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Photosynthesis and biochemical responses to elevated O₃ in *Plantago major* and *Sonchus oleraceus* growing in a lowland habitat of northern China

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ABSTRACT

A field experiment was carried out to compare the responses to ozone (O₃) in two common herbaceous plant species, *Plantago major* L. and *Sonchus oleraceus* L., by building open-top growth chambers in situ to simulate O₃ stress (+O₃, 85 ± 5 ppb, 9 hr/day for 30 days) in a lowland habitat in Inner Mongolia, Northern China. Responses to O₃ of gas exchange, chlorophyll *a* fluorescence, leaf pigment content, antioxidant capability, soluble protein content, membrane lipid peroxidation and dark respiration (R_d) were analyzed. Results showed that elevated O₃ exposure significantly reduced the light-saturated net photosynthesis (P_{Nsat}), stomatal conductance (g_s) and transpiration rate (E) in both species. Although non-significant interactive effect between species and O₃ on P_{Nsat} was analyzed, the reduction in P_{Nsat} in *S. oleraceus* might be due primarily to the higher fraction of close PSII reaction centers and impaired activities of plant mesophyll cells as evidences by decreased maximum efficiency of PSII photochemistry after dark adapted state (F_v/F_m) and unchanged intercellular CO₂ concentration (C_i). Besides, biochemical analysis showed that *S. oleraceus* had lower antioxidant ability compared to *P. major*. As a result, *S. oleraceus* was damaged to the larger extent in terms of lipid peroxidation and visible O₃ injury, indicating that *S. oleraceus* was more sensitive to O₃ than *P. major*. Our results indicated that wild herbaceous plant species growing in a lowland habitat in sandy grassland were sensitive to O₃ stress and *S. oleraceus* can be considered as one of the bio-indicators for high O₃ concentration in semi-arid grassland of northern China.

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Introduction

Concentration of tropospheric ozone (O₃), a pervasive phytotoxic air pollutant, has been increasing in the past decades (Derwent et al., 2007) and its annual average value is expected to further increase over the twenty-first century reaching above 60 ppb, much higher than the internationally accepted

environmental criteria of 40–50 ppb (hourly mean value) (Vingarzan, 2004; Cape, 2008). The O₃ concentration is mainly determined by photochemical reactions with precursor pollutants (i.e., nitrogen oxidants and volatile organic compounds). Urban areas are the main sources of precursor pollutants, but the presence of high ozone levels is also recorded in rural and remote areas (Volk et al., 2006), where the increasing biogenic

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emissions of VOCs might play an important role in the formation of O_3 (Sanderson et al., 2003; Fu and Liao, 2014). The rising O_3 concentration is known to pose great threat to vegetation growth (Ashmore, 2005; Wang et al., 2005).

Generally, when O_3 levels exceed the tolerance threshold of plants, it will cause damages to plants mainly through impairing the integrity of metabolically important membranes, causing peroxidation of cellular membrane lipids or oxidation of cellular proteins (Severino et al., 2007; Biswas et al., 2008a; Pellegrini et al., 2015). The oxidative stress may cause visible injury (Bungener et al., 1999), inhibition of carbon assimilation (Biswas et al., 2008a, 2008b; Fiscus et al., 2005; Kobayakawa and Imai, 2015), senescence acceleration (Pellegrini et al., 2015; Xu et al., 2007) and reduction in net primary productivity and crop yields (Feng et al., 2003, 2015; Anav et al., 2011). Concerns for worldwide food safety, a large body of research has documented the effects of O_3 on cultivated plants (Feng et al., 2015), but limited experimental work has investigated the influence of O_3 on natural vegetation (Pfleeger et al., 2010).

A number of studies have found that wild plants are as sensitive to elevated O_3 as crops and species-specific responses exist (Agathokleous et al., 2015; Davison and Barnes, 1998; Pleijel and Danielsson, 1997), which will induce potential adverse effects on the structure and function of natural or semi-natural grassland under high O_3 environment (Hayes et al., 2016; Power and Ashmore, 2002; Thwaites et al., 2006; Wedlich et al., 2012). Up to now, the effects of rising O_3 concentration are examined majorly on the intensively managed, productive artificial or semi-natural grasslands in European or Mediterranean regions (Bassin et al., 2007), but rarely on the low productive perennial grasslands, such as Mongolia plateau.

Hunshandake sandy grassland locates in a typical semi-arid region of the north hemisphere in northern China, belonging to Mongolia plateau. The landscape is composed of lowlands, typical steppe, and fixed or semi-fixed dunes. We investigated the responses to high O_3 concentration of plants growing in lowlands, which may be more sensitive to O_3 than those in other habitats as plants from high soil moisture habitats are considered to be relatively sensitive to O_3 since they may take up more O_3 (Power and Ashmore, 2002). High O_3 sensitivity has been identified in plant species belonging to genera of *Plantago* *Sonchus* (Lyons et al., 1997; Reiling and Davison, 1995; Zheng et al., 2002) and *Sonchus* (Bergmann et al., 1999; El-Khatib, 2003). In the present study, therefore, we studied the effects of high O_3 exposure on two common herbaceous plant species, *Plantago major* L. and *Sonchus oleraceus* L., in situ in the lowland habitat. We compared their O_3 sensitivity with respect of visible injury and chlorophyll *a* fluorescence and studied the related eco-physiological and biochemical mechanisms. Our research may help predict the negative effects of the rising ground-level O_3 concentration on grasslands in Mongolia plateau.

