A., 2008. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (*Triticum aestivum* L.): a meta-analysis. *Glob. Chang. Biol.* 14, 2696–2708.
- Feng, Z.Z., Pang, J., Kobayashi, K., Zhu, J., Ort, D.R., 2011. Differential responses in two varieties of winter wheat to elevated ozone concentration under fully open-air field conditions. *Glob. Chang. Biol.* 17, 580–591.
- Feng, Z.Z., Pang, J., Nouchi, I., Kobayashi, K., Yamakawa, T., Zhu, J., 2010. Apoplastic ascorbate contributes to the differential ozone sensitivity in two varieties of winter wheat under fully open-air field conditions. *Environ. Pollut.* 158, 3539–3545.
- Feng, Z.Z., Paoletti, E., Bytnerowicz, A., Harmons, H., 2015. Ozone and plants. *Environ. Pollut.* 202, 215–216.
- Feng, Z.Z., Sun, J., Wan, W., Hu, E., Calatayud, V., 2014. Evidence of widespread ozone-induced visible injury on plants in Beijing, China. *Environ. Pollut.* 193, 296–301.
- Finnan, J.M., Jones, M.B., Burke, J.I., 1998. A time-concentration study on the effects of ozone on spring wheat (*Triticum aestivum* L.): 3: effects on leaf area and flag leaf senescence. *Agric. Ecosyst. Environ.* 69, 27–35.
- Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant Cell Environ.* 28, 997–1011.
- Fiscus, E.L., Reid, C.D., Miller, J.E., Heagle, A.S., 1997. Elevated CO_2 reduces O_3 flux and O_3 -induced yield losses in soybeans: possible implications for elevated CO_2 studies. *J. Exp. Bot.* 48, 307–313.
- Gelang, J., Pleijel, H., Sild, E., Danielsson, H., Younis, S., Sellden, G., 2000. Rate and duration of grain filling in relation to flag leaf senescence and grain yield in spring wheat (*Triticum aestivum*) exposed to different concentrations of ozone. *Physiol. Plant.* 110, 366–375.
- Gillespie, K.M., Rogers, A., Ainsworth, E.A., 2011. Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). *J. Exp. Bot.* 62, 2667–2678.
- Gillespie, K.M., Xu, F., Richter, K.T., McGrath, J.M., Markelz, R.J.C., Ort, D.R., Leakey, A.D.B., Ainsworth, E.A., 2012. Greater antioxidant and respiratory metabolism in field-

- grown soybean exposed to elevated O₃ under both ambient and elevated CO₂. *Plant Cell Environ.* 35, 169–184.
- Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207–212.
- Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 125, 189–198.
- Inada, H., Kondo, T., Akhtar, N., Hoshino, D., Yamaguchi, M., Izuta, T., 2012. Relationship between cultivar difference in the sensitivity of net photosynthesis to ozone and reactive oxygen species scavenging system in Japanese winter wheat (*Triticum aestivum*). *Physiol. Plant.* 146, 217–227.
- Kangasjarvi, J., Jaspers, P., Kollist, H., 2005. Signalling and cell death in ozone-exposed plants. *Plant Cell Environ.* 28, 1021–1036.
- Kangasjarvi, J., Talvinen, J., Utriainen, M., Karjalainen, R., 1994. Plant defense systems induced by ozone. *Plant Cell Environ.* 17, 783–794.
- Kollist, H., Moldau, H., Mortensen, L., Rasmussen, S.K., Jorgensen, L.B., 2000. Ozone flux to plasmalemma in barley and wheat is controlled by stomata rather than by direct reaction of ozone with cell wall ascorbate. *J. Plant Physiol.* 156, 645–651.
- Li, P., Calatayud, V., Gao, F., Uddling, J., Feng, Z., 2016. Differences in ozone sensitivity among woody species are related to leaf morphology and antioxidant levels. *Tree Physiol.* <http://dx.doi.org/10.1093/treephys/tpw042>.
- Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* 148, 350–382.
- Long, S.P., Bernacchi, C.J., 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *J. Exp. Bot.* 54, 2393–2401.
- Long, S.P., Drake, B.G., 1991. Effect of the long-term elevation of CO₂ concentration in the field on the quantum yield of photosynthesis of the C3 sedge, *Scirpus olneyi*. *Plant Physiol.* 96, 221–226.
- Luwe, M., Heber, U., 1995. Ozone detoxification in the apoplast and symplast of spinach, broad bean and beech leaves at ambient and elevated concentrations of ozone in air. *Planta* 197, 448–455.
