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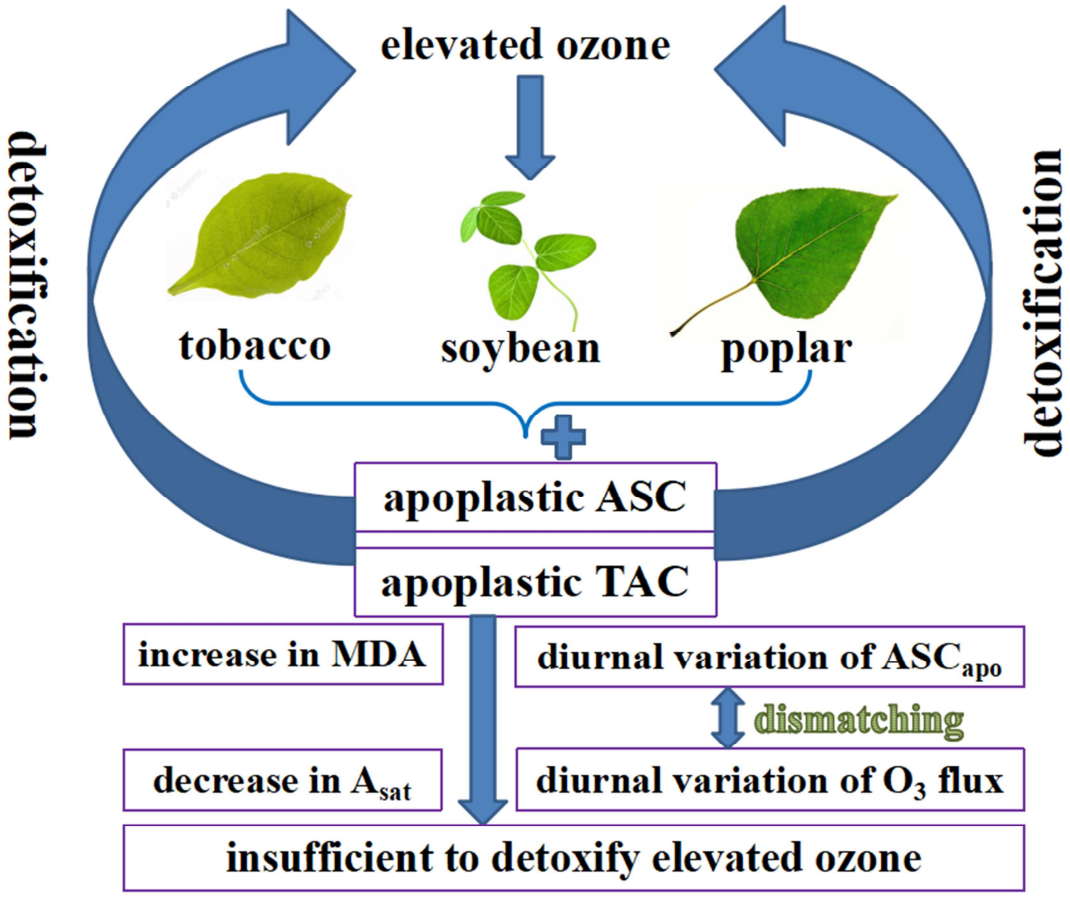

Title: Increase of apoplastic ascorbate induced by ozone is insufficient to remove the negative effects in tobacco, soybean and poplar

Capsule: Apoplastic ascorbate was induced by high ozone

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Abstract

Apoplastic ascorbate (ASC_{apo}) is an important contributor to the detoxification of ozone (O_3). The objective of the study is to explore whether ASC_{apo} is stimulated by elevated O_3 concentrations. The detoxification of O_3 by ASC_{apo} was quantified in tobacco (*Nicotiana* L), soybean (*Glycine max* (L.) Merr.) and poplar (*Populus* L), which were exposed to charcoal-filtered air (CF) and elevated O_3 treatments (E-O_3). ASC_{apo} in the three species were significantly increased by E-O_3 compared with the values in the filtered treatment. For all three species, E-O_3 significantly increased the malondialdehyde (MDA) content and decreased light-saturated rate of photosynthesis (A_{sat}), suggesting that high O_3 has induced injury/damage to plants. E-O_3 significantly increased redox state in the apoplast (redox $state_{apo}$) for all species, whereas no effect on the apoplastic dehydroascorbate (DHA_{apo}) was observed. In leaf tissues, E-O_3 significantly enhanced reduced-ascorbate (ASC) and total ascorbate (ASC+DHA) in soybean and poplar, but significantly reduced these in tobacco, indicating different antioxidative capacity to the high O_3 levels among the three species. Total antioxidant capacity in the apoplast (TAC_{apo}) was significantly increased by E-O_3 in tobacco and poplar, but leaf tissue TAC was significantly enhanced only in tobacco. Leaf tissue superoxide anion ($O_2^{\bullet-}$) in poplar and hydrogen peroxide (H_2O_2) in tobacco and soybean were significantly increased by E-O_3. The diurnal variation of ASC_{apo}, with maximum values occurring in the late morning with lower values experienced in the afternoon appeared to play an important role in the harmful effects of O_3 on tobacco, soybean and poplar.

Keywords: Apoplast, Ascorbate, Detoxification, Ozone, Plants, Reactive oxygen species, Total antioxidant capacity.

Introduction

Ground-level ozone (O_3) is an air pollutant detrimental to crop and ecosystem productivity (US EPA, 2013; LRTAP Convention, 2017). Modeling studies indicate that current ambient O_3 concentrations have significantly decreased the yields of major crops like rice, wheat, soybean, potato, barley and bean by 5-19% (Feng and Kobayashi, 2009; Osborne et al., 2016; Mills et al., 2018a,b). Gaseous O_3 penetrates plant leaves through open stomata and dissolves in the apoplastic fluid. Once dissolved, the O_3 molecule reacts with H_2O and solutes to produce several harmful reactive oxygen species (ROS), including hydroxyl radical, singlet oxygen and hydrogen peroxide (Kanofsky and Sima, 1995; Vainonen and Kangasjärvi, 2014; Krasensky et al., 2017). Perception of ROS from its degradation in the apoplast activates several signal transduction pathways that regulate the responses of the cells to the increased oxidative load (Kangasjärvi et al., 2005). The responses include changes in cellular redox homeostasis, perception by apoplastic proteins, oxidative damages of membranes, and transport of apoplastic hydrogen peroxide across the plasma membrane through aquaporins (Vainonen and Kangasjärvi, 2014). The interaction between O_3 and plant tissues is driven mainly by three distinct processes: changes in external O_3 concentration, O_3 uptake and O_3 detoxification. The diurnal pattern of detoxification does not necessarily match the diurnal patterns of external O_3 concentration and O_3 uptake (Heath et al., 2009; Wang et al., 2015), which are responsible for injury/damage to vegetation (Musselman et al., 2006; Mishra and Agrawal, 2015).

