Physiological and visible injury responses in different growth stages of winter wheat to ozone stress and the protection of spermidine

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ABSTRACT

The open top chamber (OTC) method was used in a farmland to study the influence of different levels of O3 concentrations (40 ppb, 80 ppb and 120 ppb) on the enzymatic activity and metabolite contents of the antioxidation system of the winter wheat leaves during the jointing, heading and milk stage. The protective effect of exogenous spermidine (Spd) against the antioxidation of winter wheat under the O3 stress was investigated. With the increasing O3 concentrations and fumigation time, the injuries of the winter wheat leaves were observed to be more serious. For instance, when the O3 concentration reached 120 ppb, the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and nitrate reductase (NR) in the jointing stage decreased by 50.3%, 64.9%, 75.5% and 92.9%, respectively; peroxidase (POD) and glutathione reductase (GR) increased by 45.1% and 80.5%, respectively; the contents of malondialdehyde (MDA), ascorbic acid (AsA) and reduced glutathione (GSH) increased by 314.3%, 8.4% and 31.7%, respectively; and the soluble protein (SP) content decreased by 47.5%. The O3 stress also had significant impact on the contents of proline (Pro), NO3−–N and NH4+–N of the winter wheat leaves. During the heading stage, when the O3 concentration was 40 ppb and 80 ppb, the content of Pro was 163.9% and 173.2% higher than that in the control group, respectively. But under 120 ppb, it was decreased by 42.4%. Exogenous application of Spd increased the activities of SOD, POD, CAT, APX and GR, as well as the contents of GSH and SP, but decreased the contents of MDA and AsA. This indicates that Spd is an effective antioxidant to relieve the O3 stress on winter wheat leaves, thereby might be applicable to protect winter wheat from the harm of O3.

Keywords: Winter wheat, ozone, injury, antioxidation system, spermidine

doi: 10.5094/APR.2015.067

1. Introduction

The extensive use of fossil–fuel and nitrogen–based fertilizers has dramatically increased emissions of nitrogen oxides (NOx) and volatile organic compounds (VOCs) into the atmosphere. It was predicted that the concentration of O3 in the atmosphere will be continuously rising and the pollution is expanding (Fishman, 1991). The surface layer O3, as one of the most important atmospheric pollutants, has been increasing and became the focus of worldwide researchers and the public (Vingarzan, 2004; Selin et al., 2009). The monitoring data of O3 concentration in 86 sites in the rural and remote regions of 35 states in US showed that the days with a daily average over 80 ppb numbered 21.1 d each year; and the highest daily average of three months was 54 ppb (McCready and Andersen, 2000). Recently, researchers reported that the surface layer one–hour average O3 concentration in Beijing–Tianjin–Tangshan region, Yangtze River Delta and other regions reached as high as 150 ppb (Shao et al., 2006).

Many studies reported that the O3 stress might cause the following negative effects to plants: injury, retarded growth, decreased stomatal conductance of leaves, lower photosynthesis rate, inhibited growth of plant height and leaf area, accelerated aging, disordered metabolism of carbon and nitrogen and crop yield reduction (Feng et al., 2003; Kontunen–Soppela et al., 2007; Feng et al., 2008; Mills et al., 2009; Wittig et al., 2009; Zhu et al., 2011; Avenery et al., 2013). Huang et al. (2012) studied the influence of O3 on the visible injury of rice leaves, nitrogen metabolism, contents of saccharides and proteins in rice grains.

Van Dingenen et al. (2009) have estimated the risk to crop damage caused by surface ozone based on two types of exposure indicators (seasonally mean daytime ozone concentration, and seasonally accumulated daytime ozone concentration above 40 ppb). It was suggested that crops have great responses to cumulative exposures to O3 in the range 50–87 ppb in the USA and 35–60 ppb in Europe (Mills et al., 2007). Field experiment studies in European Open Top Chambers Programme (EOTCP) showed that crops yield losses from O3 pollution occurred by 5–10% and deteriorated crop quality. And also larger losses are expected in the future (Grunhage et al., 2012; Debaje, 2014). Nataliya et al. (2011) estimated economic losses for wheat, rice, maize, and soybean for China, South Korea and Japan to be up to 9% for the cereal crops and 23–27% for soybean or 5 billion dollars for all crops. In Europe, the standard for the protection of vegetation against ozone damage is expressed as a critical level of accumulated ozone concentration above a threshold of 40 ppbV which should not be exceeded during the growing season (3 ppm h for agricultural crops, 5 ppm h for forests) (Van Dingenen et al., 2009).

