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Cadmium pollution enhanced ozone damage to winter wheat: biochemical and physiological evidences

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Abstract

Combined effects of ozone (O_3) and cadmium (Cd) on growth and physiology of winter wheat (*Triticum aestivum* L. cv. JM22) were determined. Wheat plants were grown without or with Cd and exposed to charcoal-filtered air (< 10 ppb O_3) or elevated O_3 (80 ± 5 ppb, 7 hr/day) for 20 days. Results showed that O_3 considerably depressed light saturated net photosynthetic rate (–20%), stomatal conductance (–33%), chlorophyll content (–33%), and total biomass (–29%) without Cd. The corresponding decreases were further enhanced by 45%, 56%, 60% and 59%, respectively with Cd, indicating a synergistic effect of O_3 and Cd on wheat. Ozone significantly increased the activity of superoxide dismutase (46%), catalase (48%) and peroxidase (56%). However, great increases in malondialdehyde (MDA) content (2.55 folds) and intercellular CO_2 concentration (1.13 folds) were noted in O_3+Cd treatment compared to control. Our findings demonstrated that the increased anti-oxidative activities in wheat plants exposed to O_3+Cd might not be enough to overcome the adverse effects of the combination of both pollutants as evidenced by further increase in MDA content, which is an important indicator of lipid peroxidation. Precise prediction model on O_3 damages to crop should be conducted to ensure agricultural production security by considering environmental constraints in an agricultural system in peri-urban regions.

Key words: antioxidant activity; cadmium; plant growth; ozone stress; *Triticum aestivum* L.

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Introduction

Current ozone (O_3) levels in many countries are high enough to reduce growth and yield of crops including winter wheat (Kobayashi et al., 1995; Zheng et al., 1998; Chameides et al., 1999; Jin et al., 2001). Previous studies estimated that yield loss caused by O_3 pollution would become larger (e.g., 9%–18% for wheat globally) by 2020 (Aunan et al., 2000; Wang and Mauzerall, 2004; Wang et al., 2005, 2007; Liu et al., 2009; van Dingenen et al., 2009). Soil contamination by heavy metals also turns into another pressing environmental issue in the world, especially in developing countries, where rapid economic growth normally accelerates industrialization, urbanization as well as agricultural modernization (Huang et al., 2007; Rodriguez et al., 2007). Cadmium (Cd), a toxic heavy metal, is principally dispersed into agricultural soil through utilization of phosphate fertilizers, sewage and industrial wastewater for irrigation (Wagner, 1993; Sanità di Toppi and Gabbrielli, 1999; Jamali et al., 2007).

The combined ozone and Cd pollution may cause further physiological and biochemical changes in crops, and hence result in growth inhibition and yield loss. O_3 is a phytotoxic air pollutant to crops. According to the previous investigations (Barnes et al., 1990; Kangasjarvi et al., 1994; Pell et al., 1997), O_3 induces visible foliar injury and damage to the photosynthetic apparatus, and reduces photosynthetic rate. Such kinds of changes accelerate plant senescence (Grandjean and Fuhrer, 1989; Pell et al., 1997; Ojanpera et al., 1998) and finally lead to growth reduction and yield loss (Grandjean and Fuhrer, 1989; Ollerenshaw and Lyons, 1999; Pleijel et al., 2000; Feng et al., 2008). Physiologically, an elevated Cd has been reported to affect crops at different levels, inducing damages in membrane structure, water and ion uptake and inhibition in photosynthetic processes (Lata, 1991; Wójcik et al., 2005). However, except for sunflowers (Di Cagno et al., 2001), cress and lettuce (Czuba and Ormrod, 1974, 1981), there are very few investigations on the responses of crops to O_3 combined with Cd pollution. Besides, one of

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the main limitations of previous research works is that leaf biochemistry has not been integrated or correlated with physiological and growth data in explaining the observed interaction between O_3 and Cd. Therefore, the integrative effect of O_3 and Cd pollution on crops especially on winter wheat still remains unclear.

In peri-urban regions, the cadmium-affected farmlands are now being to expose to numerous stresses, especially O_3 pollution (Lam et al., 2005; Wang et al., 2005; Li et al., 2007; Shi et al., 2008). Since both O_3 and Cd may lead to physiological and biochemical changes and yield loss, it is possible that the two pollutants together may interact on crop metabolism. After passing through the stomatal pore, O_3 reacts with molecules in the intercellular space, leading to produce reactive oxygen species (ROS), which can severely compromise the cell membrane (Ashmore and Marshall, 1999). Similarly, Cd after absorbed by roots can induce membrane damage, inhibition or inactivation of enzymes and disruption of electron transport due to the induction of oxidative stress and replacement of elements such as Fe, Mn, and Zn, which are essential cofactors of many enzymes (Tukaj et al., 2007). We therefore hypothesize that O_3+Cd treatment may have a synergistic effect on biochemical and physiological aspects in a cultivar of winter wheat. Our findings may be useful in predicting wheat performance under elevated O_3 and Cd pollutions that commonly occur in an agricultural system in peri-urban regions.

1 Materials and methods

1.1 Plant culture

The experiment was conducted in the Eco-farming Research Station at Shandong Agricultural University (35°26′34″N, 117°49′13″E), Pingyi County, Shandong Province, China. Seeds of winter wheat (*Triticum aestivum* L. cv. JM22) were sown in quartz sand. On day 7 after planting, 20 seedlings were individually transported into pots (30 cm × 20 cm × 12 cm, length × width × height) containing quartz sand and Hoagland nutrient solution. Meanwhile, the plants were homogeneously divided into two groups (each contains 8 pots). One group was added with 0.5 mmol/L Cd^{2+} (+Cd) through dissolving $CdCl_2{\cdot}6H_2O$ into the nutrient solution, another was added with the same volume of distilled water in stead of Cd (–Cd). The plants were grown in six open top chambers (OTCs, 2.4 m in diameter, and 1.8 m in height, in field) receiving charcoal-filtered (CF) air (< 10 ppb O_3) for 3 days to adapt chamber environments before starting O_3 treatment. After the preliminary period, plants were exposed to O_3 fumigation. The gas diffusion system of the OTCs was constructed on the basis of the method described by Uprety (1998). The OTCs were continuously ventilated by fans so that air shifted completely when O_3 was dispersed into OTCs. The temperature in OTCs was 20–33°C and the average photosynthetic photon flux density (PPFD) was 600 μmol/(m^2·sec) at canopy height during 14 hr photoperiod. The maximum PPFD in the chambers was 1500 μmol/(m^2·sec) and relative humidity (RH) was 75%. Seasonal temperature fluctuated from 18 to 30°C, and seasonal RH varied from 48% to 96%.