Antioxidant compounds in the apoplast are a first line of defense against O_3 by scavenging ROS, so that O_3 injury is attenuated (Lyons et al., 1999; Turcsányi et al., 2000; Wang et al., 2015). The key antioxidant in the apoplast is ascorbate, which accounts for less than 10% of the leaf ascorbate pools (Noctor and Foyer 1998; Pignocchi and Foyer 2003; Dumont et al., 2014; Yendrek et al., 2015), but plays important roles in antioxidantive defense, particularly via the ascorbate-glutathione (AsA-GSH) cycle. Genetic evidence indicates that the most important biosynthesis of ascorbate is carried out via the D-mannose/L-galactose pathway (Ishikawa and

Shigeoka, 2008). If the capacity for ROS detoxification by ascorbate in the apoplast and leaf tissue is overwhelmed by high O_3, ROS cause oxidative damage to plasma membranes and cytoplasm and then induces injury or damage to vegetation (Castagna and Ranieri, 2009; Hossain et al., 2015).

Some studies have indeed reported that apoplastic ascorbate (ASC_{apo}) detoxifies a considerable portion of O_3 under relevant conditions, e.g. bean exposed to 450-650 ppb O_3 for 3.5 h (Moldau et al., 1997) and durum wheat with ambient O_3 exposure (maximum values, 40-50 ppb from 12 to 17h) for 14 d (De la Torre 2008). A strong positive correlation between O_3 sensitivity and ASC_{apo} content was found in different species or cultivars, such as *Plantago major* cultivars (Barnes et al., 2000), tobacco (Sanmartin et al., 2002), snap bean ecotypes (Burkey et al., 2003), wheat (Feng et al., 2010) and leguminous crops (Yendrek et al., 2015). Also, Sanmartin et al. (2002) found that over-expressing ASC_{apo} oxidase increased O_3 sensitivity in tobacco exposed to 100 ppb O_3. Further evidence supporting the involvement of ascorbate (ASC) in O_3 tolerance was derived from *Arabidopsis* mutant *vtc1* studies, in which the *vtc1* mutant containing only 30% of leaf ASC in the wild-type and 23% of the ASC_{apo} level, showed higher sensitivity to O_3 than the wild-type plant (Conklin et al., 1997).

However, some other studies have questioned the efficiency of ASC_{apo} in O_3 detoxification. For example, wheat exposed to 75-100 ppb O_3 showed O_3 flux to plasmalemma is controlled by stomata rather than by direct reaction of O_3 with cell wall ascorbate (Kollist et al., 2000). D'Haese et al. (2005) and Van Hove et al. (2001) reported that ASC_{apo} does not contribute to the differential O_3 tolerance of two clones of *Trifolium repens* L and *Populus* L exposed to 60 ppb O_3 and ambient O_3, respectively. Booker et al. (2012) and Cheng et al. (2007) demonstrated that the apoplastic ascorbate pool is mostly oxidized in *Arabidopsis* and soybean, respectively, and therefore could not serve as an effective antioxidant. Thus, we proposed that ASC_{apo} detoxification depends on O_3 concentration and species.

De la Torre (2008) and Wang et al. (2015) found that the diurnal variations of ASC_{apo} in wheat could be caused by the daily variations of ambient O_3 concentrations. Luwe (1996) also reported that in beech (*Fagus sylvatica*) leaves, ASC_{apo} levels were

positively correlated with ambient O_3 concentrations with delays 3 to 7d. Ambient O_3 concentrations, especially in China, are high enough to induce negative effects on plants (Feng et al., 2014). There is insufficient evidence to explain the stimulating effect of high O_3 on ASC_{apo} by the ambient O_3 concentration only. Furthermore, few studies focused on investigating the direct stimulation of high O_3 on ASC_{apo} using charcoal-filtered air (CF) treatments with very low O_3 concentrations as a control. Even if they did, their results were controversial and limited to one species in each study, for example in soybean (*Glycine max* (L.) Merr.) (Cheng et al., 2007) and *Plantago major* (Lyons et al., 1999). Thus, it is necessary to further explore whether ASC_{apo} is stimulated by high O_3 concentrations directly along with antioxidant capacity.

Considering the importance and uncertainty of the role of ASC_{apo} in the detoxification of O_3, we studied three species (i.e. tobacco, soybean and poplar) under CF and elevated O_3 (E-O_3, non-filtered ambient air plus 40 ppb) concentrations. Our aim in this study was to explore whether ASC_{apo} is stimulated by high O_3 concentrations, and to test if the response is common among the species. We also tried to clarify the apoplastic antioxidant system capacity to detoxify O_3 by comparing with the antioxidant system in leaf tissue in their responses to O_3. In addition, we investigated malondialdehyde (MDA), superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) contents in leaf tissues to see whether the detoxification of O_3 by ASC_{apo} is sufficient to remove the negative effect of elevated O_3.

2. Materials and methods

2.1. Plant material

We used three species including tobacco ‘NC89’ (*Nicotiana* L), soybean ‘ZH37’ (*Glycine max* (L.) Merr.) and the hybrid poplar clone ‘546’ (*P. deltoides* cv. ‘55/56’×*P. deltoides* cv. ‘Imperial’) in the experiment considering their sensitivity to O_3 (Mills et al., 2007; Shang et al., 2017). Plants of tobacco and poplar were individually planted into 20 L plastic pots filled with local loamy soil. Seeds of tobacco ‘NC89’ were germinated in the nursery firstly on 15 April 2017. After they grew to about 10 cm in

height, we transplanted the seedlings into pots. Three seeds of soybean per pot were planted in pots directly on 8 May 2017. Rooted cuttings of poplar clone '546' were raised in 1-L peat container firstly on 10 April 2017 and then transplanted to pots when the plants were 30 days old with ca 28 cm in height. The soil was excavated from farmland at 2-10 cm depth, sieved through a 0.2 mm pore size screen and carefully homogenized. All plants were irrigated manually 3-4 times per week to keep soil moisture uniform and close to field capacity.