After entering into the plant, O3 induces the generation of reactive oxygen species (ROS), including H2O2, superoxide radicals (O2−) and ·OH. The ROS damages the membrane system of the plants and aggravates the peroxidation of the membrane lipid, leading to physiological function disorder of plants, especially photosynthesis process (Pasqualini et al., 2002; Calatayud et al., 2003). Malondialdehyde (MDA) is an end–product of the radical–initiated oxidative decomposition of polysaturated fatty acids; therefore, it is frequently used as a biomarker of oxidative stress.
An enhanced level of lipid peroxidation, as indicated by higher MDA content, can show an oxidative stress under the effect of a high O₃ concentration. During the natural adaptation process, plants also develop a series of antioxidation system. By increasing the activity of the antioxidation system, the stress resistance of plant could be enhanced, which relieves the injury of oxidation (Hofer et al., 2008). The antioxidation system is composed of antioxidases and non–enzyme compounds with high reducibility. Antioxidases mainly include superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), which play an important role in the clearing of active oxygen, as well as glutathione reductase (GR) and ascorbate peroxidase (APX) that are crucial in the ascorbate–glutathione (AsA–GSH) cycle (also called Halliwell–Asada pathway). Non–enzyme compounds include AsA, GSH and carotenoids (Car). Polymamines (PAs) are nitrogenous bases belonging to fatty group with biological activity that are produced during metabolic process. They mainly include putrescine (Put), spermine (Spm) and spermidine (Spd). PAs participates in a variety of physiological processes such as seed germination, rooting, embryogenesis, pollen tube growth and fruit formation. They have an important role in membrane stability, free radical clearing, osmotic adjustment, mineral nutrition and ageing adjustment of plants (Martin–Tanguy, 2001; Shoeb et al., 2001; Groppa et al., 2003; Kusano et al., 2008). Exogenous application of PAs increased the resistance of plants to drought (Capell et al., 2004; Yang et al., 2007), osmotic stress (Tonom et al., 2004; Legocka and Kluk, 2005) and heavy metal stress (Groppa et al., 2003; Xu et al., 2011). Spd is also PA, and its role in the growth, development and resistance of plants to environmental stresses has been reported (Zhu et al., 2006). Compared with Put and Spm, Spd is more efficient in the resistance to environmental stresses (Duan et al., 2008).

The present study investigated the influence of the increase of O₃ concentration on the injury and the antioxidation system of winter wheat leaves at different stages by open top chamber (OTC) method in farmlands. The influence of exogenous application of Spd on the changes of the physiological indexes of winter wheat was also studied. The objective was to clarify the influence of O₃ stress on the antioxidation system of winter wheat leaves and the relief mechanism of Spd on O₃ stress injuries.

2. Materials and Methods

2.1. Experiment site

The experiment site is located in the Seed Management Station of Changping, Northwest Beijing (40°12′N, 116°8′E), China. The station is characterized by continental monsoon climate and four distinct seasons. The mean annual temperature is 11.8 °C. The mean annual sunshine is 2684 hours, and the frost–free season lasts for about 200 days. The basic physicochemical properties of soil are as follows: organic matter contents 16.4 g kg⁻¹ soil; total nitrogen 0.9 g kg⁻¹ soil; available phosphorus 38.1 mg kg⁻¹ soil; available potassium 102.1 mg kg⁻¹ soil; and soil pH 8.3.

2.2. Plant materials

The variety used in the experiment was *Triticum aestivum* L. Beijing 9549, provided by Beijing Agricultural College. The seeds were sowed on September 28, 2009. On April 26, 2010, urea was applied (225 kg ha⁻¹). The field management was coherent to that of the local farms during the entire growth season of winter wheat.