1. Materials and methods

1.1. Experimental site and plant species

The present research was conducted at an experimental site of Hunshandake sandland, Ecosystem Research Station of Chinese Academy of Sciences (42°54′39.2″N, 116°01′07.5″E,

1300 m elevation) locating at the middle of Xilingole league of Inner Mongolia autonomous region of China. Climatically, the research site belongs to the continental zone, characterized by cool summers and severe winters. Mean annual temperature is 1.5°C with mean monthly temperature ranging from −17.8°C in January to 18.7°C in July. The mean annual precipitation is 365 mm, 60%–80% of which falls during the growing season from May to August. The annual evaporation is about 2000 mm, much higher than annual precipitation. The frostless period lasts approximately 100 days from June to August.

The targeted species are *P. major* and *S. oleraceus*, which are two common herbaceous plant species naturally growing in the lowland habitat dominated by perennial grasses. Prior to the year (2010) of the experiment, the studied area had been used for sheep grazing with a stocking rate of 1.4 sheep unit per hectare for 4 months each year for 10 years. Soil characteristics were measured in pooled samples of five 5-cm-diameter soil cores (0–20 cm) in each selected area for OTC construction on June 14, 2010. Average soil moisture and soil pH value were 17.5% and 9.0, respectively. Soil organic matter, total N, total P and total K were 6.70, 1.40, 0.18 and 17.6 g/kg, respectively.

1.2. Ozone treatments in situ

On June 15, 2010, six open-top chambers (OTCs, octagonal base, 2.0 m in diameter, and 1.8 m in height) were constructed on the selected lowland with relatively identical vegetation. For either targeted species, there were at least 15 plants inside each OTC. We selected and labeled 6 plants for measurements with similar growth status and locus inside each OTC. The OTCs were ventilated continuously (24 hr/day) with air that had passed through activated charcoal filters (CFs) attached to fan boxes. The gas dispensing system of the OTC was constructed following the methodology described by our previous report (Biswas et al., 2008a). The plants inside the OTCs received charcoal-filtered air for two weeks until O_3 treatments. Irrigation (approximate to 10 mm precipitation) was carried out every week.

On July 1, 2010, O_3 concentration in three randomly selected OTCs was artificially elevated. Ozone was generated by electrical discharge O_3 generator (CFY20, Makes Co., Beijing, China) using pure oxygen and then mixed with charcoal-filtered air to the OTCs. The other three OTCs with continuously charcoal-filtered air were taken as control (CF, $[O_3] < 10$ ppb). O_3 concentration inside OTCs was continually monitored at approximately 10 cm above plant canopy using a UV absorption ozone analyzer (MOT400, Yueke Technology Ltd., Shenzhen, China). Since local photosynthetic photo flux density (PPFD) can rise up to above 1000 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$ by 08:00 am, daily O_3 treatment was started from 08:00 am to 17:00 pm. The O_3 concentration inside the + O_3 chambers on clear days averaged (85 ± 5) ppb, which approximately tripled the average ambient O_3 concentration (32 ± 10 ppb). The O_3 exposure treatment lasted for 30 days from July 1 to July 30, 2010. O_3 exposure was stopped on rainy days (totally 6 days) and during the gas exchange measurement. Therefore, the value of AOT40 (accumulated O_3 exposure over the threshold of 40 ppb) in + O_3 chambers during the experiment period was approximately 9.72 ppm•hr. The PPFD in the chambers was approximately 1800 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$. The temperature was 8–14°C (night) to 22–32°C (day) and the relative

humidity (RH) ranged from 36% (day) to 100% (night) inside the OTCs during the experiment.

1.3. Visible O₃ injury

Before the start of O₃ treatment on June 30, 2010, we marked the upper two fully developed leaves in targeted plants as young leaves and the other lower leaves as old leaves. At the end of O₃ treatment on July 30, 2010, i.e., 30 days after O₃ exposure, we assessed the visible O₃ injury by investigating the percentage of necrotic mottle or chlorosis area on both young and old leaves of targeted plants in control and O₃ fumigated chambers by placing a transparent plastic grid above the plant and counting intersections of the grid that represented leaf surface area (Li et al., 2011).

1.4. Gas exchange measurements

On morning (08:30–11:30 am) of July 29, 2010, area-based light-saturated net photosynthesis (P_{Nsat}), stomatal conductance (g_s), transpiration rate (E), and intercellular CO₂ concentration (C_i) were measured on the last fully developed leaf of each labeled plant ($n = 6$) of either studied species using an infrared gas analysis instrument (LI-6400, Li-COR Inc., Lincoln, NE, USA). The ambient air with CO₂ concentration of 360 ppm was used. The sampling leaf was illuminated with a maximum photosynthetic photon flux density (PPFD) of 1500 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$ by internal light emitting diode light source. Relative humidity was maintained at 70% and leaf temperature was set at 25°C in the leaf chamber. The same leaves measured P_{Nsat} were kept under darkness for 30 min to avoid the post-illumination burst in respiration before dark respiration measurements (Balaguer et al., 1995). The leaf chamber was maintained at 0 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$ during respiration measurement and CO₂ efflux was recorded 30 min when the data was stable. Gas exchange rates were expressed on the projected leaf area basis. O₃ exposure was stopped during gas exchange measurement.

