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Combined cadmium and elevated ozone affect concentrations of cadmium and antioxidant systems in wheat under fully open-air conditions

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ABSTRACT

Pollution of the environment with both ozone (O_3) and heavy metals has been steadily increasing. An understanding of their combined effects on plants, especially crops, is limited. Here we studied the effects of elevated O_3 on oxidative stress and bioaccumulation of cadmium (Cd) in wheat under Cd stress using a free-air concentration enrichment (FACE) system. In this field experiment in Jiangdu (Jiangsu Province, China), wheat plants were grown in pots containing soil with various concentrations of cadmium (0, 2, and 10 mg kg^{-1} Cd was added to the soil) under ambient conditions and under elevated O_3 levels (50% higher than the ambient O_3). Present results showed that elevated O_3 led to higher concentrations of Cd in wheat tissues (shoots, husk and grains) with respect to contaminated soil. Combined exposure to Cd and elevated O_3 levels strongly affected the antioxidant isoenzymes POD, APX and CAT and accelerated oxidative stress in wheat leaves. Our results suggest that elevated O_3 levels cause a reduction in food quality and safety.

1. Introduction

The average ozone (O_3) concentration in the troposphere has continuous increased along with carbon dioxide (CO_2) due to global change [1]. More recent studies suggested that further globally increase in O_3 concentrations is expected, including in several parts of Asia [1,2]. The available data in China have confirmed the worrisome situation [3,4]. The monitoring in the Yangtze Delta in 1999 and 2000 showed that average daytime O_3 levels in May were between 60 and 79 ppbv [4]. Zhou [5] ever reported that 6.1–10.4% of the hourly mean O_3 concentrations have exceeded 60 ppbv, and the peak O_3 concentration was as high as 196 ppbv in the Yangtze Delta.

Tropospheric O_3 is a noticeable air pollutant, and the recent research observed that elevated O_3 decreased photosynthetic rates and chlorophyll content, reduced ascorbate in the apoplast and leaf tissue, increased lipid oxidation and accelerated leaf senescence of agricultural crops [6–13]. Together, these events contribute to crops losses and reduced growth [6–8,14–19]. The result of Avnery et al. [17] indicated that elevated O_3-induced global yield reductions ranged, from 8.5 to 14% for soybean, 3.9 to 15% for wheat, and 2.2 to 5.5% for maize. Experiments conducted in China have also shown that elevated O_3 concentrations may lead to wheat and rice losses [5,7,14,18–20].

Heavy metals increased their concentrations in the environmental as consequences of agriculture, some industrial, mining and waste disposal [21–25]. Heavy metals are toxic to plants, animals, and humans at different concentrations, and are known to cause significant environmental damage and human health problems [26–28]. Ormrod [29] ever reported that after pea plants were treated with Ni and exposed to 50 pphm O_3 for 6 h in a cube plexiglass chamber, higher Ni concentrations were found in the plants than in the controls not exposed to O_3. Similar observations of stimulation of Cd uptake in cress shoots after O_3 exposure have been reported by Czuba and Ormrod [30]. As Cagno et al. [30] reported, Cd plus O_3 treatment had a synergic effect on photosynthesis and ascorbate-dependent defences in sunflower. These studies highlight the need for a better understanding of the mechanisms by which elevated O_3 and heavy metals jointly affect plant growth, development, and uptake of metals, especially from the viewpoint of the food safety.

Among heavy metals, Cd is probably the most harmful [31]. It is well known that Cd can enter the food chain via crops uptake from contaminated soils, and pose serious threats to human health [32–36]. According to statistics, the farmland polluted by Cd in China has reached 20×10^4 ha and produces 14.6×10^8 kg of

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agricultural products every year [36]. In the future, further increases in O_3 levels and soils contamination with Cd are coexisted in China; therefore, an understanding of their combined effects on plants, especially crops, is crucial. However, whether elevated O_3 in the future, through its effect on soil Cd, will have a significant impact on the food quality and food safety is not clear.