1.2 Ozone fumigation

The O_3 treatments including elevated O_3(+O_3) and CF air control were performed in six OTCs. O_3 was electronically discharged with an O_3 generator (CF-KG1, Sumsun EP Hi-Tech., China), and randomly dispensed into three of the six chambers. A manual mass flow controller was employed to regulate the flow of O_3-enhanced air to the OTCs. O_3 concentrations in the OTCs were continuously monitored at plant canopy with an UV absorption O_3 analyzer (Model 205, 2B Tech. Inc., USA), which was calibrated with an O_3 monitor (ML 9810B, Eco-Tech., USA). The O_3 concentrations inside the elevated O_3 chambers were maintained at (80 ± 5) ppb for 7 hr/day (9:00–16:00) from day 11 to day 30 after planting; meanwhile, the control plants were exposed to charcoal-filtered air only. Water lost by evapotranspiration was replenished every day during the experimental runs.

1.3 Leaf visible injury

Visible injuries were observed on the fourth youngest leaf from the main stem after both Cd and O_3 treatments on day 31 after planting. Five plants for both –Cd and +Cd treatments from elevated O_3 and CF air chambers were sampled. The percentage of damaged area (mottled or necrotic) on the leaves was calculated by placing a transparent plastic gird above the plant and counting intersections of the grid that represented leaf surface area.

1.4 Gas exchange measurement and leaf sampling

On day 20 after treatment, the recently fully expanded leaf was selected for gas exchange measurement with a portable gas exchange fluorescence system (GFS-3000, Walz, Germany). The leaf cuvette environment was controlled at PPFD of 1500 μmol/(m^2·sec) using internal light source, RH 50% and temperature 30°C. Airflow was set at 750 μmol/sec and CO_2 concentration maintained at 380 μmol/mol. Variables of the gas exchange measurement included area-based light saturated net photosynthetic rate (A_{sat}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E).

The middle part of recently fully expanded leaves without visible damage in both elevated O_3 and ambient air chambers was excised and placed in liquid nitrogen immediately for biochemical analysis. Samples were transferred to an ultra-freezer at –80°C until assay. The plants were harvested, divided into shoot and root and dried at 75°C for 48 hr for dry mass. Part of shoots and roots was rinsed with distilled water for Cd determination before being dried.

1.5 Determination of chlorophyll content

Chlorophyll was extracted from frozen leaf samples (0.2 g) in 95% ethanol for 48 hr in the dark at 4°C. The extract was then assayed for chlorophyll with the absorption spectra (Arnon, 1949).

1.6 Lipid peroxidation and antioxidant enzymes activities

Frozen leaf samples (0.5 g) were homogenized in a pre-chilled mortar placed on ice with 5 mL of 0.05 mol/L potassium phosphate buffer (pH 7.8) containing 8.5% (*V*/*V*) of 0.2 mol/L KH_2PO_4 and 91.5% of 0.2 mol/L K_2HPO_4. The homogenate was centrifuged at 4000 × *g* for 20 min at 4°C. Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content (Kramer et al., 1991). A mixture of 1 mL supernatant and 2 mL of 0.6% thiobarbituric acid (TBA) was boiled for 15 min, cooled and centrifuged for 10 min at 4000 r/min. The absorbance was read at 600, 532 and 450 nm using an UV/Visible light spectrophotometer (UV-365, Shimadzu, Japan). Peroxidase (POD) was determined through measuring the oxidation of guaiacol. The assay mixture contained 50 mL of 0.1 mol/L NaCl (pH 6.0), 28 μL guaiacol and 19 μL of 30% H_2O_2. The absorbance was recorded five times at 470 nm with 30 sec intervals. Variation of absorbance per minute per gram fresh weight symbolized POD activity. Superoxide dismutase (SOD) activity was determined according to the methodology of Giannopolitis and Ries (1977). The supernatant was desalted by Sephadex G-25 gel filtration to remove interfering materials and served as crude enzyme extract. One unit of SOD activity (U) was defined as the amount of crude enzyme extract required for inhibiting the reduction rate of nitro-blue tetrazolium (NBT) by 50%. Catalase (CAT) activity was determined following the method of Aebi (1984). CAT activity was determined as variation of absorbance per minute per gram fresh weight.

1.7 Determination of cadmium concentration

The determination of Cd concentration was conducted based on the nitric-perchloric acid (4:1, *V*/*V*) digestion of three tissue samples. Cadmium concentration was determined by ICP-OES (ICPS-7500, Shimadzu, Japan).

1.8 Statistical analysis

Each dependent variable was analyzed separately using two-way ANOVA of SPSS package (Ver. 15, SPSS, Chicago, USA) to determine cadmium and ozone effects on the measured variables. Turkey method was used to assess pair-wise comparisons among treatments. Differences between treatments were considered significant if $P \leqslant 0.05$.

2 Results and discussion

2.1 Cadmium concentration and distribution in tissues

As shown in Table 1, a large increase in Cd concentration in the whole plants was noted in +Cd treatment. Cd concentration was much higher in the roots than in the shoots (13 folds and 11 folds in CF treatment and $+O_3$ treatment, respectively). At combination of Cd and O_3, both Cd uptake and concentration in leaves (22%) and roots (7%) were significantly higher compared with Cd treatment without O_3.