2.2. Experimental site and O_3 treatments

The experiment was conducted in open-top chambers (OTCs) in the field at Tangjiapu village (40°45′N, 115°97′E), Yanqing County, Northwest of Beijing, China. Each species was subjected to two O_3 treatments: charcoal-filtered air (CF) in which ~ 60-70% of the O_3 in the ambient air was filtered, and elevated O_3 (E-O_3, non-filtered ambient air plus an extra 40 ppb O_3 during fumigation hours). The O_3 fumigation was conducted from 10 June to 22 September 2017 for 10 h (from 08:00 to 18:00) daily except during period of rain. The addition of 40 ppb to ambient concentrations has previously been applied in different studies (e.g. Gao et al., 2017; Yuan et al., 2016). The accumulated O_3 concentration over an hourly threshold 40 ppb (AOT40) reached about 20 ppm.h (Table 1), which is a realistic value frequently observed in many polluted regions in China, e.g. Changping (AOT40=17 ppm.h, Yuan et al., 2015), and a bit lower than about 30 ppm.h in other places in China (Li et al., 2018).There were three chamber replicates for every O_3 treatment. The daily average air temperatures inside and outside the OTCs were 22.4 and 20.5 °C during ozone fumigation hours, respectively. For each species, five to seven plants were grown in each OTC. We used an electrical discharge O_3 generator (HY003, Chuangcheng Co. Jinan, China) to generate ozone from pure oxygen. The O_3 concentrations were monitored continuously in the centre of the OTCs by an UV absorption O_3 analyser (Model 49i-Thermo, Thermo Scientific, Massachusetts, USA).

At the same time as the OTC experiment, additional 10-12 plants for soybean and poplar were grown in ambient air conditions to investigate the diurnal variations

of ASC_{apo}, considering many leaves required in the similar position.

2.3. Gas exchange

Gas exchange parameters including light-saturated rate of photosynthesis (A_{sat}) and stomatal conductance (g_s) were measured with a portable leaf photosynthesis system with Li-6400-02B LED light source (Licor-6400, LI-COR Inc., Lincoln, NE, USA) according to Dai et al. (2017). One to two plants were randomly selected in each chamber, and one fifth or sixth leaf from the top of plants was selected in each plant for the gas exchange measurement. The system controlled saturating PPFD at 1200 µmol m^{-2} s^{-1}, block temperature at 30 °C, the CO_2 concentration of air entering the leaf cuvette at 400 ppm using CO_2 cylinder, flow rate at 500 µmol s^{-1} and the relative humidity at 50-60%.

2.4. Sampling and leaf tissue extraction

After the gas exchange measurement, we took leaf samples from the same plants at approximately 10:00 a.m. on 29 July (tobacco), 6 August (soybean) and 8 August (poplar) 2017 at CF air and E-O_3 (Table 1). We presumed that ASC_{apo} reached the maximum level at around 10 a.m. based on experience, which was confirmed by the measurement of diurnal change in ASC_{apo} (see 2.7). For the leaf tissue measurements, the leaves were quickly stored in liquid N until extraction of antioxidants and assessment of lipid peroxidation. For apoplastic analyses, the leaves were sampled and extracted following the approach described below.

Table 1. The date, time, O_3 concentration at sampling and AOT40 (accumulated O_3 over an hourly concentration threshold of 40 ppb during daylight hours as specified by LRTAP Convention, 2017) for the period when the tobacco, soybean and poplar species were grown in charcoal-filtered air (CF) and elevated O_3 (E-O_3) in OTCs.

Species	Treatments	Sampled date	Sampled time	O_3 concentration	AOT40

				(ppb)	(ppm h)
Tobacco	CF	29 July	10:00 am	17.2	2.30
	E-O_3	29 July	10:00 am	96.6	17.2
Soybean	CF	6 August	10:00 am	11.6	2.50
	E-O_3	6 August	10:00 am	74.3	20.5
Poplar	CF	8 August	10:00 am	5.37	2.51
	E-O_3	8 August	10:00 am	64.2	21.0

202

203 **2.4.1. Isolation of apoplastic fluid**

204 The infiltration-centrifugation method described previously (Feng et al., 2010;
205 Wang et al., 2015) was used to extract ASC_{apo}. The same leaves used for
206 photosynthesis measurements were cut into several segments with a length of about 4
207 cm, and then washed with distilled water, blotted dry and weighed. The segments
208 were vacuum infiltrated in 40 mL 100 mM KCl using a 50-mL polyethylene syringe
209 in an intercellular fluid extractor (NS-AFE-1, Pulanta Co. Suzhou, China), and kept at
210 a constant pressure. After being vacuum infiltrated, we removed excess KCl solution
211 from the infiltrated leaf surface and re-weighed the tissue. The intercellular wash fluid
212 (IWF) was recovered from the infiltrated leaf tissue by centrifugation at 2300, 4000
213 and 9000 *g* for tobacco, soybean and poplar, respectively, at 4 °C. 50 µL of 6% (w/v)
214 meta-phosphoric acid was added to IWF (supernatant) to stabilize ascorbate. After the
215 collection of IWF, the aliquot was weighed as soon as possible. We used glucose
216 6-phosphate (G6P) to detect the presence of cytoplasmic contamination (Burkey et al.,
217 2006). Individual IWF samples were excluded from analysis if a G6P signal was
218 observed.

219

220 **2.4.2. Extraction of leaf tissue**

221 Frozen leaf tissue stored in the liquid N was ground with a mortar and pestle and
222 extracted in a buffer containing 6% (w/v) metaphosphoric acid and 0.2 mM
223 diethylenetriaminepentaacetic acid (Burkey et al., 2006). The extraction buffer was

prepared fresh each day and used in a ratio of 10 mL g^{-1} FW. The homogenate was subjected to centrifugation at 18,000 *g* for 20 min at 4 °C. Extracted supernatants were immediately assayed for the antioxidants and oxidation products in leaf tissue.