Wheat is one of the important crops in the world, providing nutrition to a large proportion of the world's population, including China. Modem wheat (*Triticum aestivum* L.) is recognized as one of the most O_3-sensitive crops [9,14,37]. With the aim of predicting future food safety, here we present our study of the combined effect of elevated O_3 and Cd levels on wheat (*Triticum aestivum* L.) using the free-air concentration enrichment (FACE) system in farm fields in Jiangsu Province, China. In this study, different levels of Cd were artificially contaminated to soil in pots. The objective of this work was (1) to investigate the impact of elevated O_3 on Cd concentration in the wheat tissues (shoot, husk, and seed) grown on contaminated soils with three levels Cd; (2) to assess the combined effect of elevated O_3 and Cd level on the antioxidant enzymes in wheat shoot. To our knowledge, no such study has been conducted to date.

2. Materials and methods

2.1. Site description

The O_3-FACE system was established in the town of Xiaoji, Jiangdu County, Jiangsu Province, China (119°42′E, 32°35′N). A rice–wheat rotation system prevails. The region has a subtropical marine climate with mean annual precipitation of 918 mm, mean annual temperature of 14–16 °C, a total annual sunshine duration of >2000 h, and a frost-free period of approximately 220 days. The soil is Shajiang Aquic Cambosols with a sandy–loamy texture, and Cd concentrations is 0.13 mg kg^{-1}.

2.2. O_3-FACE system

The O_3-FACE system has been described by Shi et al. [18]. Briefly, this system consists of plots with elevated O_3 levels and plots with ambient O_3 levels. Each plot has an area of 240 m^2. The target O_3 concentration for elevated O_3 level plots was 50% higher than the ambient O_3 in order to simulating the tropospheric O_3 level in the 2100. Each of the elevated plots was separated from the other plots by at least 70 m to avoid cross-contamination. In the elevated O_3 level plots, the plants were grown within a 14-m in diameter octagonal ring, which consisted of eight 6-m ABS pipes. Ozone was generated by an O_3 generator (KCF-BT0.2, Jiangsu Koner Ozone Co., Ltd.) from pure O_2 to form about 5% O_3 in 95% O_2, pressurized by an air compressor, and released into the air via tiny holes in the ABS pipes of the ring at about 0.5 m above the canopy height. The quantity and direction of the O_3 release was controlled by a proportional integral derivative algorithm for computer feedback that compares achieved O_3 concentration to the target O_3 concentration of 1.5 × ambient O_3 concentration with an O_3 monitor (Thermo Electron 49i, Thermo Scientific Co., USA), a data logger-controller (Campbell CR 10X, Campbell Scientific Co., USA), an anemometer, and a wind vane. The O_3 fumigation began at 9:00 a.m. and continued to 4:00 p.m. until harvest, except the occasions such as rain or wet leaves (see Shi et al. [37] for details). Plants in the plots within the octagon rings were sampled at least 2 m from the ABS pipes to avoid the much higher O_3 concentrations near the release holes. In ambient O_3 plots, plants were grown under ambient O_3 concentrations without the ring structures. Further details of O_3 exposure have been reported by Feng et al. [11,12].

2.3. Crop cultivation

Two FACE plots and ambient plots were used in this experiment. Yangmai 14 (*Triticum aestivum* L.) is one of the most common cultivar in China, and was chosen as test cultivar. Ten thousand grams of dry soil collected from a local farm was placed in each plastic pot (20 cm diameter and 35 cm height). Specified amounts of Cd in the form of a dissolved solution of $Cd(NO_3)_2$ were added and thoroughly mixed into the soil as: 0, 2 and 10 mg kg^{-1}, respectively. The treatment with no added Cd acted as the control. Each treatment was applied to six replicate pots. Three pots were randomly placed in two FACE plots, and another three pots were randomly placed in two ambient plots. The seeds of cultivars were manually sown at a density of ten seedlings per pot. After germination of the seeds, the pots were placed into FACE plots and ambient plots. The cultivation, and crop health measures, were similar to those used by local farmers. The first seeds were planted on 15 November, 2007 and the plants were harvested on 29 May 2008, and O_3 fumigation began on 5 March 2008 at tillering stage of wheat and continued to the harvest. The second sowing was on 17 November 2008, with harvest on 31 May 2009, and O_3 fumigation began on 1 March 2009 continued to the harvest.

Wheat of the first year were sampled at the grain maturity stage (29 May 2008) for analysis of Cd in grains. And those of the second year were sampled at the panicle initiation (20 April 2009) and grain maturity stages (31 May 2009). At the panicle initiation, antioxidant isoenzymes and Cd levels in wheat shoots were analyzed, and the tissue used for biochemical analyses was frozen in liquid N_2 and stored at −80 °C until analysis. At grain maturity stages of the 2009, the plants of wheat divided into three parts (shoots, husks, and grains), and the Cd levels in shoots, husks, and grains were analyzed.