Table 1 Cadmium concentrations in winter wheat (JM22)

		Cd concentration (μg/g dw)	
		Shoot	Root
CF	–Cd	0.02 ± 0.01 c	1.44 ± 0.27 c
	+Cd	27.56 ± 1.01 b	356.73 ± 6.11 b
$CF+O_3$	–Cd	0.05 ± 0.01 c	1.57 ± 0.22 c
	+Cd	33.66 ± 1.08 a	382.99 ± 3.75 a

Data are presented as mean of three replications ± SE. Means in each column not followed by the same letter are significantly different from each other at $P \leqslant 0.05$.
CF: charcoal-filtered air; O_3: 80 ± 5 ppb; –Cd: without Cd; +Cd: Cd stressed.

2.2 Visible injury and growth parameters

Ozone and Cd caused visible injuries (necrotic stipples and chlorosis) which were higher in Cd pollution (3.62 folds) than in O_3 treatment (1.45 folds). Although O_3+Cd treatment showed higher visible injury (3.02 folds), the increment of visible injuries in O_3+Cd treatment was not significantly differed from +Cd treatment (Fig. 1D). Plant height decreased significantly compared to control due to the presence of Cd and/or O_3, appearing 14%, 26% and 31% decreases in O_3, O_3+Cd, and Cd treatment, respectively (Fig. 1A). Root length, shoot mass, root mass and total mass displayed 12%, 25%, 36% and 29% reduction, respectively in $+O_3$ treatment, however, those figures reached to 52%, 38%, 73%, and 50%, respectively in +Cd treatment (Fig. 1B, 2A, 2B, and 2D). A significantly synergistic interaction between O_3 and Cd was noted. For instance, plants subjected to O_3 had only 12% reduction in root length compared with CF, nevertheless, such reduction was enhanced to 60% in O_3+Cd treatment (Fig. 1B). Similar responses were observed in relative water content (RWC), shoot mass, root mass and total mass (Figs. 1C, 2A, 2B, and 2D). Only significant differences in terms of plant height and visible injury were detected in the interaction between O_3 and Cd, indicating a synergistic effect of O_3 and Cd on plant height and visible injury (Table 2). Relative water content (Fig. 1C) was significantly reduced only in plants grown in the presence of Cd. However, no further effects were recorded in plants grown under combination of O_3 and Cd.

A synergistic effect of O_3 and Cd treatments was observed as documented by higher declines in shoot mass, root mass, total mass, root length and RWC, but not in plant height. Induction of growth inhibition and shifts in biomass partitioning had been confirmed in response to O_3 and Cd by previous investigations (Fuhrer et al., 1992; Kobayashi et al., 1995; Meyer et al., 2000; Sandalio et al., 2001; Fiscus et al., 2005; Tukaj et al., 2007; López-Millán et al., 2009). In this study, a strong decline in root/shoot ratio was observed in +Cd treatment (–56%), but greater in O_3+Cd treatment (–67%, Fig. 2C), indicating a synergistic effect of O_3 and Cd stresses. A synergistic effect on gas exchange, chlorophyll contents, biomass accumulation and biomass partitioning corroborated the earlier investigations (Czuba and Ormrod, 1981; Welfare et al., 1996; Di Cagno et al., 2001). Root growth was affected severely than shoot, which was also consistent with Warwick and Taylor