2.5. Determination of antioxidants

The spectrophotometric method according to Luwe and Heber (1995) was used to determine ASC and DHA contents in IWF and leaf tissue extracts. Initial absorbance of the extract was measured at 265 nm in 100 mM K-phosphate buffer (pH 7.0), and then independently monitored following the addition of 1 U mL^{-1} ascorbate oxidase (AO) or DL-dithiothreitol (DTT) for measuring ASC or DHA, respectively. We used an extinction coefficient of 14 mM^{-1} cm^{-1} for calculating ASC at 265 nm (Nakano and Asada, 1981). The redox state of ascorbate was calculated as ASC/(ASC+DHA).

Total antioxidant capacity (TAC) was determined following the ferric reducing antioxidant power (FRAP) assay, which offers a putative index of the ability to resist oxidative damage (Benzie and Strain, 1996), expressed as Fe^{2+} equivalents (µmol Fe^{2+} g^{-1} FM).

2.6. Determination of MDA, $O_2^{\bullet-}$ and H_2O_2

Malondialdehyde (MDA) content was determined to estimate lipid peroxidation by 2-thiobarbituric acid-reactive metabolite (TBA) according to Heath and Packer (1968). The equation of C_{MDA} (mmol L^{-1}) = $6.45\times(OD_{532}-OD_{600})-0.56\times OD_{450}$ was used to calculate the content.

Superoxide anion ($O_2^{\bullet-}$) contents were measured by the hydroxylamine oxidation method according to Wang and Luo (1990). Samples (~0.02 g fresh leaves) were ground in liquid N and extracted with 2 mL of 65 mM sodium phosphate buffer (pH 7.8). The samples were centrifuged at 5000 *g* at 4 °C for 10 min and the supernatant was collected. All the extracts were implemented in an ice bath. A mixture of 1 mL supernatant, 0.9 mL 65 mM phosphate buffer (pH 7.8) and 0.1 mL 10 mM hydroxylamine was prepared, and then incubated at 25 °C for 20 min in a reaction

system composed of 0.5 mL mixture, 0.5 mL 17 mM p-aminobenzenesulfonic acid and 0.5 mL 7 mM alpha-naphthylamine. After the reaction the solution was shaken with an equal volume of n-butanol. The n-butanol absorbance was measured spectrophotometrically at 530 nm, using phosphate buffer as a blank. The extinction coefficient of nitrite (NO_2^-) is 4×10^4 M^{-1} cm^{-1}. The $O_2^{\bullet-}$ contents were calculated from NO_2^- contents based on the equation: $NH_2OH + 2O_2^{\bullet-} + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O$.

The hydrogen peroxide (H_2O_2) content was measured according to Gay and Gebicki (2000). 2 mL reaction system consisted of the appropriate volumes of reagents, 25 mM H_2SO_4, 100 µM xylenol orange (XO) and 250 µM ferrous iron to make sure the final pH was 1.8 ± 05. After 30 min in the dark, the absorbance was determined at 560 nm with XO/Fe^{2+} as a blank. The extinction coefficient of H_2O_2 is 5×10^5 M^{-1} cm^{-1}.

2.7. Diurnal variations of apoplastic ascorbate

We also measured diurnal changes of ASC_{apo} and redox $state_{apo}$ in the soybean and poplar plants grown in ambient air. We took samples every two hours between 08:00 and 16:00 on 24 July and 21 August 2017 for soybean, and on 25 July and 6 September 2017 for poplar. These dates were set before and after the measurement of plants grown in OTCs (Table 1) so as to confirm that the sampling at 10 a.m. captured antioxidants at the peak ASC_{apo}. We took leaf samples, extracted apoplastic fluid, and determined ASC_{apo} in the same way as described for the plants grown in OTCs.

2.8. Statistical analysis

The data of the measurements were averaged by chamber and subjected to the Student's *t*-test for the effect of O_3 treatment on individual traits for each species. When Levene's statistic for homogeneity of variance showed a significant heterogeneity ($P \leq 0.05$), we conducted Welch's test for treatment means and noted as such in reporting the results below. Because the sampling was not conducted on the same day for all species, we did not test for the interaction between O_3 treatment and species. Analysis of variance (ANOVA) was conducted to test the differences for

diurnal variations of apoplastic ascorbate between times of the day for each species. $P \leq 0.05$ was considered statistically significant. We used JMP software (SAS Institute, Cary, NC, USA) for the statistical analyses.