1.5. Chlorophyll *a* fluorescence analysis

On July 30, 2010, chlorophyll *a* fluorescence analysis was carried out in pre-dawn hours when sunlight (PPFD < 1 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$) was too low to induce photosynthetic processes using a pulse amplitude modulation fluorescence analyzer (Mini-PAM, Heinz Walz, Effeltrich, Germany) in the mid region of the last fully developed leaf of each labeled plant ($n = 6$) of either studied species in each OTC. The minimum fluorescence (F_0) was determined with modulated light which was sufficiently low (< 1 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$) not to induce any significant variable fluorescence (F_v). The maximum fluorescence (F_m) was determined with a 0.8 sec saturating pulse at 8000 $\mu\text{mol}/(\text{m}^2\cdot\text{sec})$. The difference between F_m and F_0 was calculated as variable fluorescence ($F_v = F_m - F_0$). The maximum efficiency of photosystem II photochemistry in the dark-adapted state (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_0) / F_m$.

1.6. Leaf sampling and pigment content analysis

On July 30, 2010, the last fully developed leaves exhibiting no visible O₃ injury were harvested from the labeled plants in

both control and O₃-fumigated chambers. Leaf samples were frozen in liquid nitrogen immediately after excision and kept in a refrigerator at –40°C to be analyzed. Five samples per species from each chamber were analyzed for biochemical parameters and content/activity of each parameter was calculated on the fresh weight (fw) basis.

Pigment was extracted from frozen leaf samples (0.2 g) in 20 mL 95% ethanol in the dark for 48 hr at 4°C. The extract absorbance at 663, 646, 470 nm was read. Concentrations of chlorophyll *a* ($C_{\text{Chl-a}}$, mg/L), chlorophyll *b* ($C_{\text{Chl-b}}$, mg/L), and carotenoids (C_{Car} , mg/L) were calculated by Eqs. (1)–(3) provided by Arnon (1949):

$$C_{\text{Chl-a}} = 12.21A_{663\text{ nm}} - 2.81A_{646\text{ nm}} \quad (1)$$

$$C_{\text{Chl-b}} = 20.13A_{646\text{ nm}} - 5.03A_{663\text{ nm}} \quad (2)$$

$$C_{\text{Car}} = (1000A_{470\text{ nm}} - 3.27C_{\text{Chl-a}} - 104C_{\text{Chl-b}}) / 229 \quad (3)$$

1.7. Determination of ascorbic acid (AsA) and malondialdehyde (MDA) content

Frozen leaf samples (0.4–0.5 g) were homogenized in a pre-chilled mortar on ice with 0.8 mL of 6% (W/V) trichloroacetic acid (TCA). The mixture was continuously homogenized to fine powder and stood on ice for another 15 min. The homogenate was quantitatively transferred to a 2 mL reaction vessel on ice and adjusted to a volume of 2 mL with 6% (W/V) TCA. The homogenate was centrifuged at 15,600 $\times g$ (4°C) for 5 min. The supernatant was transferred to a new ice-chilled reaction vessel and AsA concentration was immediately assayed following the method of Kampfenkel et al. (1995). The absorbance was read at 525 nm using distilled water as reference using a spectrophotometer (UV-160A; Shimadzu Scientific Instruments, Columbia, MD, USA).

MDA content was analyzed according to the modified method of Heath and Packer (1968). Leaf samples (0.4–0.5 g) were homogenized in a pre-chilled mortar and pestle with 2 mL ice-cold 6% (W/V) TCA and centrifuged at 15,600 $\times g$ for 5 min. Assay mixture containing 0.7 mL aliquot of supernatant and 2 mL of 6% (W/V) TCA was water-bathed at 95°C for 30 min and then rapidly cooled in an ice-bath. After centrifugation (10,000 $\times g$ for 5 min), the supernatant absorbance at 532, 600, 450 nm was read. Concentration of MDA (C_{MDA} , $\mu\text{mol/L}$) in the supernatant was calculated using the following equation: $C_{\text{MDA}} = 6.45 (A_{532\text{ nm}} - A_{600\text{ nm}}) - 0.56A_{450\text{ nm}}$.

1.8. Soluble protein and peroxidase enzyme activity

Frozen leaf samples (0.4–0.5 g) were homogenized in a pre-chilled mortar on ice with 5 mL of 0.1 mol/L potassium phosphate buffer (pH 7.0) containing 1% (W/V) polyvinylpyrrolidone and 0.1 mmol/L EDTA-Na₂. The homogenate was centrifuged at 12,000 $\times g$ for 20 min and the supernatant was used for protein and enzyme extraction. Using the Coomassie brilliant blue G-250 dyeing method according to Bradford (1976), soluble protein content was calculated by comparing the standard curve utilizing bovine serum albumin as standard. The activity of peroxidase (EC 1.11.1.7) was assayed using the modified method of Chance and Maehly (1955). Reaction mixture (3 mL) was composed of

0.1 mol/L potassium phosphate buffer (pH 7.0), 20 mmol/L guaiacol and 1 mmol/L H_2O_2 . The reaction was initiated by adding 0.02 mL enzyme. The optical density at 470 nm was recorded. The linear initial reaction rate was used to estimate the activity, which was expressed in μmol of guaiacol dehydrogenation product (GDHP) formed per mg protein per min using the extinction coefficient of 26.6 per mmol/L per cm.