2.3. Ozone fumigation

In–situ ozone fumigation was carried out on winter wheat with the self–made open–top fumigation system which consisted of the open–top box, gas distribution system, air blower, ozone generator, and O₃ concentration control system and ozone analyzer (Figure S1). With skeleton made of reinforcing steel bar, the open–top box was manufactured into an octahedron with a 45° contracted aperture on top. The outside of the box was covered with transparent polyethylene thin film. The box had a side length of 1 m and a height of 2.7 m, with a coverage area of approximate 4.8 m². The ozone was generated from the medical pure oxygen (99.5%) through high–pressure discharge process in the ozone generator (SK–CFG–3, Jinan Sankang Envi–Tech Co., Ltd.). The oxygen flow rate was adjusted with mass flow meter (GF317, Aalborg Industries Inc.) and Kingview industrial control software (MCGS 6.2, Beijing Kunlun Industrial Control Technology Development Co., Ltd.) to control the ozone concentration. The ozone concentration in the box and in ambient atmosphere was continuously monitored with two ozone analyzers (Model 49c, Thermo Electron Co., Franklin, MA) (Huang et al., 2012). The standard deviation of the actual test data of ozone homogeneity was below 4.4%, and the coefficient of variation was below 7.8%. The variation tendency of the sampling point and the fixed control point was consistent, which satisfied the requirement of this experiment. The light intensity, humidity and temperature in the OTC were 500–1 100 μmol m⁻² s⁻¹, 60–85% and 25–45 °C, respectively.

Four concentrations of O₃ were set up: ambient atmosphere filtered by activated carbon 5 ppb control check (CK), 40 ppb, 80 ppb and 120 ppb. Each treatment had three replicates. The O₃ fumigation on the winter wheat started on April 5, 2010. The fumigation lasted for 9 h (8:00–17:00) each day and stopped on June 12. During the O₃ fumigation, injuries of the plants were observed and recorded carefully. Fresh leaves were collected during the jointing stage (April 27), heading stage (May 13) and milk stage (June 8). For each sampling point, 15–20 leaves were randomly collected. The samples were immediately treated with liquid nitrogen and stored at −80 °C. Because the leaves in the milk stage withered largely after O₃ fumigation and few fresh leaves were left, only Nitrate reductase activity (NR activity), NO₃⁻–N, NH₄⁺–N and Pro contents were measured.

2.4. Spd application experiment

Potted planting experiment was used to study the protective effect of Spd for winter wheat under the ozone stress. The winter variety was also *Triticum aestivum* L. Beijing 9549, provided by Beijing Agricultural College. The plastic pots had a diameter of 20 cm and a height of 25 cm. The soil was the surface soil of 20 cm in the experimental site. After passing through a 2 mm sieve, the soil was homogenized and placed in the pots. Every pot contained 1.5 kg of soil. Before transplantation of the winter wheat seedlings, fertilizers (0.428 g kg⁻¹ urea, 0.323 g kg⁻¹ CaHPO₄·2H₂O and 0.247 g kg⁻¹ K₂SO₄) were added to the soil. In each pot, 10 winter wheat seedlings of 10 cm tall at the same growth status were transplanted. After the survival of the seedlings was confirmed, six seedlings were retained for each pot. The winter wheat seedlings were transferred to the OTC for O₃ fumigation and Spd spraying.

Under the O₃ fumigation of 120 ppb, different concentrations of Spd were sprayed: distilled water (control), 0.25 mmol L⁻¹ Spd, 0.50 mmol L⁻¹ Spd and 0.75 mmol L⁻¹ Spd. Each treatment had four replicates. Spd was sprayed at 8:00 am and 6:00 pm every day by the foliar application method. The application amount was 50 mL for each pot every time. The spraying lasted for 2 weeks. Then, 15 to 20 fresh leaves were randomly collected. The samples were immediately treated with liquid nitrogen and stored at −80 °C.