2.4. Determination of Cd

After harvest, the plants were thoroughly washed with tap water and then with deionized water, and were then oven-dried to a constant weight at 70 °C. The dried samples were ground, weighed, and digested with concentrated $HNO_3/HClO_4$ (4:1, v/v) [38]. The Cd concentration in the digested solution was analyzed by atomic absorption spectroscopy (Thermo Sollar M6, USA).

2.5. Isoenzyme assay

Enzyme extraction was prepared according to method of García-Limones et al. [39]. Isoenzyme patterns were resolved by native polyacrylamide gel electrophoresis using a high-throughput mini-protein 3 electrophoresis system (Bio-Rad, USA) (8% separating gel, and 5% stacking gel). Crude enzyme, corresponding to 23.2 μg of total soluble protein, mixed with 50% glycerin and 0.1% bromophenol blue, was loaded onto each lane. The antioxidant isoenzymes were separated by electrophoresis at a constant voltage of 70 V until the separating gel was reached, and then at 100 V. The running buffer was 25 mM Tris, 192 mM glycine (pH 8.3).

Superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) isoenzymes in the gels were visualized as described by García-Limones et al. [39]. Catalase (CAT) isoenzyme in the gel was visualized as described by Verma and Dubey [40]. Representative photographs were taken with a Canon camera. Integrated intensities of activity bands were quantified using Image J software (National Institutes of Health, USA). And then enzyme activities were determined based on integrated intensities of isozymes' bands according to Barabás et al. [41] and Biemelt et al. [42].

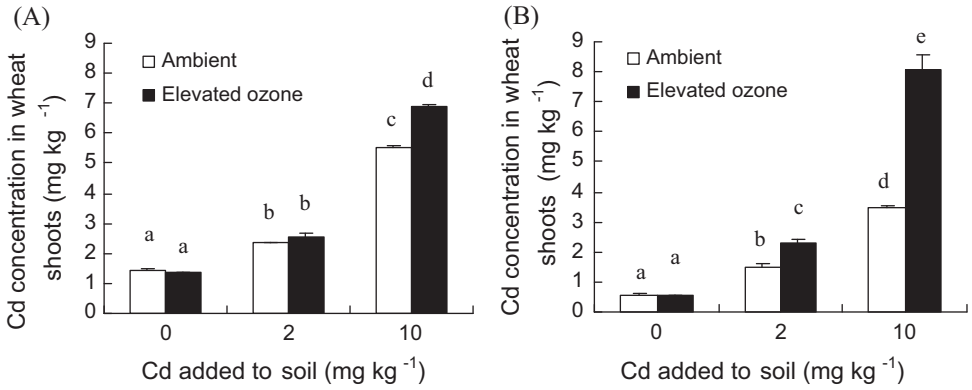

Fig. 1. Cadmium concentration in wheat shoots (A) at the panicle-initiation growth stage, and (B) at grain maturity of 2009. The lower-case letters above the bars indicate the statistical differences of the treatments; those with the same letter are not significantly different according to Student's-*t* test ($p < 0.05$).

2.6. Statistics

Data were expressed as means ± standard deviation ($n = 2$, n represents two plots) and statistical analysis was performed using the SPSS software program (SPSS Inc., Chicago, IL, Version 16.0). The data were analyzed with a two-way analysis of variance (ANOVA) approach, that is, Cd treatment (three levels) and O_3 treatment (two levels). The Student's-*t* test was also performed to distinguish among treatments. Differences between treatments were taken to be statistically significant when they occurred at $p < 0.05$.

3. Results

3.1. Effects of elevated O_3 on the Cd concentration in wheat shoots

Significant differences in the Cd concentrations were found in the shoots, depending upon soil Cd level and O_3 level (Fig. 1; Table 1). Elevated O_3 levels resulted in higher Cd concentrations in shoots of plants grown in soils contaminated with Cd (Fig. 1). At the panicle-initiation stage (Fig. 1A), the Cd concentration in shoots of plants grown under elevated O_3 levels with 10 mg kg^{-1} Cd in the soil was 24.7% higher than in shoots of wheat grown under ambient O_3 levels with 10 mg kg^{-1} Cd. At the grain maturity growth stage (Fig. 1B), the Cd concentration in shoots of plants grown under elevated O_3 levels with 2 or 10 mg kg^{-1} Cd in the soil was 53.9% and 131% higher than in shoots of wheat grown under ambient O_3 levels with 2 or 10 mg kg^{-1} Cd.

