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Diurnal variation of apoplastic ascorbate in winter wheat leaves in relation to ozone detoxification

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Besides stomatal closure, biological detoxification is an important protection mechanism for plants against ozone (O_3). This study investigated the diurnal changes of ascorbate (a major detoxification agent) in the apoplast and leaf tissues of winter wheat grown under ambient air field conditions. Results showed the reduced ascorbate in the apoplast (ASC_{apo}) exhibited a peak in late morning or midday, mismatching with either the maximum external O_3 concentrations in mid-afternoon or the maximum stomatal O_3 uptake between late morning and mid-afternoon. In contrast, the ASC in leaf tissues remained stable throughout the day. The investigations conducted in a Free-Air Concentration Elevation of O_3 system confirmed that the diurnal variations of the ASC_{apo} were induced more by the daily variations of O_3 concentrations rather than the cumulative O_3 effects. In conclusion, the O_3-stress detoxification should be a dynamic variable rather than a fixed threshold as assumed in the stomatal flux-based O_3 dose metrics.

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1. Introduction

Tropospheric ozone (O_3) is one of the most critical air pollutants, and its current concentration in many regions, particular East Asia, has elicited adverse effects on crop growth and yield (Avnery et al., 2011; Calvete-Sogo et al., 2014; Feng and Kobayashi, 2009; Feng et al., 2014; Tang et al., 2013; Wilkinson et al., 2012). The phytotoxicity of O_3 to plants derives primarily from its oxidative damage to the plasma membranes (Schraudner et al., 1997). Heath et al. (2009) reviewed the interaction of O_3 with plant tissues and proposed three distinct processes: “external O_3 concentration”, “uptake”, and “detoxification”. Specifically, the ambient O_3 generated from photolysis of NO_2 is taken up, via stomata, into apoplastic spaces of the leaves. Following the entry into leaf interior, O_3 dissolves in the apoplastic liquid and produces a series of reactive oxygen species (ROS), triggering antioxidant responses (Langebartels et al., 2002).

With regards to the first line of defense against O_3, ascorbate is the most representative one in the apoplast (Dizengremel et al., 2008). The apoplastic ascorbate accounts for up to 5% of the whole leaf ascorbate pools (Noctor and Foyer, 1998) and appeared to be sufficient to detoxify a significant portion of O_3 under environmentally relevant conditions (Chameides, 1989; Lyons et al., 1999; Plochl et al., 2000; Frei et al., 2010). Also, most evidence has shown a close positive correlation between O_3 tolerance and apoplastic ascorbate contents in different plants (Burkey et al., 2003, 2006; Feng et al., 2010; Frei et al., 2010; Zheng et al., 2000) with an exception in *Trifolium repens* (D'Haese et al., 2005). Therefore, the ascorbate especially in the apoplast has been used to represent the capacity of O_3 detoxification (Heath, 2008).

To quantitatively estimate O_3 effects on plants, relationships between the relative biomass/yield and different O_3 indices have been developed on the basis of results from controlled O_3 fumigation experiments under near-field conditions (Mills et al., 2011). So far, the stomatal O_3 flux-based approach has been assumed superior to the exposure-based ones by taking into account the biological and climatic factors (Buker et al., 2007; Feng et al., 2012; Mills et al., 2011). However, the stomatal O_3 flux-based approach

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does not consider dynamic changes of O_3 detoxification in plants, and may provide biased estimation of the O_3 damages (Massman et al., 2000).