2.5. Measurement of physiological indexes

Enzyme extraction: 0.5 g fresh leaves and 10 mL of 50 mmol L⁻¹ phosphate buffer (pH 7.8, containing 1% vinyl pyrrolidone) were added together with a small amount of quartz. The leaves were ground to homogenate on an ice bath. The samples were centrifuged at 15 000 r min⁻¹ for 4 min and the supernatant was collected for preservation under low temperature.
SOD activity was assayed in a 3-mL reaction mixture. The absorbance of solution was tested by measurement of its capacity of inhibiting the photochemical reduction of nitro–blue tetrazolium (NBT) at 560 nm (Chen and Pan, 1996). The POD activity was measured by the guaiacol method (Beffa et al., 1990). The CAT activity was measured with the ultraviolet spectrophotometer (Aebi and Packer, 1984). For the GR assay, the reaction mixture contained 0.82 mL of 1 mM oxidized glutathione, 0.08 mL of 2 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 0.1 mL enzyme extract. The consumption of NADPH was followed as the decrease in absorbance at 340 nm during the first minute of the reaction (Krivosheeva et al., 1996). The NR activity was measured in vivo by reference to the method of Tachibana et al., (1991). The AsA content was measured according to Krivosheeva et al. (1996). The measurement of MDA content was referred to the TBA colorimetry (Heath and Packer, 1968). The SP content was measured by Coomassie brilliant blue G-250 staining method. The measurement of NO$_3$–N and NH$_4$–N contents was referred to the method of Lu et al. (2004). The Pro content was measured by acid–ninhydrine method (Li et al., 2000).

2.6. Statistical analysis and processing

SPSS 13.0 and Origin 8.0 software were used for statistical analysis of data for SOD, CAT, POD, APX, NR, GR, MDA, AsA, GSH, SP, Pro, NO$_3$–N and NH$_4$–N at different O$_3$ and Spd treatments. Differences were considered significant at $p<0.05$.

3. Results

3.1. Visible injury of winter wheat leaves

The influence of O$_3$ stress on the foliar symptoms of winter wheat is shown in Figure 1. Under the O$_3$ fumigation at different concentrations, winter wheat leaves in the jointing stage showed no visible injury. During the heading stage, the O$_3$ stress caused apparent injury to the leaves. Particularly, under the highest concentration of O$_3$ fumigation (120 ppb), the leaves were the first to exhibit visible injury. First, brown spots appeared at the two sides of veins and at the tip of the leaves; then, the area of the spots began to expand; and rust spots could be observed on the leaves. During the late stage of O$_3$ fumigation (i.e. the milk stage), the entire plant of winter wheat was withered, indicating a serious injury caused by the O$_3$ stress. The winter wheat leaves under the O$_3$ concentration of 40 ppb showed injury 15 days later than that of 120 ppb. In this study, the former did not show obvious injury until the heading stage. The symptoms were also less serious than the 120 ppb treatment. The O$_3$ fumigation caused the retardation of spike growth and the earlier ripening. Therefore, the biomass and yield were reduced.

![Figure 1. Effects of elevated ozone concentrations on foliar symptom of winter wheat at different growing stages.](image)

3.2. Enzyme activities in winter wheat leaves under ozone stress

During the jointing stage of winter wheat, there was no significant difference for POD activity of the leaves in different O$_3$ treatments. In the heading stage, the POD activity showed an increasing tendency under O$_3$ stress (except for the treatment of 80 ppb) (Figure 2). When the O$_3$ concentration was 120 ppb, the POD activity was 210.7% higher than that of the control. The SOD activity decreased with the increasing O$_3$ concentration ($p<0.05$) in the jointing stage. When the O$_3$ concentration was 40, 80 and 120 ppb, the SOD activity decreased by 27.5%, 48.2% and 50.3%, respectively, compared to the control. In the heading stage, the SOD activity under O$_3$ concentrations of 40 and 120 ppb decreased significantly compared with the control. However, the SOD activity under 80 ppb was increased.