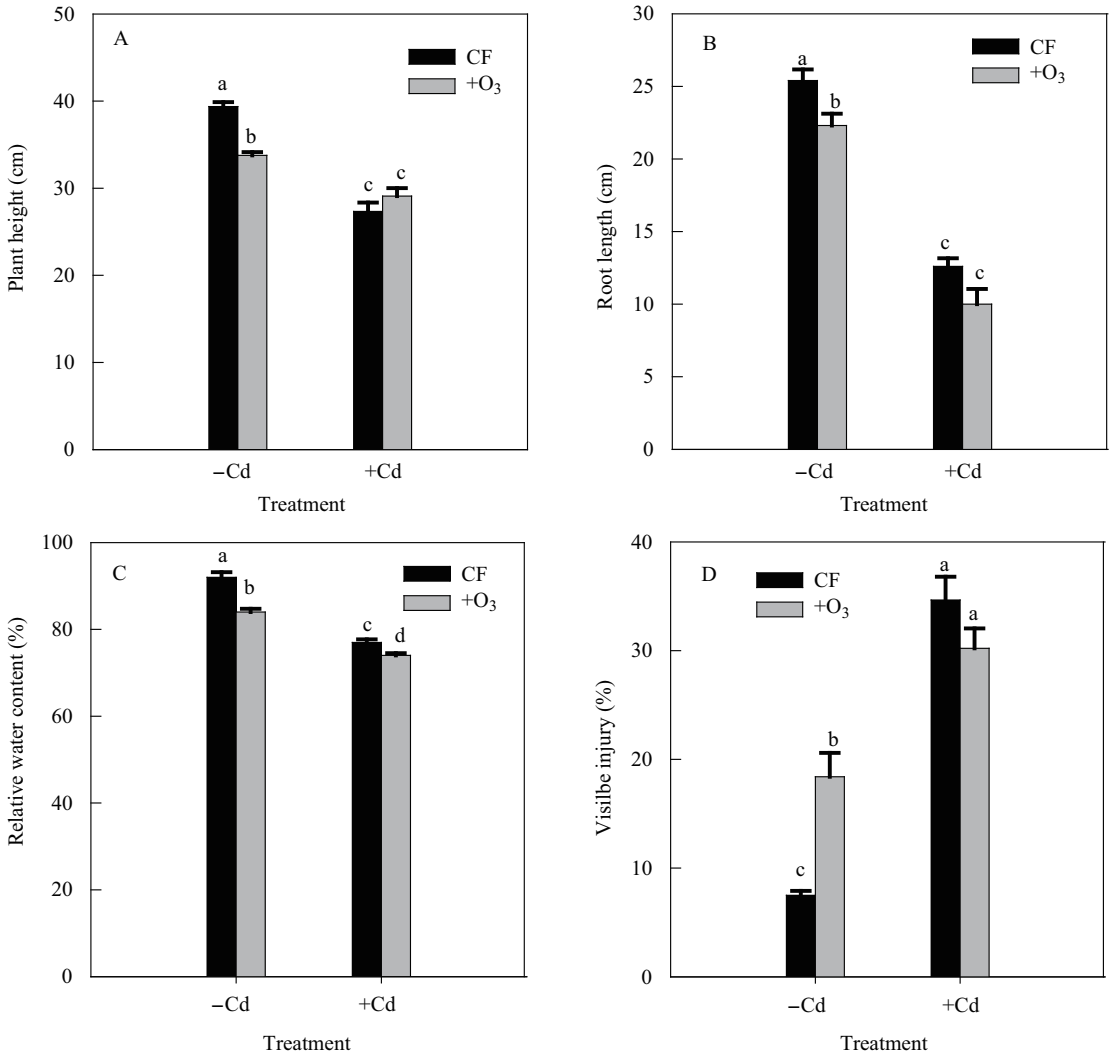

Fig. 1 Plant height (A), root length (B), relative water content (RWC) (C) and visible injury (D) of winter wheat in response to O_3 and Cd stress. Columns with different letters indicate significant differences at $P \leqslant 0.05$.

Table 2 Ozone and Cd combined effects on plant growth and leaf development

	Relativity							
	Plant height (cm)	Root length (cm)	Visible injury (%)	Water content (%)	Shoot mass (g/plant)	Root mass (g/plant)	Root/Shoot ratio	Total mass (g/plant)
O_3 stress								
CF	33.35	19.00	28.39	84.50	1.07	0.36	0.30	1.43
O_3	31.43	16.15	27.44	79.00	0.89	0.27	0.28	1.17
Cd stress								
–Cd	36.57	23.85	13.73	88.00	1.21	0.54	0.44	1.75
+Cd	28.22	11.30	32.15	75.50	0.82	0.16	0.18	0.97
Source of variation								
O_3	*	**	n.s.	n.s.	n.s.	*	*	*
Cd	***	***	***	*	*	***	***	***
$O_3 \times$ Cd	***	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.

Data are presented as mean of three replications. n.s., *, **, *** not significant, significant at $P \leqslant 0.05$, $P \leqslant 0.01$ $P \leqslant 0.001$, respectively.

(1995). The status of biomass partitioning was also altered in response to O_3 and a possible reason might be that ozone induced a decline in phloem loading in root (Welfare et al., 1996). Because either stress can cause severe growth inhibitions and reductions. But the responses of plants to combined treatment were not substantially different from that to either O_3 or Cd stress alone (Di Cagno et al., 2001). Therefore, it is difficult to separate the effect of O_3 on growth from cadmium stress.

2.3 Gas exchange

Light saturated net photosynthetic rate (A_{sat}) decreased in either $+O_3$ or +Cd treatment. In plants exposed to O_3, the reduction was 20% in comparison with the control; further remarkable decrease (–33%) was induced by Cd (Fig. 3A). It was found that the reduction in A_{sat} was found to be larger in plants subjected to O_3+Cd treatment than that in either $+O_3$ or +Cd treatment alone, indicating