3. Results

3.1. Diurnal variations of apoplastic ascorbate

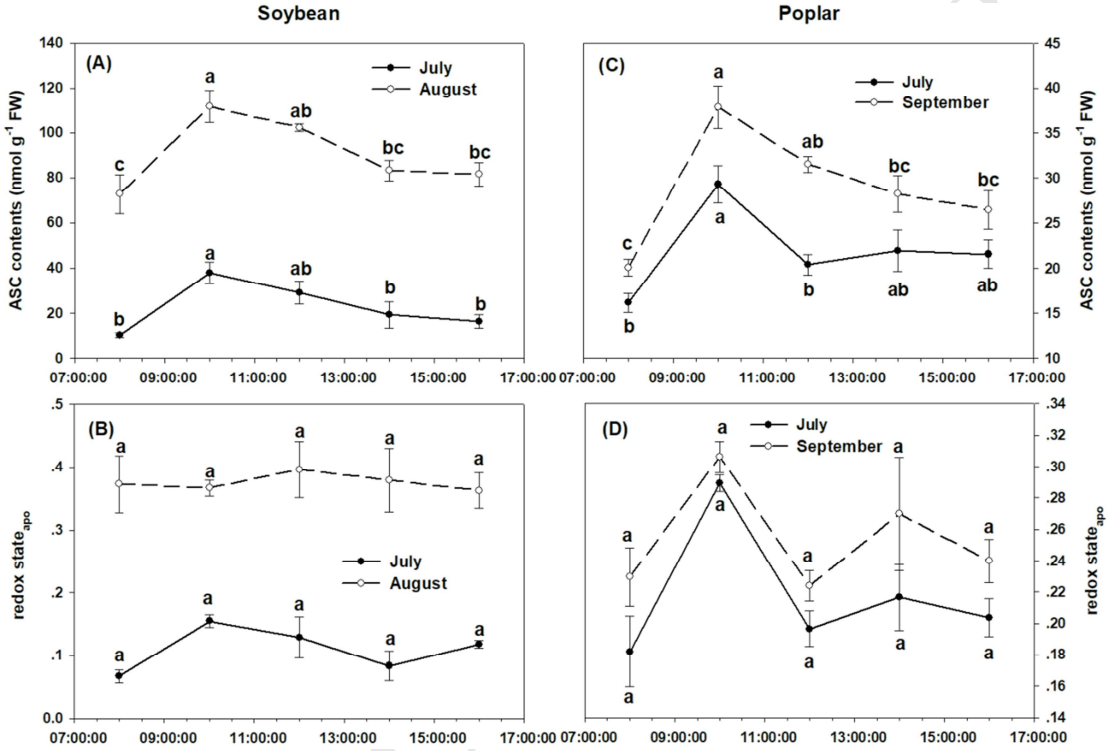


Fig. 1. Diurnal variations of reduced ascorbate (ASC_{apo}, A and C) and redox state (redox $state_{apo}$, B and D) in apoplast of soybean (measured on 24 July and 21 August, respectively, A and B) and poplar (measured on 25 July and 6 September, respectively, C and D) grown in ambient air conditions. Different letters indicate significant differences between times of the day for each species at each sampling day (mean ± SE, Tukey test, $P \leq 0.05$, n = 3-5).

For soybean and poplar grown in ambient air conditions, the diurnal variation of ASC_{apo} showed that the peak of ASC_{apo} occurred in late morning, i.e. approximately 10:00 am, which did not coincide with either the time for when the maximum external O_3 concentrations or the maximum stomatal O_3 flux occurred (Fig. 1A and C, Fig. S1). However, no significant diurnal variation in redox $state_{apo}$ was found (Fig. 1B and D).

3.2. Effect of E-O_3 on MDA and A_{sat}

The MDA in leaf tissue was significantly increased by E-O_3 for all species, in which the increases were 97.0%, 65.3% and 63.4% for tobacco, soybean and poplar, respectively (Fig. 2A). However, E-O_3 significantly decreased A_{sat} in tobacco, soybean and poplar by 46.1%, 53.5% and 30.5%, respectively, compared to CF (Fig. 2B).

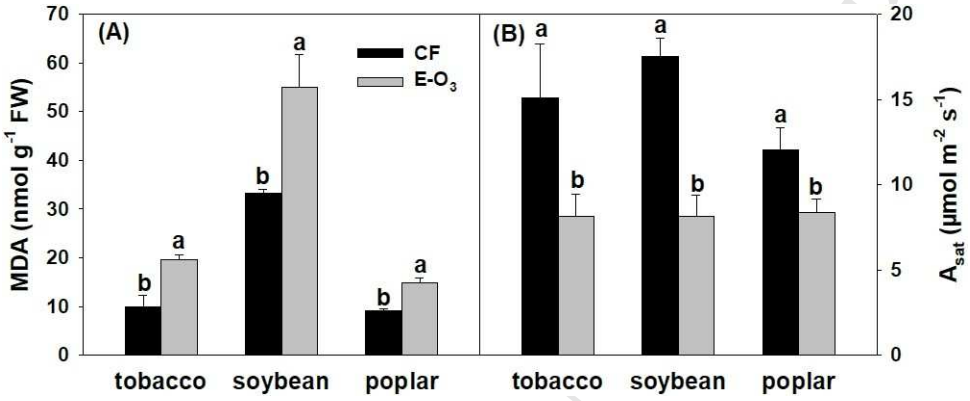
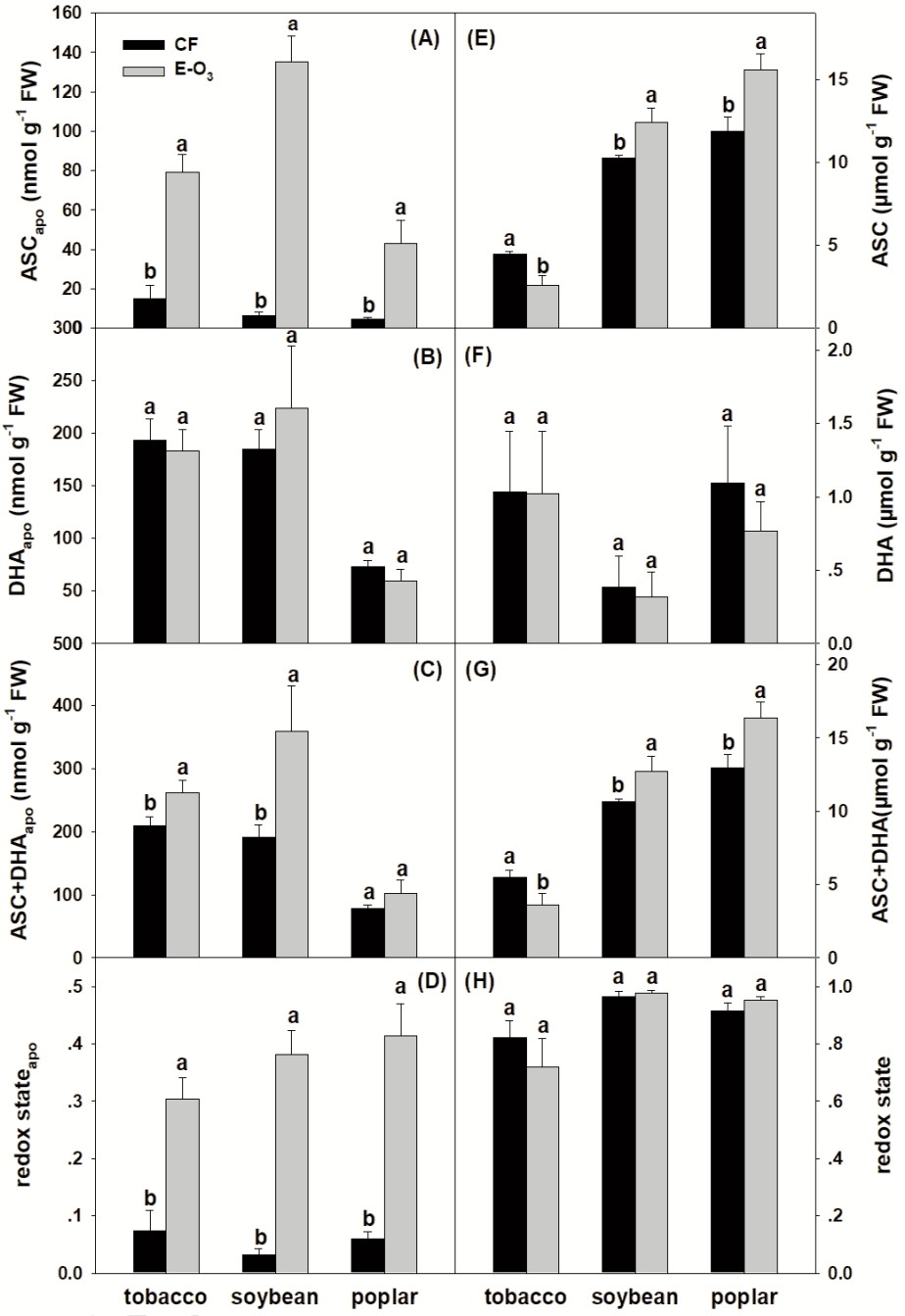