Under the treatment of low O$_3$ concentration (40 ppb) in the jointing stage, the CAT activity showed no significant difference from the control group. But under the O$_3$ concentrations of 80 and 120 ppb, the CAT activity was decreased significantly by 57.1% and 64.9%, respectively. In the heading stage, the CAT activity displayed a clear decreasing tendency ($p<0.05$) with the increasing O$_3$ concentrations. During the jointing stage, a low concentration of O$_3$ (40 ppb) significantly increased the APX activity. But with the increasing O$_3$ concentration, the APX activity tended to decrease. Most of the APX activity showed no significant difference from the control in the heading stage except that it increased by 104.5% under the O$_3$ concentration of 80 ppb.

During each stage, the GR activity under 40 and 80 ppb treatments had no significant difference as compared to the control. Under O$_3$ concentration of 120 ppb in the jointing stage, the GR activity was increased by 80.5% as compared to the control (Figure 2). But when the O$_3$ concentration was 120 ppb in the heading stage, the GR activity was lowered by 70.4%. The O$_3$ stress caused the NR activity to be significantly lower than that of the control. With the increasing O$_3$ concentration, the NR activity decreased constantly (Figure 4). When the O$_3$ concentration was 40, 80 and 120 ppb, the NR activity decreased by 45.5%, 68.9% and 92.9% in the jointing stage; by 58.0%, 78.8% and 93.5% in the heading stage; by 90.9%, 90.0% and 93.2% in the milk stage, respectively, as compared with the control.

3.3. Metabolite contents in winter wheat leaves under ozone stress

The MDA content of winter wheat leaves under low O$_3$ concentrations had no significant difference from that of the control, in both jointing and heading stages. But under the high concentration of O$_3$ (120 ppb), the MDA content was increased by 314.3% and 65.0% during the jointing and heading stages, respectively. When the O$_3$ concentration was 120 ppb in the jointing stage, the SP content had a significant decrease by 47.5%. In the heading stage, the SP content under O$_3$ stress was significantly higher than that of the control ($p<0.05$).

In the jointing stage, the AsA content was increased when the O$_3$ concentration was 40 ppb and 120 ppb, while it was decreased under the O$_3$ concentration of 80 ppb (Figure 3). In the heading stage, the AsA content under 120 ppb was increased by 63.8% ($p<0.05$). There were significant increases of the GSH content ($p<0.05$) during the jointing stage. During the heading stage, the GSH content was increased under 40 ppb O$_3$. But both the O$_3$ stress of 80 and 120 ppb caused a significant decrease of the GSH.
Figure 2. Effects of elevated ozone on enzyme activities in winter wheat leaves. Error bars represent standard deviations. Asterisks above bars indicate significant differences between O₃ concentrations and CK treatment at p<0.05.

Figure 3. Effects of elevated ozone on metabolite contents in winter wheat leaves. Error bars represent standard deviations. Asterisks above bars indicate significant differences between O₃ concentrations and CK treatment at p<0.05.
The Pro content of winter wheat leaves was significantly higher in the heading stage than in the jointing and the milk stages. During the initial period of O₃ fumigation (i.e., the jointing stage), the Pro content tended to increase with the rise of O₃ concentration (Figure 4). When in the heading stage, the Pro content increased from 228.0 µg Pro g⁻¹ FW (control treatment) to 601.6 µg Pro g⁻¹ FW under O₃ concentration of 40 ppb, and to 622.8 µg Pro g⁻¹ FW under O₃ concentration of 80 ppb. The increase magnitudes were 163.9% and 173.2%, respectively. Later, it decreased dramatically to 131.4 µg Pro g⁻¹ FW, by 42.4% under O₃ concentration of 120 ppb. During the milk stage, the Pro content under the O₃ stress was significantly lower than that of the control.

During different growth stages, the NO₃⁻–N had no significant changes as compared to the control under the O₃ concentration of 40 ppb and 80 ppb. However, when the O₃ reached 120 ppb, the NO₃⁻–N content of the leaves was lower than that of the control by 18.7%, 10.1% and 17.0%, respectively (Figure 4). During the jointing stage, the NH₄⁺–N content showed no significant difference between O₃ treatments. Except for the decrease of NH₄⁺–N under O₃ stress of 120 ppb, the other O₃ treatments produced no significant influence in the heading stage. However, during the milk stage, the O₃ fumigation caused a significant decrease of NH₄⁺–N. When the O₃ was 40, 80 and 120 ppb, the NH₄⁺–N content was decreased by 13.1%, 28.0% and 46.0%, respectively.