1.9. Statistical analysis

The experimental design consisted of three replicates, each containing one elevated O_3 and one CF air chamber with 6 plants per replicate for either species. Data for each dependent variable were analyzed using General Linear Models of SPSS package (version. 11, SPSS, Chicago, IL, USA). Means of each parameter were compared between treatments using one-way ANOVA. Interactions between O_3 treatment and species were analyzed using two-way ANOVA. Pearson's correlation test was used to explore the correlations between light-saturated net photosynthesis and other physiological or biochemical parameters. Differences between treatments were considered significant if $p \leq 0.05$.

2. Results

2.1. Visible O_3 injury

No visible injury was observed on leaves of CF plants. However, high O_3 exposure caused visible injury on leaves of both species, with more serious symptoms being observed in old leaves compared to young leaves (Fig. 1). The visible O_3 injury showed as necrotic mottle in *P. major* and as chlorosis in *S. oleraceus*. Meanwhile, the visible O_3 injury was more serious in *S. oleraceus* (67% in old leaves and 10% in young leaves) than *P. major* (26% in old leaves and 5% in young leaves).

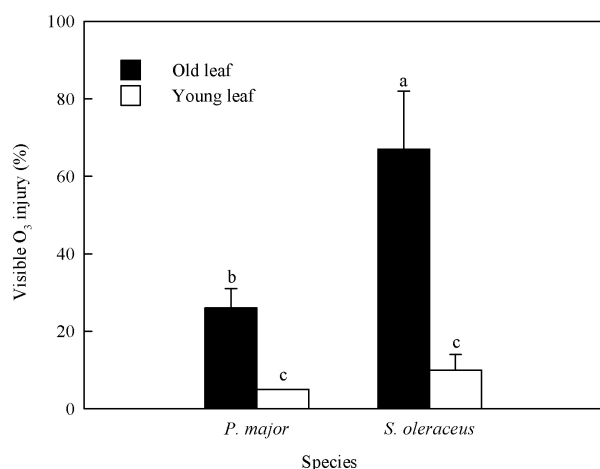


Fig. 1 – Visible O_3 injury on the old and the young leaves in two herbaceous species after O_3 exposures (charcoal-filtered air (CF) and elevated ozone ($+\text{O}_3$)). Error bars show SD, $n = 3$. Values with different letters are significantly different ($p < 0.05$). SD: standard deviation.

2.2. Gas exchanges and chlorophyll a fluorescence

Under clean air condition, non-significant difference ($p > 0.05$) was observed in the light-saturated net photosynthesis (P_{Nsat}), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) between the two species, but the average E was significantly higher in *P. major* ($7.58 \text{ mmol}/(\text{m}^2\text{-sec})$) than in *S. oleraceus* ($5.89 \text{ mmol}/(\text{m}^2\text{-sec})$) (Fig. 2). High O_3 exposure significantly decreased P_{Nsat} , g_s and E ($p < 0.001$) (Fig. 2a–c) in both species. There was no significant difference in the reductions in P_{Nsat} and E between the species ($p > 0.05$), but the reduction in g_s was much larger in *P. major* (–61%) than in *S. oleraceus* (–45%). *P. major* under high O_3 exposure exhibited significantly lower C_i ($p < 0.05$), while O_3 -treated *S. oleraceus* showed similar C_i values compared to CF ($p > 0.05$) (Fig. 2d). There was no significant interaction effect ($p > 0.05$) between O_3 and species on P_{Nsat} , g_s and E , but significant interaction effect ($p < 0.01$) was analyzed on C_i .

Statistic analysis showed that there was significant negative effects of O_3 on the maximum efficiency of photosystem II photochemistry in the dark-adapted state (F_v/F_m) (Fig. 3), but the reduction reached significant level only in *S. oleraceus* ($p < 0.05$), not in *P. major* ($p > 0.05$).

2.3. Pigment content

High O_3 exposure caused significant negative effects on photosynthetic pigment content of both species (Fig. 4). *S. oleraceus* had significantly lower Chl content than *P. major*, but their reductions caused by high O_3 showed similarly as indicated by no significant interaction effect ($p > 0.05$) on Chls between species and O_3 (Fig. 4a). No significant change in Chl-a/Chl-b ratio was observed in *P. major*, but considerable reductions in *S. oleraceus* (Fig. 4b). Leaves of *P. major* possessed higher levels of carotenoid (Car) content compared to *S. oleraceus*. Elevated O_3 exposure caused significant reductions in Car content (Fig. 4c), with non-significant difference ($p > 0.05$) between *P. major* (–17%) and *S. oleraceus* (–23%).

2.4. Malondialdehyde (MDA) content and soluble protein content

MDA content in leaves of both herbs under $+\text{O}_3$ treatment were significantly increased (Fig. 5a). The increment in leaves of ozonated-*S. oleraceus* (112%) was higher than in *P. major* (89%), but the difference did not reach significant level ($p > 0.05$). High O_3 exposure caused marginal decrease ($p < 0.10$) in soluble protein content in leaves of *S. oleraceus* (–18%), but no significant difference was observed between O_3 -treated *P. major* and CF-plant (Fig. 5b). Marginal interaction effect ($p < 0.10$) between species and O_3 on soluble protein content was analyzed.