Fig. 2. Effect of E-O_3 on malondialdehyde (MDA, A) and light-saturated rate of photosynthesis (A_{sat}, B) in leaf tissue in tobacco, soybean and poplar species grown in charcoal-filtered air (CF) and elevated O_3 (E-O_3). Bars show means across OTCs and the vertical lines show SD (n = 3). Different letters indicate significant difference between CF and E-O_3 for each species ($P \leq 0.05$) detected with the Student's t-test.

3.3. Effect of E-O_3 on ascorbate contents



317

318 **Fig. 3.** Effect of E-O_3 on reduced ascorbate (ASC_{apo} and ASC), dehydroascorbate
319 (DHA_{apo} and DHA), total ascorbate ($ASC+DHA_{apo}$ and ASC+DHA) and redox state
320 ($redox\ state_{apo}$ and redox state) in the apoplast (A-D) and leaf tissue (E-H) of tobacco,
321 soybean and poplar species grown in charcoal-filtered air (CF) and elevated O_3 (E-O_3).
322 Bars show means across OTCs and the vertical lines show SD (n = 3). Different
323 letters indicate significant difference between CF and E-O_3 for each species ($P \leq 0.05$)
324 detected with the Welch's test for ASC_{apo} in poplar (A) and the Student's t-test for all
325 other measurements.

E-O_3 significantly increased ASC_{apo} content in all species. The increases were 4.22, 20.4 and 8.16-fold for tobacco, soybean and poplar exposed to 96.6, 74.3 and 64.2 ppb O_3, respectively (Fig. 3A). Furthermore, ASC_{apo} values were very low for all the species growing in charcoal-filtered (CF) chambers (Fig. 3A), where the O_3 concentrations were low (Table 1). The effect of E-O_3 on DHA_{apo} content was not significant (Fig. 3B). The ASC+DHA_{apo} content was increased significantly at E-O_3 by 25.5% in tobacco and 87.8% in soybean, but not significantly affected in poplar (+31.7%) (Fig. 3C). Similar to ASC_{apo}, the redox $state_{apo}$ was significantly increased at E-O_3 for all species (Fig. 3D); the increases were 3.13, 10.7 and 5.86-fold for tobacco, soybean and poplar, respectively.

E-O_3 significantly increased ASC content in the leaf tissue of soybean by 21.1% and poplar by 31.4%, but significantly decreased ASC content in tobacco by 42.2% (Fig. 3E). Similar to DHA_{apo}, DHA content in leaf tissue was not significantly affected by E-O_3 (Fig. 3F). Similar to ASC, ASC+DHA content was increased by E-O_3 in soybean (+19.7%) and poplar (+26.2%), but significantly decreased in tobacco (-34.5%) (Fig. 3G). However, E-O_3 had no significant effect on the redox state in leaf tissue of any of species (Fig. 3H).

3.4. Effects of E-O_3 on TAC content

TAC_{apo} was increased by E-O_3 in tobacco and poplar by 179% and 89.6%, respectively, but not for soybean (+62.1%) (Fig. 4A). However, E-O_3 only significantly increased TAC in leaf tissue of tobacco by 47.1%, but no significant effects on soybean (-6.7%) and poplar (-6.9%) were seen (Fig. 4B).

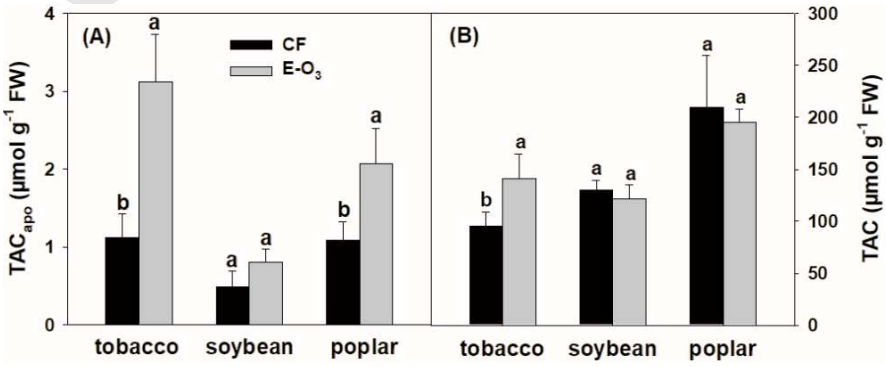


Fig. 4. Effect of E-O_3 on total antioxidant capacity in the apoplast (TAC_{apo}, A) and

leaf tissue (TAC, B) of tobacco, soybean and poplar grown in charcoal-filtered air (CF) and elevated O_3 (E-O_3). Bars show means across OTCs and the vertical lines show SD (n = 3). Different letters indicate significant differences between O_3 treatments for each species ($P \leq 0.05$) detected with the Student's t-test.