3.4. Enzyme activities in winter wheat leaves after Spd application

Both the 0.25 mmol L⁻¹ and 0.75 mmol L⁻¹ Spd increased the SOD activity of winter wheat leaves as compared with the control (Figure 5). The application of Spd also significantly increased the activities of POD, CAT and APX. In comparison with the control, when the Spd concentration was 0.25, 0.50 and 0.75 mmol L⁻¹, the POD activity of the winter wheat leaves was increased by 22.6%, 90.0% and 200.0%, respectively. The CAT activity was increased by 21.4%, 31.4% and 40.6%, respectively. The APX activity was increased by 183.8%, 164.2% and 191.0% respectively. Meanwhile, the application of Spd of 0.25 and 0.75 mmol L⁻¹ resulted in the increase of the GR activity by 25.2% and 43.7%, respectively.

3.5. Metabolite contents in winter wheat leaves after Spd application

It can be seen from Figure 6 that when the concentration of Spd increased from 0.25 mmol L⁻¹ to 0.75 mmol L⁻¹, the MDA content decreased by 9.7% to 42.5%. Statistical analysis indicated that the application of Spd of 0.50 and 0.75 mmol L⁻¹ significantly lowered the MDA content. This means that the application of Spd at these concentrations relieves, to a certain degree, the membrane lipid peroxidation of the winter wheat leaves. The exogenous application of Spd also had influence on the SP content of winter wheat leaves. Figure 6 showed that, in comparison with the control, the application of Spd of 0.25 and 0.50 mmol L⁻¹ significantly increased the SP content of winter wheat leaves (p<0.05) by 24.0% and 41.5%, respectively. The application of Spd significantly decreased the AsA content. Under the application of Spd of 0.25, 0.50 and 0.75 mmol L⁻¹, the AsA content decreased by 13.5%, 16.1% and 14.9%, respectively, in comparison with the control. Despite that the difference between the Spd treatment of 0.50 mmol L⁻¹ and the control treatment was not significant, the difference in GSH content of 0.25 and 0.75 mmol L⁻¹ treatment was significant as compared with the control treatment (p<0.05).

![Figure 4](image-url)  Effects of elevated ozone on NR activity, NO₃⁻–N, NH₄⁺–N and Pro content in winter wheat leaves. Error bars represent standard deviations. Asterisks above bars indicate significant differences between O₃ concentrations and CK treatment at p<0.05.
4. Discussion

4.1. Injury of winter wheat under O₃ stress

Our results found that O₃ stress caused serious visible injury to winter wheat leaves. Brown spots appeared at two sides of the veins and at the leaf tip; the leaves turned yellow; the spike growth was retarded and the ripening stage was advanced. During the initial period of O₃ fumigation (jointing stage), no visible injury was found in winter wheat leaves. With the prolongation of the fumigation time and higher O₃ concentrations, the injury symptoms became more and more manifest. Many researchers have reported the injury caused by O₃ stress on crops (Mills et al., 2009; Iyer et al., 2013). One of the results of an Open–Top Chamber experiment on the responses of two wheat cultivars exposed to ozone showed that visible symptoms were present as mild chlorotic spots in the wheat leaf samples. A meta–analysis of 53 peer–reviewed chamber studies showed that elevated O₃ decreases leaf photosynthetic rate by 20% and grain yield by 29% in wheat plants compared with those grown in carbon–filtered air. The O₃ concentration of 70 ppb caused the withering and the emergence of necrosis spots on the leaves of Medicago truncatula within 6 days (Iyer et al., 2013). This demonstrated the O₃ pollution would cause influence on the growth and development of a wide variety of plants in the terrestrial ecosystems.