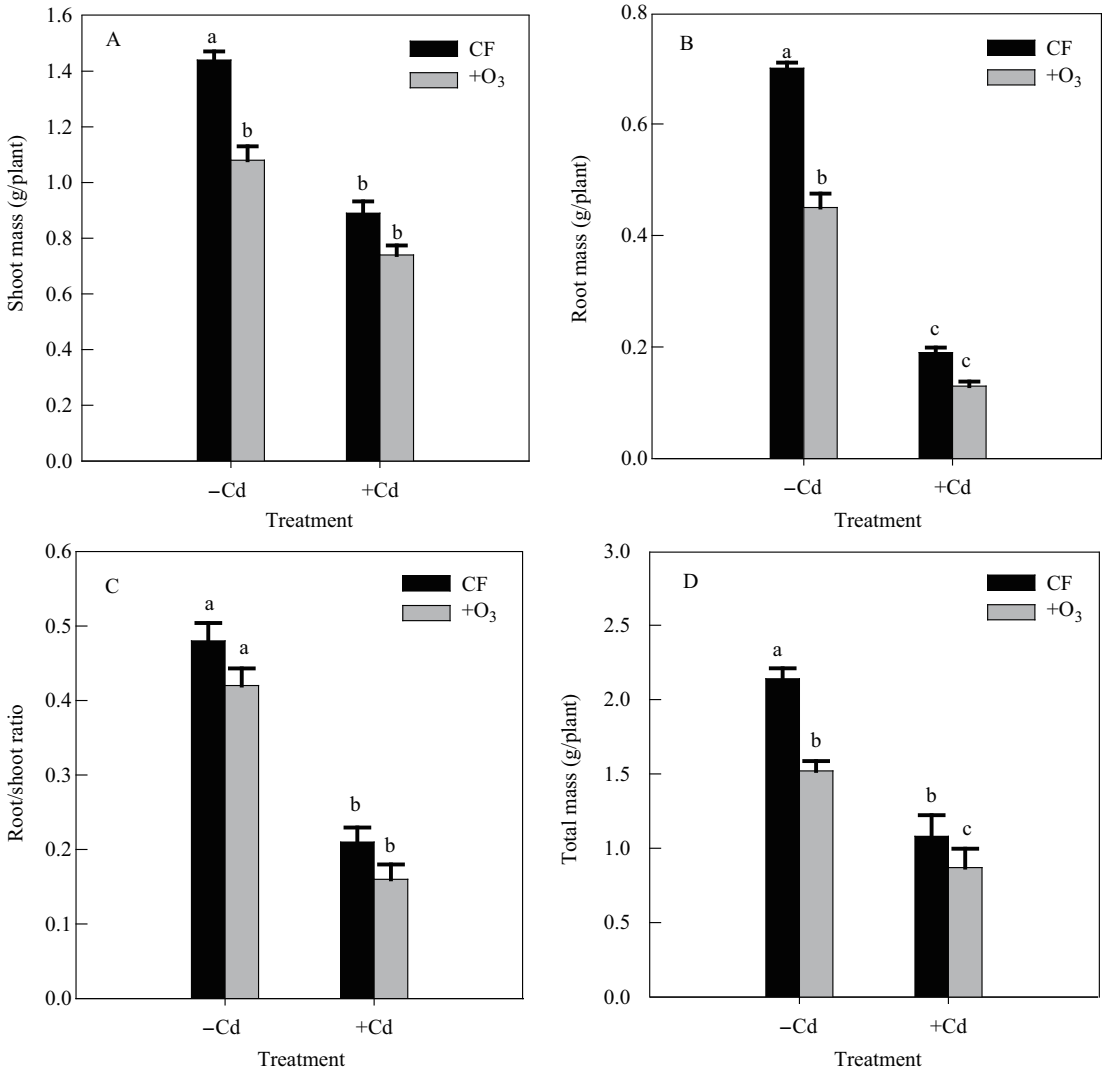

Fig. 2 Shoot mass (A), root mass (B), root/shoot ratio (C) and total mass (D) of winter wheat in response to O_3 and Cd stress. Columns with different letters indicate significant differences at $P \leqslant 0.05$.

a synergistic interaction between O_3 and Cd. Similar interactions were noted for g_s, E and growth parameters. The reduction in g_s and E in O_3+Cd treatment was 8% lower than that in +Cd treatment compared to control (Fig. 3B and 3D). Stomatal conductance was 33%, 48% and 56% lower in plants exposed to +O_3, +Cd and O_3+Cd treatments, respectively, relative to control (Fig. 3C). All gas exchange parameters except C_i significantly differed ($P < 0.05$) between +Cd and O_3+Cd treatments and significant interactions for those parameters were also detected between O_3 + Cd. Both A_{sat} and C_i in O_3-treated plants did not significantly decrease in comparison with Cd-treated plants (Table 3).

Modern spring wheat was found to be more sensitive to O_3 than wild wheat (Barnes et al., 1990). Higher sensitivity to O_3 in recently released cultivars was induced by higher g_s in the investigation on 20 winter wheat cultivars released over the past 60 years in China (Biswas et al., 2008). This indicates that high g_s induces more damage. O_3 uptake in Cd-treated wheat plants should be lower than that in non-treated plants due to lower g_s, resulting in less serious O_3-induced oxidative damage, and hence lower relative loss in Chls and A_{sat} in O_3+Cd treatment than in +Cd treatment. Similar findings were documented with O_3 and salinity (Vozzo et al., 1995; Maggio et al., 2007), or SO_2 and salinity (Ma and Murray, 1991; Huang and Murray, 1993; Huang et al., 1994). However, our results revealed that the reductions in A_{sat}, g_s and E were further enhanced in O_3+Cd treatment. Thus, the scenario of this study deserved careful consideration and this occurrence might involve two reasons. Firstly, the reduction of A_{sat} might be mainly because of the impaired activity of mesophyll cells, not the stomatal limitation, as indicated by the increased C_i and MDA content in O_3 and/or Cd-treated plants (Fig. 3 and Table 3) (Calatayud et al., 2003). Dysfunction of chloroplast membrane due to peroxidation of membrane lipid might also induce the loss in A_{sat} as shown by enhanced increase in MDA content (Table 3). Secondly, Cd concentration of nutrient solutions in +Cd treatment (0.5 mmol/L) was high enough to cause serious detrimental impact of Cd and the adverse affect of O_3 appeared to be relatively mild in the presence of both O_3 and Cd, which should also be taken into consideration (Hassan, 2004).

In this study, significantly enhanced reductions in A_{sat}, g_s and E have been found under O_3+Cd treatment (Table 3). It is reasonable to consider that the combined