2.5. Anti-oxidant ability and dark respiration

High O_3 exposure caused profound increase ($p < 0.001$) in ascorbic acid (AsA) content (Fig. 6a) and peroxidase (POD) activity (Fig. 6b) in leaves of both species compared to CF-plants. There was significant interaction effect ($p < 0.05$) on AsA between Species and O_3 , where the increments in ozonated-*P. major* (252%) was considerably higher than that in *S. oleraceus* (153%). There was non-significant difference ($p > 0.05$) between the

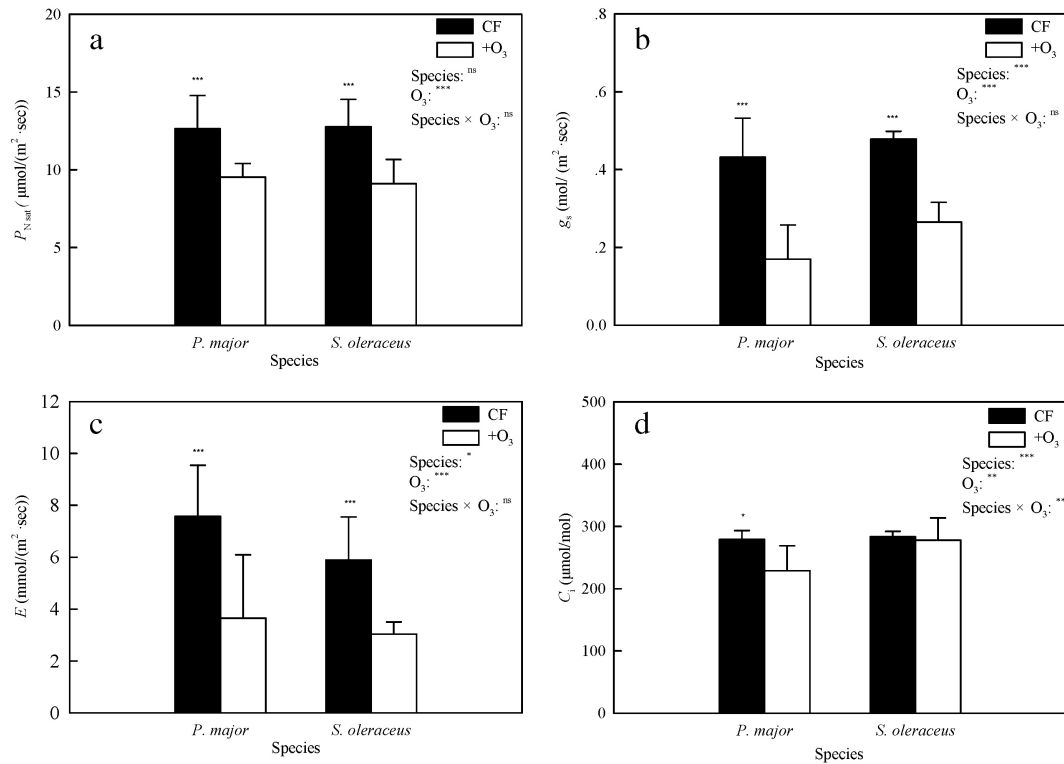


Fig. 2 – Effects of O₃ on light-saturated net photosynthesis (P_{Nsat}) (a), stomatal conductance (g_s) (b), transpiration rate (E) (c) and intercellular CO₂ concentration (C_i) (d) in two herbaceous species after O₃ exposures (charcoal-filtered air (CF) and elevated ozone (+O₃)). Error bars show SD, n = 3. The asterisks *, **, and *** denote effects by species, O₃ and species × O₃ interactions were significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. ns: not significant. SD: standard deviation.

increments in POD activity in both species compared to CF-plants (*P. major*, 131% and *S. oleraceus*, 99%). Dark respiration (R_d) was also considerably stimulated ($p < 0.001$) (Fig. 6c); the

increment was higher in *S. oleraceus* (137%) than in *P. major* (68%), although non-significant difference ($p > 0.05$) was analyzed.

2.6. Correlations between the relative reduction in P_{Nsat} and the relative changes in other physiological/biochemical parameters

The relative reduction in P_{Nsat} was analyzed to be positively correlated with the relative changes in F_v/F_m ($p = 0.043$), AsA content ($p = 0.010$), POD activity ($p = 0.002$) and soluble protein content ($p = 0.006$), but adversely correlated with the relative changes in g_s ($p = 0.045$), C_i ($p = 0.003$), R_d ($p < 0.001$) and MDA ($p = 0.002$) (Table 1). Non-significant relationships ($p > 0.05$) were analyzed between the relative decrease in P_{Nsat} and the relative changes in pigments content (Table 1).

3. Discussion

Our field experiment results showed that high O₃ exposure (AOT40 approximately 9.72 ppm·hr) caused considerable negative effects on ecophysiological processes and even foliar injury to the target herbaceous species growing in a lowland in Mongolia plateau, but the effects were species-specific. During the experiment, we also observed visible O₃ injury in other plant species including grasses, which showed symptoms later than the two examined herbs (data not show). Our results suggested that the future predicted high O₃ concentration may be a great threat to the primary production and species diversity in the semi-arid natural grasslands.