3.2. Effects of elevated O_3 on the Cd concentration in wheat husks

Significant differences in the Cd concentrations were found husks of the wheat, depending upon soil Cd level and O_3 level (Fig. 2; Table 1). The Cd concentration in husks of plants grown at elevated O_3 levels with 10 mg kg^{-1} Cd in the soil was 79.4% higher than in tissues of wheat grown under ambient O_3 levels with 10 mg kg^{-1} Cd.

3.3. Effects of elevated O_3 on the Cd concentration in wheat grains

Elevated O_3 levels resulted in higher Cd concentrations in grains of wheat grown in soils contaminated with Cd, and the results of two years have not significant difference (Fig. 3; Table 1). At grain maturity in the first harvest, the Cd concentrations in wheat grains from plants grown under elevated O_3 levels with 10 mg kg^{-1} Cd in the soil increased by 31.6% compared to that grown under ambient O_3 levels with 10 mg kg^{-1} Cd. However, the Cd concentration in grains was not significantly affected by elevated O_3 levels when the plants were grown at control and 2 mg kg^{-1} Cd. A similar trend was detected from the second year, and the Cd concentration in wheat grains of plants grown at elevated O_3 levels with 10 mg kg^{-1} Cd in the soil was 44.5% higher than in tissues of wheat grown under ambient O_3 levels with 10 mg kg^{-1} Cd.

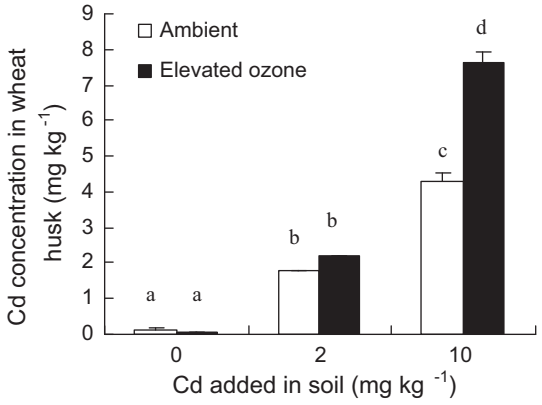

Fig. 2. Cadmium concentration in wheat husks at grain maturity of 2009. The lower-case letters above the bars indicate the statistical differences of the treatments; those with the same letter are not significantly different according to Student's-*t* test ($p < 0.05$).

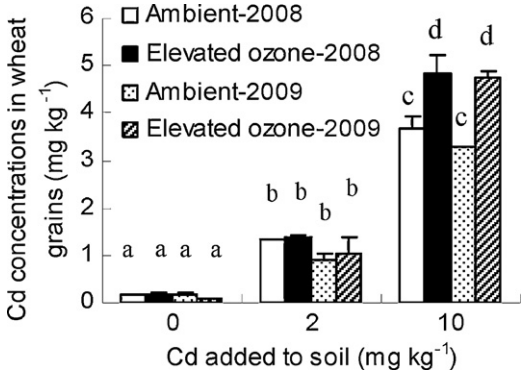

Fig. 3. Cadmium concentrations in wheat grains (A) from the 2008 and (B) 2009 harvest. The lower-case letters above the bars indicate the statistical differences of the treatments; those with the same letter are not significantly different according to Student's-*t* test ($p < 0.05$).

Table 1
Analysis of variance of interactive effects of Cd and elevated O_3 on investigated variables in wheat cultivars Yangmai 14.

		Cd	O_3	Cd × O_3
Cd concentration in shoots	Panicle-initiation stage of 2009	0.000	0.000	0.000
	Grain maturity stage of 2009	0.000	0.000	0.000
Cd concentration in husks	Grain maturity stage of 2009	0.000	0.000	0.000
Cd concentration in grains	Grain maturity stage of 2008	0.000	0.007	0.008
	Grain maturity stage of 2009	0.000	0.001	0.001
POD in shoots	Panicle-initiation stage of 2009	0.003	0.011	0.000
APX in shoots	Panicle-initiation stage of 2009	0.020	0.853	0.008
CAT in shoots	Panicle-initiation stage of 2009	0.013	0.072	0.003
SOD in shoots	Panicle-initiation stage of 2009	0.308	0.918	0.152