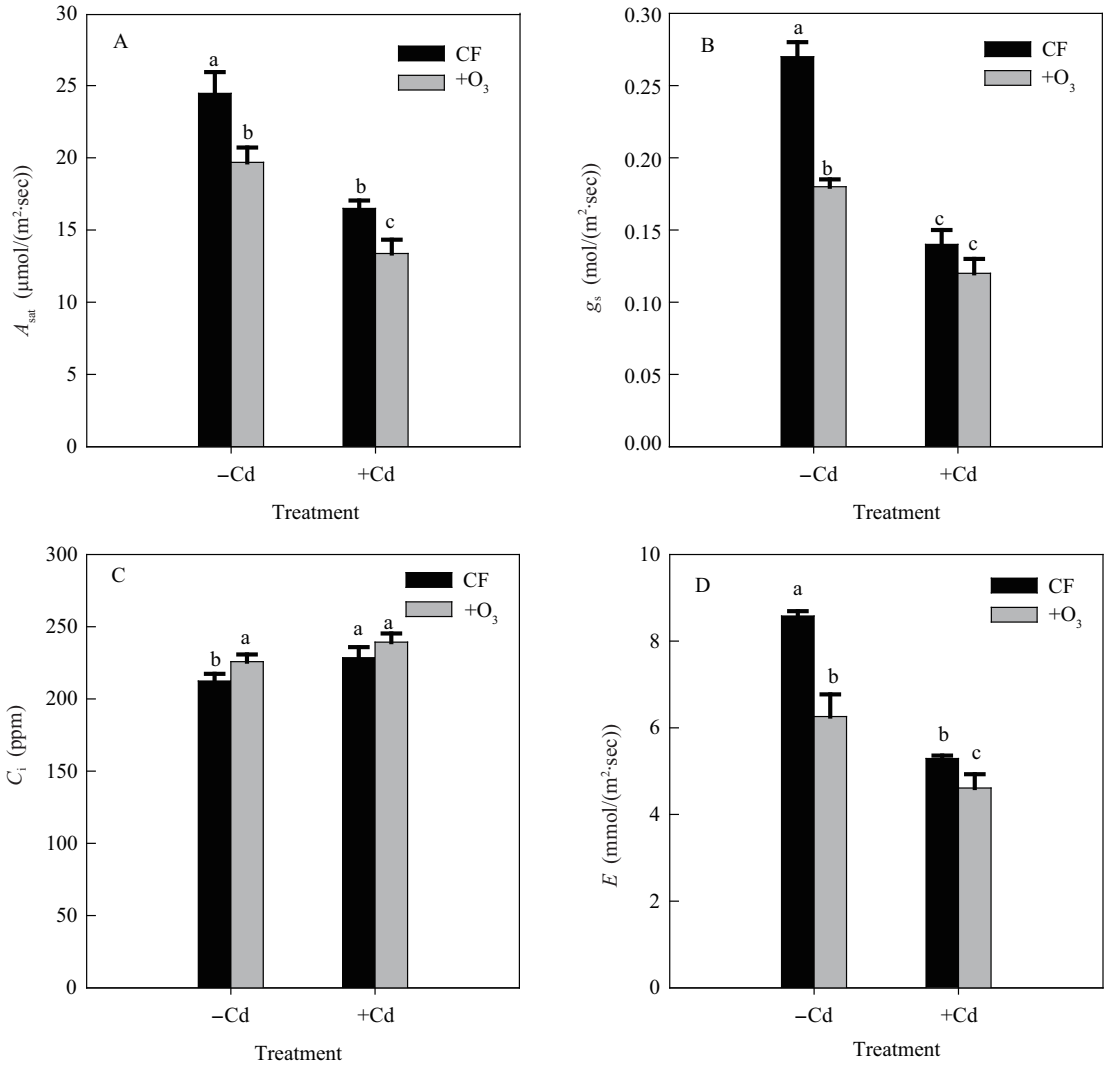

Fig. 3 Light saturated net photosynthetic rate (A_{sat}) (A), stomatal conductance (g_s) (B), CO_2 concentration in intercellular (C_i) (C), and transpiration rate (E) (D) of recently fully expanded leaves of winter wheat in response to O_3 and Cd stress (0.5 mmol/L $CdCl_2$). Columns with different letters indicate significant differences at $P \leqslant 0.05$.

Table 3 Effects of ozone and cadmium stress on leaf physiological and biochemical parameters

	A_{sat} (μmol/(m^2·sec))	g_s (mol/(m^2·sec))	C_i (ppm)	E (mmol/(m^2·sec))	POD (ΔA_{470}/(min·g fw))	SOD (U/g fw)	CAT (ΔA_{240}/(min·g fw))	MDA (μmol/g fw)
O_3 stress								
CF	20.48	0.21	220.50	6.94	202.24	187.47	12.25	3.69
O_3	16.53	0.15	232.53	5.45	268.32	314.74	16.78	4.42
Cadmium stress								
–Cd	22.07	0.23	219.105	7.42	180.65	178.52	10.17	2.68
+Cd	14.93	0.13	233.92	4.95	289.91	323.68	18.86	5.43
Source of variation								
O_3	n.s.	**	n.s.	*	**	n.s.	*	n.s.
Cd	***	***	*	***	***	**	*	**
$O_3 \times$ Cd	**	***	n.s.	***	*	**	n.s.	n.s.

Data are present as mean of three replications. n.s., *, **, *** are not significant, significant at $P \leqslant 0.05$, $P \leqslant 0.01$, $P \leqslant 0.001$, respectively.

effects of O_3 and Cd were synergistic for A_{sat} and g_s. From an agricultural point of view, this might be an important finding, since O_3 and Cd now coexist in agricultural regions.

2.4 Chlorophyll content

The effects of O_3 and/or Cd on chlorophyll (Chl-*a* and Chl-*b*) are illustrated in Fig. 4. Chl-*a* and Chl-*b* contents decreased significantly (–30% and –40%) in leaves exposed to O_3, with more notable reductions (–50% and –70%) in +Cd treatment and the largest decline (–53% and –73%) in O_3+Cd (Fig. 4A and 4B). Chl-*a*/Chl-*b* ratios were higher under O_3+Cd treatment than in the control, indicating that O_3 and Cd together damaged Chl-*b* more seriously than Chl-*a*. The increased Chl-*a*/Chl-*b* ratio was also observed in the interaction of both stresses (Fig. 4D).

Decrease in photosynthesis was accompanied by significant declines in chlorophyll contents and growth parameters. The additive effects of O_3 and Cd were observed on Chls, Chl-*a* and Chl-*b* in presence of both stresses. The reduction in Chl-*b* was generally higher than that in Chl-*a*, resulting in a higher Chl-*a*/Chl-*b* ratio in $+O_3$, +Cd and O_3+Cd treatments. Negative effects on Chls in $+O_3$ and +Cd treatments were also observed previously (Meyer et al., 2000; Mobin and Khan, 2007). The increase in the ratio of Chl-*a*/Chl-*b* has been considered associated with changes in pigment composition of photosynthetic apparatus, which have been considered as evidence of the adaptive mechanism of chloroplasts and green part of plants to endure the adverse conditions (Loggini et al., 1999). Such a phenomenon might be beneficial, or even adaptive for the plants under O_3 and/or Cd stress.

2.5 Antioxidant enzymes and lipid peroxidation

The activity of POD in plants in $+O_3$ and +Cd treatments was generally higher (i.e., 56% and 86%, respectively) relative to control, showing higher sensitivity of POD to both stresses compared with other antioxidant enzymes (Fig. 5A, 5B, and 5C). Plants exposed to O_3 exhibited significant ($P< 0.01$) differences in the levels of POD activity between CF and $+O_3$ treatments, while this difference was considerably ($P < 0.001$) higher in O_3+Cd treatment (Fig. 5). None significant difference in SOD activity was found between CF and $+O_3$ treatment in –Cd plants. MDA content was much higher in +Cd treatment (2.25 folds over control) than in $+O_3$ treatment (39% higher), with the maximum value (2.55 folds) being found in O_3+Cd treatment. Nevertheless, only significant ($P > 0.05$) differences in the activity of POD and SOD were discovered in the combined O_3 + Cd treatment, indicating a synergistic effect of these two stresses on the two enzyme activities (Table 3).