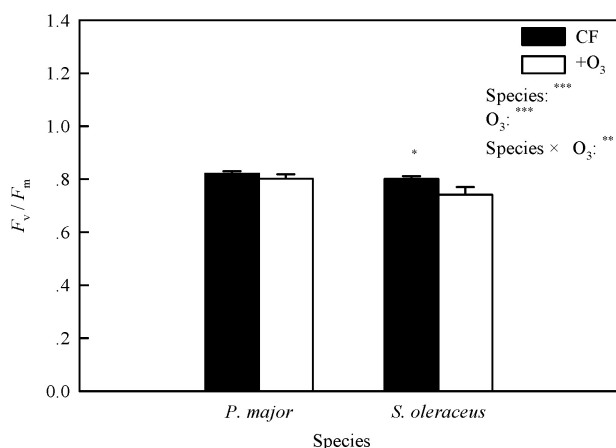


Fig. 3 – Effects of O₃ on maximum efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m) in two herbaceous species after O₃ exposures (charcoal-filtered air (CF) and elevated ozone (+O₃)). Error bars show SD, n = 3. The asterisks *, **, and *** denote effects by species, O₃ and species × O₃ interactions were significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. ns: not significant; SD: standard deviation.

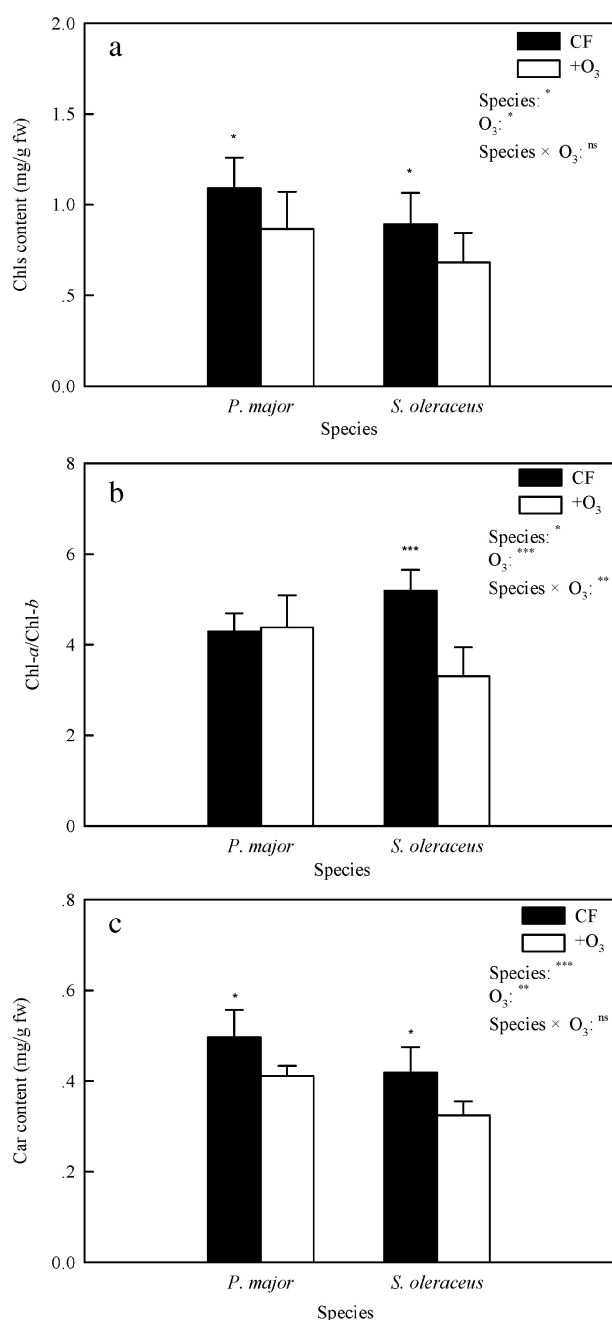


Fig. 4 – Effects of O₃ on contents of total chlorophyll (Chl) (a), Chl-a/Chl-b (b) and carotenoids (Car) (c) in two herbaceous species after O₃ exposures (charcoal-filtered air (CF) and elevated ozone (+O₃)). Error bars show SD, n = 3. The asterisks *, **, and *** denote effects by species, O₃ and species × O₃ interactions were significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. ns: not significant.

We observed that *S. oleraceus* exhibited visible O₃ injury earlier and more serious than *P. major*, indicating that *S. oleraceus* may be more sensitive to O₃ than *P. major*, as the most sensitive species to O₃ usually showed serious visible O₃ damage symptoms (Biswas et al., 2008b; Furlan et al., 2007; Pina and Moraes, 2010; Warwick and Taylor, 1995). Moreover, significant reduction in F_v/F_m was observed in ozonated-*S.*