3.4. Effects of Cd and elevated O_3 on antioxidant isoenzymes in wheat shoots

Patterns of antioxidant isoenzymes bands in wheat shoots in the panicle-initiation growth stage of the second year were shown Fig. 4. Except for POD, the APX, CAT, and SOD activities in wheat shoots were not significantly affected by elevated O_3 alone (Table 1 and Fig. 4). And POD, APX and CAT activities in wheat shoots were significantly affected by Cd alone, and this effect was aggravated by the interaction between elevated O_3 and Cd levels (Table 1 and Fig. 4). At 10 mg kg^{-1} Cd alone, the POD, APX, and CAT activities in wheat shoots were induced, but at combined 10 mg kg^{-1} Cd and elevated O_3 levels, the activities were lower than with the control. The activities of SOD in wheat shoots were not significantly affected by Cd or elevated O_3.

4. Discussion

In the present study, our data showed that elevated O_3 levels resulted in an increased concentration of Cd in wheat plants grown on Cd-contaminated soils, and that the antioxidant isoenzymes POD, APX and CAT in wheat shoots were strongly affected by combined exposure to Cd and elevated O_3 levels, and thereby leading to a reduction in food quality and safety.

Throughout the first and second wheat growing seasons, the wheat grown on elevated O_3 plots with 10 mg kg^{-1} Cd in the soil showed an increased concentration of Cd in the plant tissues compared to wheat grown on ambient plots; but in control and most of 2 mg kg^{-1} Cd treatments, the concentration of Cd in the plant tissues grown on elevated O_3 did not differ significantly from ambient. In uncontaminated soils with elevated O_3 levels, Pleijel et al. [43] also reported that no significantly effect were observed in wheat grain Cd concentrations in an open top chamber (OTC) experiment; Zhang et al. [44] observed that elevated O_3 increase the K and P concentrations in wheat grain in the FACE system. Ormrod reported that after pea plants were treated with Ni and exposed to 50 pphm O_3 for 6 h in a cube plexiglass chamber, higher Ni concentrations were found in the plants than in the controls not exposed to O_3 [29]. Similar observations of stimulation of Cd uptake in cress shoots after O_3 exposure have been reported by Czuba and Ormrod [30], who postulated that O_3 stimulation of Cd uptake could contribute to a dual mechanism of toxicity in tissues, especially when Cd concentrations were high. High concentrations of both Cd [45] and O_3 [46] contribute to the breakdown of plant macromolecules and proteins in leaves. Czuba and Ormrod [30] proposed that the action of both of these agents could induce enough serious injury to increase permeability and allow freer ion movement in tissues. Besides, many studies have shown that elevated O_3 levels reduced crops growth and yields [7,8,18–20], including a study using a same FACE system that reported that elevated O_3 levels reduced the grain yield of hybrid rice Shanyou 63, Liangyoupeijiu, and Wuyujing 3 by 17.5%, 15.0% and 6.3%, of winter wheat (*Triticum aestivum* L.) by 20%, respectively [7,18,20]. Then, considering that the elevated O_3 reduce biomass production, the concentration of a particular element in plants will (i) decrease if the same amount of the element is taken up as under ambient O_3 levels, leading to dilution phenomena; (ii) not change if the enhancement in uptake of the element is similar to the increase in biomass; or (iii) increase if the amount of element taken up is higher than the biomass response, leading to accumulation. The higher concentrations of Cd in wheat observed in our study were probably due to the change in biomass under elevated O_3 conditions. However, evidence supporting the above two explanation is lacking. Further studies are needed to characterize the mechanisms of how O_3 increase higher concentration of heavy metal in plant tissues.