3.5. Effect of E-O_3 on $O_2^{\bullet-}$ and H_2O_2 contents in leaf tissue

E-O_3 significantly increased $O_2^{\bullet-}$ content of poplar by 18.4%, but no significant effect on tobacco (+18.8%) and soybean (+45.6%) was found (Fig. 5A). In addition, E-O_3 significantly increased H_2O_2 content of tobacco and soybean by 26.2% and 82.0%, respectively, whereas had no effect for poplar (Fig. 5B).

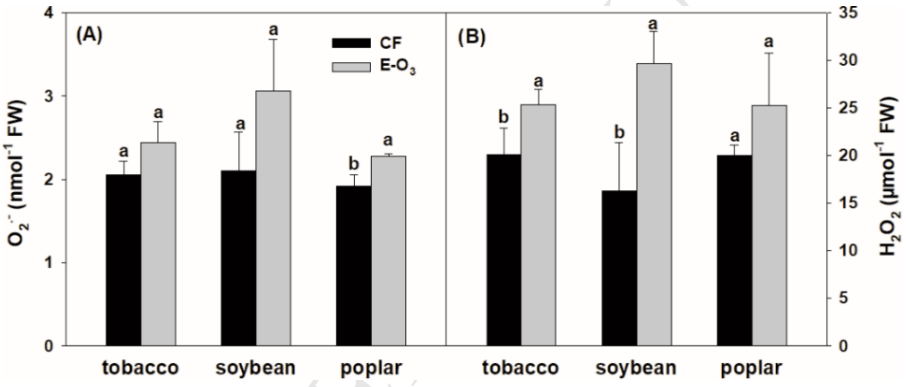


Fig.5. Effect of E-O_3 on the $O_2^{\bullet-}$ and H_2O_2 contents in leaf tissue of tobacco, soybean and poplar grown in charcoal-filtered air (CF) and elevated O_3 (E-O_3). Bars show means across OTCs and the vertical lines show SD (n = 3). Different letters indicate significant differences between O_3 treatments for each species ($P \leqslant 0.05$) detected with the Student's t-test.

4. Discussion

In recent decades, increased attention has been paid to the detoxification of O_3 by ASC_{apo}. Ascorbate is considered a powerful antioxidant due to its ability to donate electrons in several enzymatic and non-enzymatic reactions (Sharma et al., 2012). In our study, ASC_{apo} is more likely induced by high O_3 concentration as indicated by the significant increases in ASC_{apo} by E-O_3 in all the species compared to CF. Current results confirm previous finding for soybean (*Glycine max* (L.) Merr.) in greenhouse

(Cheng et al., 2007) and wheat (*Triticum aestivum* L.) in open air O_3 fumigation (Feng et al., 2010), i.e. ASC_{apo} was higher in elevated O_3 than control treatment. However, in another study, ASC_{apo} was reduced by E-O_3 in *Plantago major* (Lyons et al., 1999). The different results may be attributed to differences in sampling time. The sampling time in Lyons et al. (1999) was between 12:00 and 14:00 pm, whereas it was around 10:00 am in our experiment. The diurnal variations showed that the ASC_{apo} was the highest at 10:00 am and decreased with the increasing O_3 concentration in the afternoon. The diurnal pattern observation was also supported by studies on wheat (De la Torre 2008; Wang et al. 2015). Furthermore, the previous study in wheat by Wang et al. (2015) concluded that the ASC_{apo} was stimulated by the instantaneous O_3 on the basis of linear relationship between ASC_{apo} and O_3 concentrations in open air O_3 fumigation. In our study, we observed the stimulation effects on ASC_{apo} by high O_3 concentrations compared to CF treatment in all the species directly rather than inferring a relationship from a linear extrapolation. Thus, our results provide stronger evidence for the verification of the stimulation effects on ASC_{apo} by instantaneous O_3.

The mismatching between the diurnal pattern of detoxification and that of ambient O_3 concentration and uptake could induce injury/damage to vegetation (Musselman et al., 2006; Mishra and Agrawal, 2015). The accumulation of instantaneous flux over time combined with diurnal changes in detoxification results in an "effective" O_3 dose (Heath et al., 2009; Wang et al., 2015), which may not necessarily be the same as the integrated dose (the total amount of pollutant absorbed into the plant through the stomata over a specific period). Estimated differences between "effective" dose and integrated dose could result in differences in modeling estimates for vegetation injury and/or damage. If the period of optimum uptake occurs when higher ASC_{apo} contents are present, greater detoxification of O_3 may occur in comparison to the late afternoon when higher O_3 concentrations occur and less detoxification is present due to lower ASC_{apo} content. Our results indicate that the detoxification capacity by ASC_{apo} is not a constant but varies with O_3 concentrations, although the detoxification has been assumed to be constant in the stomatal O_3 flux model (Emberson et al., 2000; Mills et al., 2011). It is therefore recommend that the

406 O_3 flux model should incorporate a dynamic detoxification component that reflects
407 the temporal variation in ASC_{apo}.

408 ASC_{apo} is oxidized during O_3 exposures and results in the generation of DHA,
409 which is then transported back into the cytoplasm where it is reduced again to ASC by
410 coupled reactions involving DHA reductase and reduced glutathione (Luwe et al.,
411 1993; Horemans et al., 2000). However, in our study either DHA_{apo} or DHA was not
412 significantly affected by E-O_3 in any of the species studied. The inconsistent
413 responses of ASC and DHA may be explained by the hypothesis that DHA also
414 participates in the signal transductions across the plasma membrane, in processes
415 unrelated to the AsA-GSH cycle reactions (D´Haese et al., 2005). Importantly, in the
416 apoplast, the increase in ASC_{apo} and no change in DHA_{apo} induced by E-O_3 appear to
417 reflect the dynamic response of ASC_{apo} to the diurnal O_3 concentrations (De la Torre
418 2008; Feng et al., 2010; Wang et al., 2015). After the ASC_{apo} rise to a certain threshold
419 as the O_3 concentrations increase, the ASC_{apo} will decrease and DHA_{apo} increase when
420 the ASC_{apo} reacts with O_3. In addition, Luwe et al. (1993) found that the transport of
421 DHA back into the cytosol was slower than ASC transport into the apoplast. The
422 redox $state_{apo}$ is controlled by ascorbate oxidase (AO) and this may affect the growth
423 and O_3 resistance of plants (Pignocchi et al., 2003). It has been reported that ASC also
424 plays an essential role in the homeostasis of the intracellular redox status (Pastori et
425 al., 2003; Barth et al., 2004). Our results showed that E-O_3 not only induced a greater
426 ASC_{apo} content but also a greater redox $state_{apo}$, suggesting that the altered redox
427 $state_{apo}$ by E-O_3 may also affect signal transductions across the plasma membrane
428 (D´Haese et al., 2005). Riikonen et al. (2009) found that interactions with O_3 and
429 temperature affect the redox $state_{apo}$ in the apoplast of birch (*Betula pendula*). The
430 significant increase in TAC_{apo} of tobacco and poplar induced by E-O_3 indicated that
431 E-O_3 induced an antioxidant response including ASC_{apo}. In agreement with Cheng et
432 al. (2007), TAC_{apo} was not affected by E-O_3 in soybean. However, the ASC_{apo} content
433 was very low, even under E-O_3 in Cheng et al. (2007), which suggested that other
434 antioxidant metabolites in addition to ASC_{apo} possibly affect plant sensitivity to O_3 in
435 soybean leaves.