Ozone generates reactive oxygen species (ROS), thereby accelerates lipid peroxidation, photosynthetic pigment decomposition, decreases CO_2 assimilation and reduction in biomass accumulation after entering the cell wall and plasma membrane through stomatal pore (Pell et al., 1997; Baier et al., 2005; Fiscus et al., 2005). Although Cd is a non-redox metal unable to participate in fenton-type reactions, it has been reported that Cd may indirectly induce the generation of ROS. Therefore, the accumulation of Cd might be correlated with the generation of ROS (Sanità di

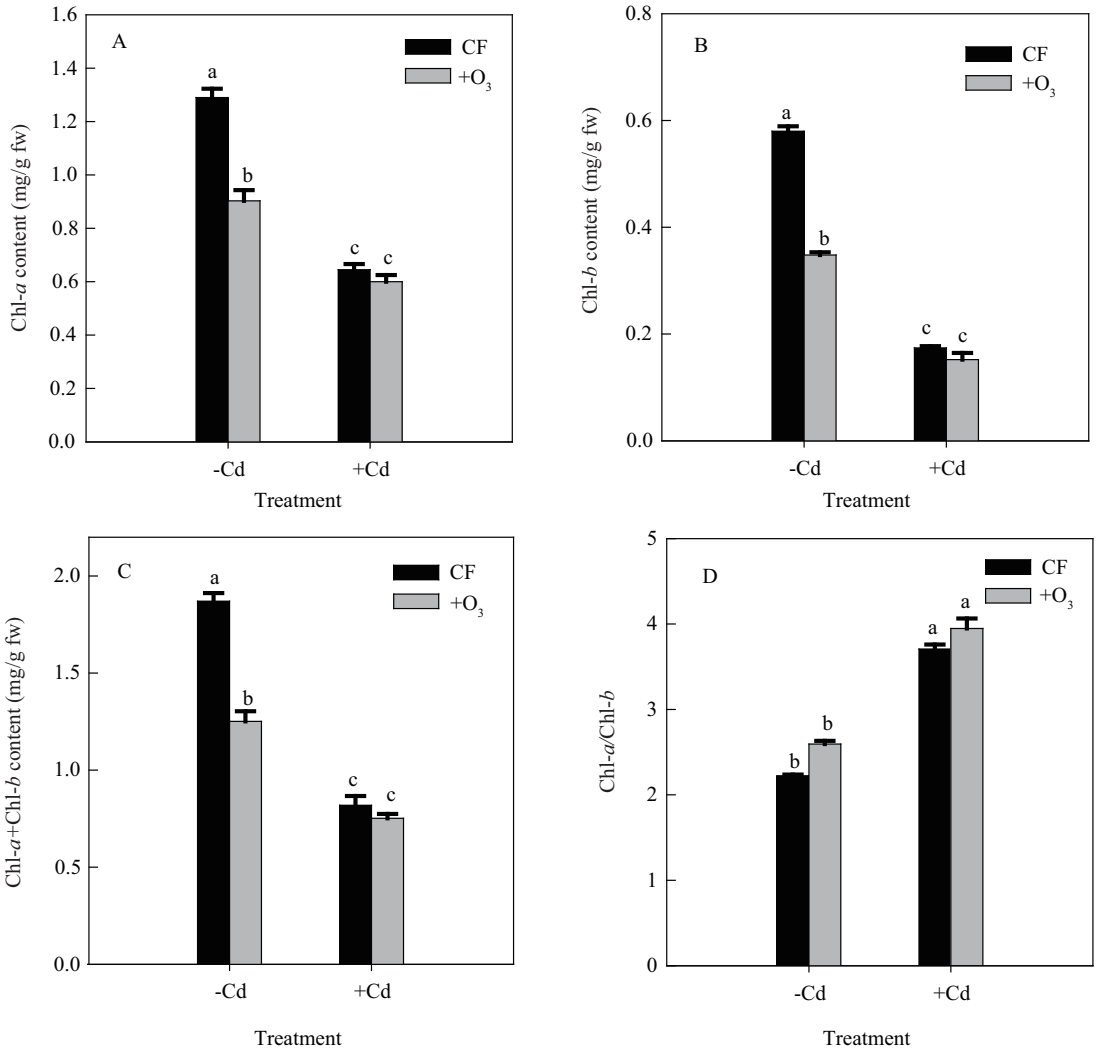

Fig. 4 Chl-*a* content (A), Chl-*b* content (B), Chl-*a* + Chl-*b* content (C) and Chl-*a*/Chl-*b* (D) of recently fully expanded leaves of winter wheat in response to O_3 and Cd stress. Columns with different letters indicate significant differences at $P \leqslant 0.05$.