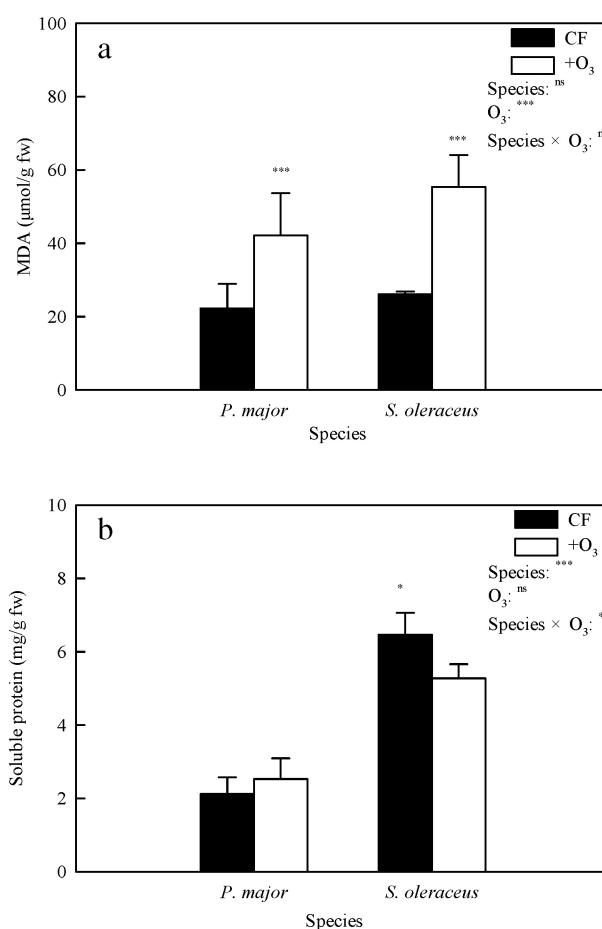


Fig. 5 – Effects of O₃ on contents of malondialdehyde (MDA) (a) and soluble protein (b) in two herbaceous species after O₃ exposures (charcoal-filtered air (CF) and elevated ozone (+O₃)). Error bars show SD, n = 3. The asterisks *, **, and *** denote effects by species, O₃ and species × O₃ interactions were significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. ns: not significant. SD: standard deviation.

oleraceus, but non-significant changes in *P. major*. Such result may confirm that *S. oleraceus* was more susceptible to O₃ damage (Biswas et al., 2008b; Calatayud and Barreno, 2004; Guidi et al., 2000; Reiling and Davison, 1992). El-Khatib (2003) also reported that *S. oleraceus* was very sensitive to O₃ (ranked as the second place among the five common Egyptian plants). Another *Sonchus* species, *Sonchus asper*, was recognized as one most sensitive species exhibiting ozone-specific symptoms even when AOT40 < 1.5 ppm·hr. (Bergmann et al., 1999). Therefore, *S. oleraceus* can be considered as one of the bio-indicators for O₃ pollution in Mongolia Plateau grassland.

Our experiment demonstrated that high O₃ exposure caused significant reductions in P_{Nsat} in both herbs, reflecting that photosynthetic processes were negatively affected (Pellegrini et al., 2013a, 2013b). The significantly inverse correlations between the decrease of P_{Nsat} and the relative reduction in g_s and C_i indicated that the down-regulation in assimilation rate was not the result of stomatal limitation, but due to a reduced mesophyll activity (Calatayud et al., 2003; Pellegrini et al., 2011). The significantly positive correlation between the relative

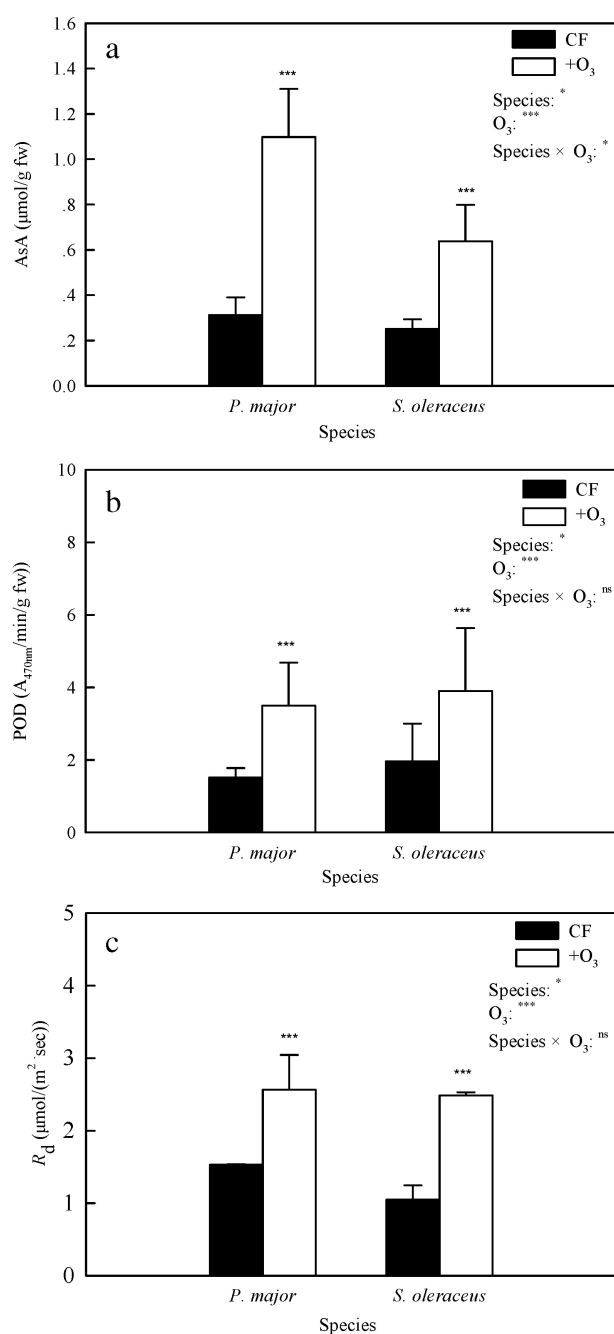


Fig. 6 – Effects of O₃ on content of ascorbic acid (AsA) (a), peroxidase (POD) activity (b) and dark respiration (R_d) (c) in two herbaceous species after O₃ exposures (charcoal-filtered air (CF) and elevated ozone (+O₃)). Error bars show SD, n = 3. The asterisks *, **, and *** denote effects by species, O₃ and species × O₃ interactions were significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. ns: not significant. SD: standard deviation.

decrease of P_{Nsat} and the relative reduction in F_v/F_m indicated that the decrease of photosynthetic rate was also partially caused by the reduction in the fraction of open PSII reaction centers. Elevated O₃ exposure significantly reduced leaf total Chl contents of both herbs, indicating that the chlorophyll binding proteins of the light harvesting complex (LHC) was

Table 1 – Correlation between relative reduction in light-saturated net photosynthesis (P_{Nsat}) and relative changes in physiological/biochemical parameters in two herbaceous species exposed to elevated O₃.