Whether elevated O_3 increase uptake of Cd or not, in the present study, the Cd concentration in wheat grains of all samples far exceeded the legal limits (wheat flour: 0.1 mg kg^{-1}) [47]; and after exposed to elevated O_3 level, the Cd concentration in wheat grains is more up to the legal limits than that of the ambient. Cadmium can accumulate in the human body and damage kidneys, bones, and reproductive system [48]. To keep the Cd levels in creatinine in urine below 1 μg Cd g^{-1} in 95% of the population by age 50, the European Food Safety Authority (EFSA) has suggested that the average daily dietary Cd intake should not exceed 0.36 μg Cd/kg body weight, which corresponds to a weekly dietary intake of 2.52 μg Cd/kg body weight [49]. For an average adult of 60 kg with a daily intake of 261.1 g rice or wheat [50], this estimated weekly dietary intake would be far exceeded by all of the wheat samples from our study grown in contaminated soils, and elevated O_3 levels significantly increased the Cd intake. In China, farmland polluted by Cd has reached 20×10^4 ha and produces 14.6×10^8 kg of agricultural products annually [36]. Furthermore, wheat is one of the important crops, and lots of population in China currently depends on wheat as staple foods. The high, toxic concentrations of Cd accumulated in crops threaten food quality and safety. This threat will increase as the CO_2 levels increase in the future.

Many environmental stresses including O_3 and Cd, generate oxidative stress in plant tissues by inducing an over-production of ROS, such as superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^\bullet OH$) [51–54]. To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymes such as POD, APX, SOD and CAT. Under normal physiological conditions, the ROS generated from metabolism of extraneous chemicals in the body can be removal well by the antioxidant defense system. However, when ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and significant oxidative damage [55]. In the present study, the change of the antioxidative defense system in wheat shoots suggested that elevated O_3 levels plus high levels of Cd had a significantly synergic effect on oxidative stress in wheat.

Di Cagno et al. [31] ever reported that O_3 fumigation induced a remarkable increase of POD activity in sunflower in the controlled environmental chamber study. Singh et al. [8] reported that the activity of CAT decreased whereas POD increased in two soybean