In leaf tissue, the ASC response to E-O_3 was different from that of ASC_{apo}. The results may be explained by the conclusion by Foyer et al. (1994) that 'the antioxidative system does not appear to anticipate the possibility of potential injury by an immediate response of the genes for the antioxidants, but rather waits to respond to actual injury', suggesting that the antioxidative responses in leaf tissues to E-O_3 may undergo a time-lag. Furthermore, ASC was reduced by E-O_3 in tobacco, but increased in soybean and poplar, which indicates a difference in antioxidative capacity to E-O_3 among the three species. The antioxidant system in tobacco leaves may also have been overwhelmed by high O_3 to some extent as suggested by the significant reduction in ASC and A_{sat}, as well as some visible O_3 symptoms on the leaves (not shown). Therefore, our results suggest that ASC_{apo} responded immediately and preferentially to high O_3 concentrations compared to ASC in leaf tissue, which is also supported by the different response between TAC and TAC_{apo}. In agreement with this finding, Turcsányi et al. (2000) found that acute O_3 treatment (150 ppb O_3 for 8 h) affected the ASC_{apo} but had no effect on the level and/or redox state of ASC in leaf tissue. In addition, the differences in ASC content were attributable to the antioxidative capacity of the different species in response to O_3 as indicated by significant difference in ASC among the three species in CF conditions ($p < 0.001$, data not shown), i.e. species differences resulted from the differences in constitutive antioxidant capacity of plants *in vivo*, rather than induced by high O_3 stimulation. The significant decrease in ASC, but significant increase in TAC of tobacco indicated that there are also other antioxidants, for example, antioxidative enzymes involving the detoxification of O_3. Effects of E-O_3 on the redox state in leaf tissue were not significant, indicating that E-O_3 did not cause gross cellular oxidative stress.

The detoxification by ASC_{apo} was insufficient in the E-O_3 treatment to protect the plants from O_3 injury, as indicated by significant increase in MDA and decrease in A_{sat}. The higher MDA contents in leaf tissue indicated an increase in lipid peroxidation by E-O_3 in all species. Furthermore, the significant increase of leaf tissue $O_2^{\bullet-}$ and H_2O_2 in some species by E-O_3 indicated that the capacity for ROS detoxification by ASC_{apo} has been overwhelmed and O_3-induced ROS has induced

oxidative damage to plasma membranes and cytoplasm (Castagna and Ranieri, 2009; Hossain et al., 2015). In the present study, ASC_{apo} was significantly increased at E-O_3 when we sampled at 10:00 am for all the species. However, the ASC_{apo} decreased with the increasing O_3 concentration in the afternoon from the diurnal variations, suggesting that the capacity to transport ASC from the cytoplasm to the apoplast is exceeded by the demand from incoming O_3 with the result that ASC_{apo} declined. The results imply that the lower potential of O_3 injury in the morning was due to the lower O_3 flux or the higher detoxification potential, i.e. higher ASC_{apo} content. In contrast, the detoxification potential by ASC_{apo} was low in the afternoon. In conclusion, the O_3 detoxification by ASC_{apo} was limited due to the limited ASC_{apo} level, i.e. the ASC_{apo} and TAC_{apo} were not sufficient to detoxify O_3 to protect plant from O_3 injury, especially when the O_3 concentration was relatively high in the afternoon (Fig. S1). The visible O_3 symptoms that occurred on the leaves of tobacco and soybean supported the findings (not shown). Oksanen et al. (2005) also demonstrated H_2O_2 accumulation extending from cell wall to cytosol and adjacent chloroplast in birch (*Betula pendula*) exposed to 2-fold ambient O_3. In addition, some studies have shown that additional apoplastic constituents, like antioxidant enzymes, polyamines, phenolics and glutathione may also play a role in the detoxification of O_3 in the leaf apoplast (Turcsányi et al., 2000; Booker et al., 2012). However, the specific detoxification mechanism and ability of them are not clear yet, and warrant further studies.

5. Conclusion

The major conclusions of our study were (1) high O_3 concentrations stimulate ASC_{apo}, which is an important contributor to the O_3 detoxification process, (2) ASC_{apo} displays a diurnal variation, and (3) the detoxification by ASC_{apo} was not sufficient to protect plants from current O_3 pollution based on the “effective” dose received during the study period, although the ASC_{apo} was significantly stimulated by E-O_3 in all the species. ASC_{apo} is induced by high instantaneous O_3 concentrations, whereas ASC contents in different leaf tissues are more likely associated with antioxidative capacity

among different species. These results provide additional information for further developing "effective" O_3 flux models, which consider the diurnal variation in plant detoxification. The findings are important to those researchers who develop models that relate to the effects of O_3 on vegetation and to those who are involved in the O_3 standard-setting process. In addition, further research is recommended to quantify the degree of O_3 detoxification by ASC_{apo} among different species.

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695

Highlights

- Apoplastic antioxidant was investigated in three species under two O_3 treatments
- Apoplastic reduced-ascorbate is induced by high instantaneous O_3 concentrations
- Apoplastic reduced-ascorbate displays a diurnal variation
- Increase of apoplastic ascorbate induced by O_3 is insufficient to protect plants from O_3