Physiological/biochemical parameter	<i>r</i>	<i>p</i> value
Stomatal conductance (g_s)	−0.613	0.045
Transpiration rate (E)	−0.270	0.420
Intercellular CO ₂ concentration (C_i)	−0.808	0.003
Maximum efficiency of PSII photochemistry in dark-adapted state (F_v/F_m)	0.618	0.043
Total chlorophyll contents (Chls)	0.330	0.267
Chlorophyll <i>a</i> /Chlorophyll <i>b</i> ratio (Chl- <i>a</i> /Chl- <i>b</i>)	0.498	0.119
Carotenoids (Car)	0.453	0.161
Ascorbic acid (AsA)	0.733	0.010
Peroxidase (POD)	0.822	0.002
Dark respiration (R_d)	−0.876	0.000
Malondialdehyde (MDA)	−0.821	0.002
Soluble protein	0.768	0.006

Changes are expressed as percent differences between O₃-exposed (+O₃) plants and CF plants, $(+O_3 - CF) / CF$, before Pearson's correlation test. CF: charcoal-filtered air; +O₃: elevated ozone.

negatively affected by the O₃-induced oxidative stress (Pellegrini et al., 2015); nevertheless, changes of Chl content may not explain the decrease of P_{Nsat} as there was no significant correlation between them, but indicated leaf senescence triggered by high O₃ exposure (Pellegrini et al., 2015).

There were no species-specific responses in P_{Nsat} to high O₃ exposure among the two herbs; however, when g_s of both herbs were considerably reduced under high O₃ exposure, the C_i was significantly reduced in O₃-treated *P. major* but unchanged in the ozonated-*S. oleraceus* compared to CF plants. This reflected that the O₃-induced non-stomatal limitation to P_{Nsat} in O₃-treated plant was larger in *S. oleraceus* than in *P. major*. In terms of g_s , the *S. oleraceus* plants had higher average value but exhibited less reduction under high O₃ exposure compared to *P. major*, suggesting that the former might absorb more O₃ than the latter (Biswas et al., 2008a). Moreover, the O₃-treated *S. oleraceus* showed significant reduction in F_v/F_m , implying that the fraction of open PSII reaction centers was reduced in *S. oleraceus*, but not in *P. major*. The considerable reduction of Chl-*a*/Chl-*b* ratio in *S. oleraceus* might indicate that its Chl-*a* was deconstructed more than Chl-*b* by elevated O₃, and this may partly explain its significant reduction in F_v/F_m , as a fraction of Chl-*a* play as PSII reaction centers. These results reflected that photosynthetic processes in *S. oleraceus* was damaged more serious than in *P. major*, which might be due to the larger O₃ flux into *S. oleraceus* plant under O₃ treatment.

Before the development of visible O₃ injury symptoms, O₃ stress causes a series of biochemical responses (Dietz, 2010). In our experiment, the O₃ pollution caused significant increase in MDA content in leaves of both herbs to a similar extent, indicating that O₃ aroused oxidative stress to their cellular membrane and led to lipid peroxidation (Li et al., 2011; Pellegrini et al., 2011). Elevated O₃ caused marginal significant decrease in soluble protein content in leaves of *S. oleraceus*, but the effect in *P. major* was much smaller. This indicated that *P. major* was less damaged by O₃ compared to *S. oleraceus* (Biswas et al., 2008a). The O₃-treated plants promoted antioxidant capacity as evidenced by the increases in AsA content and POD activity. This is

consistent with the previous studies on herbaceous species, such as *Melissa officinalis* (Pellegrini et al., 2013a, 2013b, 2015). The antioxidant capacity might depend on assimilation rate as indicated by the positive correlations between the relative increase in both AsA content ($r = 0.733$) and POD activity ($r = 0.822$) and the relative change in P_{Nsat} . The stimulated dark respiration (R_d) also indicated that the O_3 -treated plants strengthened antioxidant ability to repair the damaged cellular membrane. The higher increase in AsA content and POD activity in O_3 -treated *P. major* compared to *S. oleraceus*, indicated that *P. major* possessed stronger antioxidant capacity. Species-specific response to O_3 was found in AsA content, suggesting that AsA might play an important role in scavenging free oxygen radicals in *P. major*, as many studies found that changes in ascorbate content contributed to the differential ozone sensitivity among plant species or cultivars (Biswas et al., 2008a; Feng et al., 2010; Severino et al., 2007).

4. Conclusions

High sensitivity to O_3 and species-specific responses were examined in plants naturally growing in a lowland in Hunshandake sandy grassland of northern China. So, the near-future predicted ground-level O_3 concentration would cause considerable damage to the semi-arid grassland in Mongolia plateau. Responses of physiological and biochemical changes as well as visible injury indicated that *S. oleraceus* was more sensitive to O_3 than *P. major*, which might be due to the higher g_s and lower antioxidant ability under O_3 stress. The high sensitivity to O_3 of *S. oleraceus* implied that it can be considered as one of the bio-indicators for high O_3 concentration in semi-arid grassland of northern China.

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