- McKee, I.F., Eiblmeier, M., Polle, A., 1997. Enhanced ozone-tolerance in wheat grown at an elevated CO₂ concentration: ozone exclusion and detoxification. *New Phytol.* 137, 275–284.
- McKee, I.F., Farage, P.K., Long, S.P., 1995. The interactive effects of elevated CO₂ and O₃ concentration on photosynthesis in spring wheat. *Photosynth. Res.* 45, 111–119.
- Miller, J.D., Artica, R.N., Pell, E.J., 1999. Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. *Plant Physiol.* 120, 1015–1023.
- Morgan, P.B., Bernacchi, C.J., Ort, D.R., Long, S.P., 2004. An in vivo analysis of the effect of season-long open-air elevation of ozone to anticipated 2050 levels on photosynthesis in soybean. *Plant Physiol.* (135), 2348–2357.
- Mulholland, B.J., Craigan, J., Black, C.R., Colls, J.J., Atherton, J., Landon, G., 1997. Impact of elevated atmospheric CO₂ and O₃ on gas exchange and chlorophyll content in spring wheat (*Triticum aestivum* L.). *J. Exp. Bot.* 48, 1853–1863.
- Overmyer, K., Brosche, M., Kangasjarvi, J., 2003. Reactive oxygen species and hormonal control of cell death. *Trends Plant Sci.* 8, 335–342.
- Paoletti, E., Ranieri, A., Lauteri, M., 2008. Moving toward effective ozone flux assessment. *Environ. Pollut.* 156, 16–19.
- Pell, E.J., Eckardt, N.A., Glick, R.E., 1994. Biochemical and molecular basis for impairment of photosynthetic potential. *Photosynth. Res.* 39, 453–462.
- Pleijel, H., Danielsson, H., Gelang, J., Sild, E., Sellden, G., 1998. Growth stage dependence of the grain yield response to ozone in spring wheat (*Triticum aestivum* L.). *Agric. Ecosyst. Environ.* 70, 61–68.
- Pleijel, H., Eriksen, A.B., Danielsson, H., Bondesson, N., Sellden, G., 2006. Differential ozone sensitivity in an old and a modern Swedish wheat cultivar — grain yield and quality, leaf chlorophyll and stomatal conductance. *Environ. Exp. Bot.* 56, 63–71.
- Rao, M.V., Koch, J.R., Davis, K.R., 2000. Ozone: a tool for probing programmed cell death in plants. *Plant Mol. Biol.* 44, 345–358.
- Ribas, A., Penuelas, J., Elvira, S., Gimeno, B.S., 2005. Ozone exposure induces the activation of leaf senescence-related processes and morphological and growth changes in seedlings of Mediterranean tree species. *Environ. Pollut.* 134, 291–300.
- Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.
- Sun, J., Feng, Z., Ort, D.R., 2014. Impacts of rising tropospheric ozone on photosynthesis and metabolite levels on field grown soybean. *Plant Sci.* 226, 147–161.
- Tang, H., Liu, G., Han, Y., Zhu, J., Kobayashi, K., 2011. A system for free-air ozone concentration elevation with rice and wheat: control performance and ozone exposure regime. *Atmos. Environ.* 45, 6276–6282.
- Van Dingenen, R., Dentener, F.J., Raes, F., Krol, M.C., Emberson, L., Cofala, J., 2009. The global impact of ozone on agricultural crop yields under current and future air quality legislation. *Atmos. Environ.* 43, 604–618.
- Wang, J., Zeng, Q., Zhu, J., Chen, C., Liu, G., Tang, H., 2014a. Apoplastic antioxidant enzyme responses to chronic free-air ozone exposure in two different ozone-sensitive wheat cultivars. *Plant Physiol. Biochem.* 82, 183–193.
- Wang, J., Zhu, J., Zeng, Q., Liu, G., 2014b. Phenolic compounds and antioxidant ability responses to experimental free-air ozone exposure in two wheat cultivars. *B. Environ. Contam. Tox.* 93, 625–631.
- Zheng, Y., Shimizu, H., Barnes, J.D., 2002. Limitations to CO₂ assimilation in ozone-exposed leaves of *Plantago major*. *New Phytol.* 155, 67–78.
- Zhu, X., Feng, Z., Sun, T., Liu, X., Tang, H., Zhu, J., Guo, W., Kobayashi, K., 2011. Effects of elevated ozone concentration on yield of four Chinese cultivars of winter wheat under fully open-air field conditions. *Glob. Chang. Biol.* 17, 2697–2706.