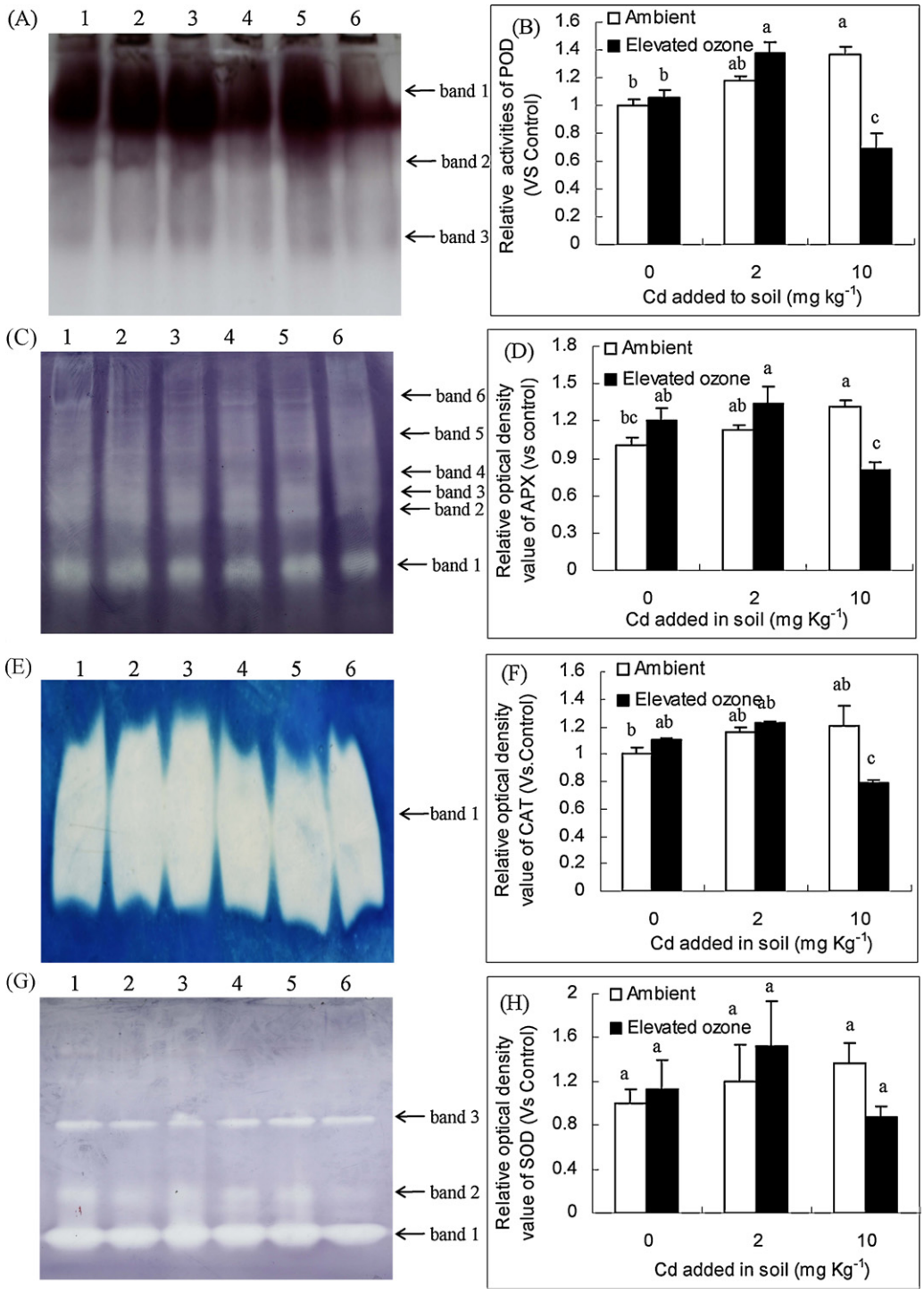

Fig. 4. Effect of Cd and elevated O_3 on antioxidative isoenzymes in wheat shoots at the panicle-initiation growth stage of 2009. (A and B) POD; (C and D) APX; (E and F) CAT; and (G and H) SOD. (A, C, E, G) Activity staining of isoenzymes in native polyacrylamide gels. Arrows indicate bands of activity. Lanes 1–3: 0 (control), 2, and 10 mg kg^{-1} Cd in the soil at ambient O_3 levels, respectively. Lanes 4–6: 0, 2, and 10 mg kg^{-1} Cd in the soil under elevated O_3 levels, respectively. (B, D, F, H) Enzyme activities determined by integrating the band intensities. The lower-case letters above the bars indicate the statistical differences of the treatments; those with the same letter are not significantly different according to Student's-*t* test ($p < 0.05$).

varieties upon exposure to O_3 in the open top chambers experiment. Rai and Agrawal [56] found that SOD and POD activities, ascorbic acid and total phenolics were higher in two rice (*Oryza sativa* L.) cultivars using open top chambers. However, in this study, POD, APX, CAT, and SOD activities in the wheat shoots were not significantly affected by elevated O_3 alone, which could be due to the plant cultivars variation. Feng et al. [11,12] ever indicated that wheat variety Yangfumai 2 (Y2) was more sensitive to O_3 than Yangmai 16 (Y16) in the same FACE system. Our study showed that Yangfumai 14 may be not sensitive to O_3 alone.

Our study also showed, while no significant change was observed for 2 mg kg^{-1} Cd or O_3 single treatment, a remarkable increase was shown in POD and APX activities in wheat shoots subjected to both stresses. The results suggested that there was an increase in the production of ROS by combined Cd and elevated O_3, and wheat plants tried to cope with the oxidative stress induced

by these two pollutants by strengthening their antioxidant capabilities, then the activities of POD and APX increased markedly. Di Cagno et al. reported similar results when sunflower plants were exposed to these two pollutants in a chamber experiment [31]. However, the APX, POD, and CAT activities were lower when plants were exposed simultaneously to a higher concentration of Cd ($10\,mg\,kg^{-1}$) and elevated O_3 levels. A possible explanation for the decrease in enzyme activities was that after simultaneously exposure to$10\,mg\,kg^{-1}$ Cd and elevated O_3, higher Cd concentrations accumulated in wheat tissue, more and more ROS could be produced, and then the increased ROS generation exceeded the ability of these enzymes to eliminate the ROS, leading to the inactivation of the enzymes. Then, elevated O_3 levels plus a high concentration of Cd in soil accelerate oxidative stress in wheat, and subsequently probably cause molecular damage in living system and reduced growth.

5. Conclusion

In conclusion, our study using a FACE system showed that elevated O_3 levels plus a high concentration of Cd had a significant synergic effect on oxidative stress in wheat leaves. Our data also showed that elevated O_3 levels lead to a higher Cd concentration in wheat grown in Cd-contaminated soils, suggesting that elevated O_3 levels cause a reduction in food quality. The data presented here were obtained from crops grown in artificially contaminated soils in pots. More data need to be collected from crops grown under a wide range of soil conditions and realistic field conditions to make better predictions on the combined effects of elevated O_3 levels and multi-metal-contaminated soils on the metal uptake by crops and thereby on their contribution to food quality and safety at the world scale.

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