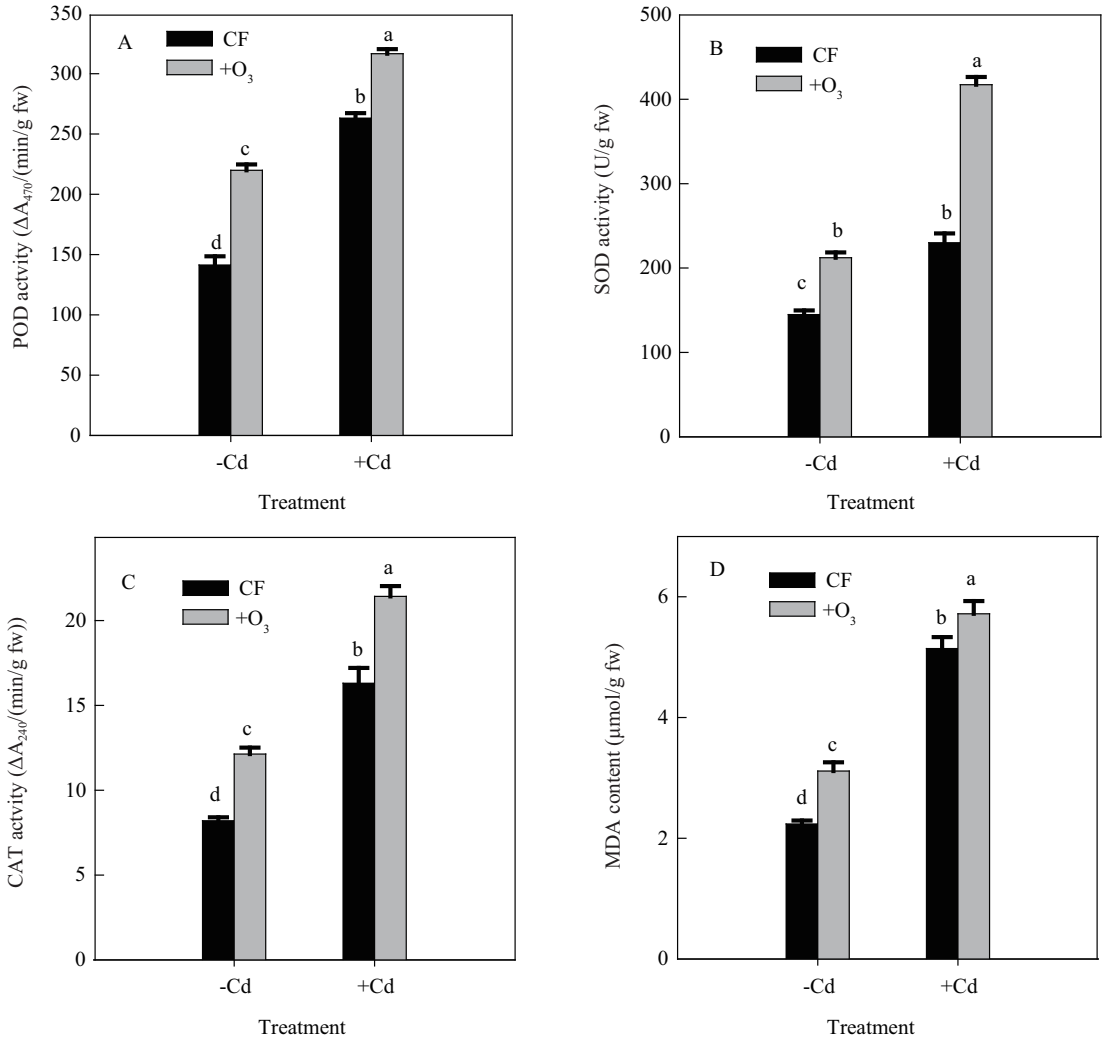

Fig. 5 Antioxidant enzymes (POD (A); SOD (B); CAT (C)) activities and MDA content (D) of winter wheat leaves in response to O_3 and Cd stress. Columns with different letters indicate significant differences at $P \leqslant 0.05$.

Toppi and Gabbrielli, 1999). Antioxidant enzymes, including SOD, CAT and POD are responsible for the removal of $O_2^{\bullet -}$ and H_2O_2, thereby preventing the formation of $^{\cdot}OH$ radicals (Sandalio et al., 2001; León et al., 2002). In the present study, O_3+Cd induced considerably higher antioxidant enzymes activities compared with the control, and significantly greater than that induced by O_3 or Cd alone, exhibiting synergistic effects of O_3 and Cd on major antioxidant enzymes (Fig. 5).

This study indicated that wheat plants reacted positively to fight against oxidative stress induced by O_3 and Cd by intensifying antioxidant activities. For instance, the activity of SOD generally increased both in O_3- and Cd-treated plants. However, a greater increase in antioxidative activities was induced under combination of both pollutants. The increase in CAT in O_3 and/or Cd treatment is also considered as an indirect evidence of enhanced anti-oxidative activities. Cd induced a decrease in CAT activity in *Pisum sativum* L. (Sandalio et al., 2001), while the sharply opposite effect was observed in *Brassica juncea* plants (Mobin and Khan, 2007). Therefore, the effects of Cd on CAT were complex and varied due to Cd concentration, plant species. In this article, CAT activity as well as POD activity increased both in O_3- and Cd-treated plants and an enhanced increment was observed in O_3+Cd treatment. Induction of MDA, which is an index of lipid peroxidation, was observed in the plants exposed to both pollutants. Whereas the further enhanced MDA content was observed in plants exposed to the combination of both pollutants, suggesting that the increased antioxidative activities might not be enough to overcome the adverse effect induced by the two pollutants. The presence of oxidative stress is further confirmed by inhibition of gas exchange, decomposition of chlorophyll, decrease in photosynthesis and reduction in biomass accumulation.

2.6 Future perspectives

Overall, synergistic effects of O_3 and Cd on gas exchange, photosynthesis, chlorophyll content, carbohydrates accumulation, and plant growth were observed in winter wheat. Cd induced further growth loss in ozonated wheat plants. From an agricultural point of view, these results might have substantially practical consequences. This study would facilitate more precise prediction model on O_3 damages to crops to ensure agricultural production security by considering agro-environmental constraints (Maggio et al., 2009), such as soil contamination by Cd in the agricultural system in peri-urban region. We consider that

further researches on the aspects of, such as, combination of O_3 and Cd effects on uptake of nutrient elements, grain filling, grain quality and yield, are necessary to provide further and fully insight of interactions between both pollutants.

3 Conclusions

(1) This study determines the combined effect of O_3 and Cd on gas exchange, growth, and antioxidant content of winter wheat. The synergistic effect of both pollutants on chlorophyll content, photosynthesis and antioxidant content improves mechanisms of growth reduction in wheat exposed to combination of O_3 and Cd .

(2) Plants that experienced high O_3 or Cd pollution exhibited increased antioxidant activities including SOD, CAT and POD, while the greatest augments were observed in O_3+Cd treatment. The further enhanced MDA content in O_3+Cd-treated plants indicated that the increased antioxidative activities might not be enough to overcome the adverse effects of both pollutants.

(3) Our findings might be important from an agricultural point of view, since the coexistence of O_3 and Cd might compromise crop production due to intensified anthropogenic activities. It's therefore necessary to conduct precise prediction on O_3 damages to crops to ensure agricultural production security by considering other crop growth constraints, such as soil contamination by Cd in an agricultural system of peri-urban regions.

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