4.2. Enzymatic activity of plant leaves under O₃ stress

The influence of O₃ stress was not significant on the POD activity during the jointing stage of winter wheat. The reason was probably that the action time of O₃ stress was too short to impose influence on the POD activity. During the heading stage, the O₃ stress caused the increase of POD activity. During the jointing stage, the SOD activity decreased significantly as the O₃ concentration increased. The CAT activity increased under low O₃ concentrations while decreased significantly under high O₃ concentrations. During the heading stage, the SOD activity was lower than that of the control when the O₃ concentration was 40 and 120 ppb. The CAT activity decreased significantly as the O₃ concentration increased. This indicates that under the O₃ stress, the membrane system of plants is seriously damaged. Under environmental stresses, the POD activity of the leaves increases significantly (Biswa et al., 2008; Singh et al., 2011), so as to facilitate the clearing of peroxides such as H₂O₂ by POD. The resistance of plants to environmental stresses is thus enhanced.

The present research found that the AsA content under the O₃ concentration of 120 ppb was increased significantly, during both the jointing and the heading stages. This indicates that AsA plays an important role in the clearing of reactive oxygen free radicals. There are some other studies also indicated that the AsA content in leaves was increased significantly under O₃ stress (Iyer et al., 2013). During the jointing stage, the GSH content was increased significantly with the increase of O₃ concentration. This was also consistent with the one reported by Iyer et al. (2013). During the heading stage, the O₃ stress of 80 and 120 ppb caused a significant decrease of GSH. During the heading stage, the AsA plays the dominant role under high O₃ concentration. Some studies indicated that glutathione, AsA and phenolic compounds were the electron donors of POD. When these substances were increased, the POD activity was also increased (de Souza and MacAdam, 1998). As the chloroplasts do not contain much CAT, the H₂O₂ generated by the disproportionation of SOD and O₂⁻ in the chloroplasts is removed by a special metabolic pathway, that is, the AsA–GSH cycle. AsA is the electron donor for APX in the cycle, which reduces H₂O₂ to H₂O (Noctor and Foyer, 1998).

In this study, the O₃ stress caused the APX activity of winter
wheat leaves to change to various degrees during different growth stages. During the jointing stage, the low O$_3$ concentration caused a significant increase of the APX activity, while a high O$_3$ concentration caused a decrease of the APX activity. During the heading stage, the O$_3$ concentration of 80 ppb caused a significant increase of the APX activity. The study of Singh et al. (2011) concluded that a low dose of oxidation stress exerted little influence on the APX activity of plant leaves. But under high oxidation stress, the APX activity was increased significantly. The oxidants such as H$_2$O$_2$ are removed in plants by the increasing APX activity. The O$_3$ concentration of 120 ppb caused a significant increase of the GR activity and a decrease of the GR activity in winter wheat leaves during the jointing stage. Other O$_3$ treatments had little influence on the GR activity. This indicates that only high concentration of O$_3$ stress can influence the GR activity, and the mechanism is complicated.

In the present study, the O$_3$ stress caused the decrease of NR activity of winter wheat leaves. The contents of NO$_3$–N and NH$_4$–N were changed. This is possibly because the O$_3$ pollution retards the growth and development of winter wheat, and various metabolic functions are affected. Hence, the NR synthesis is influenced, which in turn lowers the contents of NO$_3$–N and NH$_4$–N in winter wheat. The O$_3$ stress caused a significant decrease of NR activity in rice leaves. It has been reported that, the salt stress and heavy metal stress also decreased the NR activity in plant leaves and influenced the normal nitrogen metabolism (Singh et al., 2011).

4.3. Changes of metabolite contents in plant leaves under O$_3$ stress

During the heading stage, the Pro content of winter wheat leaves was increased by multiple times as compared to the control under O$_3$ concentration of 40 and 80 ppb, respectively. This indicates that winter wheat responds actually on physiological and biochemical levels to O$_3$ stress, which is coherent to the findings of Singh et al. (2011). When the O$_3$ concentration increased to 120 ppb, the Pro content was decreased quickly. This is possibly because the high concentration of O$_3$ damages the plant cells and enzyme structures, which blocks the synthesis of Pro. During the milk stage, the Pro content was significantly decreased with the increase of O$_3$ concentration. This is because long-term O$_3$ stress causes serious injuries to winter wheat and the synthesis of Pro is restricted. The present study demonstrated that the high concentration of O$_3$ stress caused the increase of the MDA content in winter wheat leaves, whether in jointing stage or in heading stage. This is also consistent with the findings of other researchers (Singh et al., 2011; Tiwari and Agrawal, 2011). During the jointing stage, the SP content in the leaves showed a decreasing tendency under O$_3$ stress. However, the SP content was increased with the rise of O$_3$ concentration in the heading stage. This indicates that the adaptation mechanism of winter wheat to O$_3$ stress varies from stage to stage.

4.4. Effects of exogenous application of Spd on plants under O$_3$ stress

In the present research, the exogenous application of Spd caused the decrease of MDA content in winter wheat leaves. Meanwhile, the GSH content, SOD, CAT, POD APX and GR activities were all increased. This indicates that the application of Spd relieves the injuries caused by O$_3$ stress to plants. The application of Spd caused the decrease of AsA in the winter wheat leaves. This is possibly because the increase of APX activity resulted in the transformation of more AsA from reductive state to oxidative state. The reason for the relieving of environmental stresses by Spd could be as follows: Spd induces the synthesis and accumulation of polyamines in plants, which promotes the synthesis of enzyme proteins and increases the activity of total enzymes. At the same time, polyamines can directly bind to the enzyme molecules; the activity per unit of enzyme is improved and the O$_3^-$ production rate is slowed down under O$_3$ stress. Thus, the damage of ROS to the membrane system is reduced. Meanwhile, the polycation characteristics enable Spd to non–covalently bind to acidic proteins, membrane phospholipid layer and cell wall and to maintain the stability and integrity of the membrane. Therefore, the peroxidation of the membrane lipid is slowed down. The application of 0.25 and 0.50 mmol L$^{-1}$ Spd increased the SP content in the leaves. But when the Spd concentration increased to 0.75 mmol L$^{-1}$, the SP content tended to decrease. This indicates that Spd influences SP content of leaves in a dose–dependent manner.

Given the current emission trends, surface O$_3$ is projected to rise globally by 20–25% between 2015 and 2050, and 40–60% by 2100 (Meehl et al., 2007). It is assumed in a meta–analysis that future O$_3$ is represented by a daytime concentration of 51–75 ppb during the crop growth. Compared with yield loss at current ambient O$_3$, future O$_3$ could drive a further 10% decrease in yield for soybean, wheat and rice, indicating the future O$_3$ as a significant threat against food production in the world (Ashmore et al., 2006; Feng et al. 2009). The results of this study pointed out that Spd could protect the winter wheat from O$_3$ injuries by increasing antioxidants capacities. Therefore, the effects of Spd counteracting O$_3$ damages in wheat could be well considered in alleviating potential O$_3$ damages in agricultural ecosystems. However, the specificity of different cultivars and agricultural environments should be taken into account to develop reliable and effective concentrations of Spd on O$_3$ damage.

5. Conclusions

In conclusion, our results found that O$_3$ stress caused visible injury to winter wheat leaves, and with the prolongation of the fumigation time, the injury symptoms became more and more manifest. The increase of O$_3$ concentration influenced the antioxidation activity of enzymes and the metabolite contents to various extents during different growth stages. It is a spontaneous response of plants to clear the reactive oxygen radicals generated under O$_3$ stress. This indicates that the response mechanism of winter wheat to different O$_3$ concentrations at different growth stages is complex. The exogenous application of Spd caused the increases of SOD, POD, CAT, APX and GR activities. At the meantime, it decreased the MDA and AsA contents and increased the GSH and SP contents. Spd is an effective antioxidant in relieving the O$_3$ stress and can be applied to the protection of winter wheat from O$_3$ stress.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 41071336) and the Knowledge Innovation Project of the Chinese Academy of Sciences (No. KSCX2–YW–N–41–05).

Supporting Material Available

Simulation experiment of ozone stress on winter wheat grown in the field (Figure S1). This information is available free of charge via the Internet at http://www.atmospolres.com.

References