Growing attention has been paid to considering the combination of O_3 uptake and detoxification (Dizengremel et al., 2008; Fares et al., 2010; Grantz, 2014; Grantz et al., 2013; Heath et al., 2009; Musselman et al., 2006; Massman, 2004). Besides species and developmental stages, the detoxification processes to O_3 are theoretically dependent on temporal changes of O_3 concentration and other environmental conditions, such as solar radiation, air temperature and moisture stress (Heath et al., 2009). In addition, Heath et al. (2009) suggested that diurnal patterns of detoxification do not necessarily match that of O_3 concentration. It implies that the daily maximum O_3 concentration or uptake on the one hand may not incur the maximum damage due to a high capacity of detoxification; and on the other hand, the low and medium O_3 concentration may cause considerable damages due to a weak detoxification capacity. However, this theoretical daily trend especially for the detoxification by apoplastic ascorbate is conceived on the basis of high O_3 exposures for brief periods (less than two weeks) and limited data with few sampling times e.g. twice or three times per day (Burkey et al., 2003; Kollist et al., 2000). Therefore, it is necessary to experimentally demonstrate the diurnal patterns of "external O_3 concentration", "uptake", and "detoxification" in plants growing under open air field conditions from seed germination to maturity.

In the present study, we investigated the daily variations of ascorbate contents in both apoplast and leaf tissues in the ambient air field condition, in order to test whether there exists a diurnal pattern of ascorbate in winter wheat grown in the present environment. Further, the plants exposed to additional O_3 treatment via Free-Air Concentration Elevation of O_3 (O_3-FACE) system were sampled in order to determine whether daily variations of O_3 concentrations or the cumulative O_3 effects contribute to the diurnal changes. The experiment was investigated over a long period under open air field conditions with four measuring days at different developmental stages. The obtained results will serve as a better input for the quantification of effective O_3 flux (balance between stomatal O_3 uptake and detoxification) and improve the O_3 exposure standards and critical levels for crops.

2. Materials and methods

2.1. Experimental site

The study was carried out in a paddy field in Xiaoji Town, Jiangsu Province, China (119° 42′ E, 32° 35′ N). The site has been cultivated continuously with rice–wheat or rice–rapeseed rotation for more than 1000 years. The soil type is Shajiang Aquic Cambosols with a sandy-loamy texture. The site is in the subtropical marine climatic zone with mean annual precipitation being 1100–1200 mm, mean annual temperature 16 °C, a total annual sunshine hour more than 2000 h and a frost-free period more than 230 days (Zhu et al., 2011).

2.2. Plant material

The winter wheat variety cv. Yangmai 16 (Y16) was selected in this experiment for its popularity in this region. The cultivation followed the local standard practices (Zhu et al., 2011). The wheat plants reached flowering stage on 29 April and grain maturity on 7 June, 2009. Nitrogen (210 kg N ha^{-1} of urea and diammonium phosphate) was split-applied with 60% at planning, 10% at early tillering and 30% at elongation stage, respectively. The P (90 kg P_2O_5 ha^{-1}) and K (90 kg K_2O ha^{-1}) were split-applied with 60% at planting and 40% at elongation stage, respectively.

2.3. Measurements and sampling

The daily changes of ascorbate contents in the apoplast and leaf tissues were measured in wheat plants on the four days of 28 April, 9 May, 10 May and 24 May, 2009, under the ambient air O_3 (A-O_3) condition. On the four days, the daily samplings were conducted between 8:00 h and 17:00 h with 1.5 h interval for ascorbates measurements. At the same time, the gas exchange (including photosynthetic rate and stomatal conductance) of flag leaves was measured in the field using a LI-6400 photosynthesis system (LICOR, Lincoln, NE, USA) fitted with a 6400-40 leaf chamber fluorometer (LCF) under the ambient humidity, temperature and radiation.

To determine whether daily variations of O_3 concentrations or the cumulative O_3 effects contribute to the diurnal changes of ASC_{apo}, we sampled the plants exposed to an O_3-FACE with elevated (E-O_3, targeted at ambient $[O_3] \times 1.5$) $[O_3]$ on both 9 May and 10 May following to the same protocol as ambient plants. The O_3 fumigation was intentionally ceased on 9 May, and restarted on the next day, 10 May from 9:00 a.m. The O_3-FACE fumigation began at 9:00 a.m. and continued through to the sunset. O_3 fumigation started on 1 March at tillering stage of wheat until the grain maturity. More details of the design and the performance of the system can be found in Zhu et al. (2011) and Tang et al. (2011).

2.4. Extraction of apoplastic fluid

The apoplastic ascorbate was extracted by infiltration–centrifugation method as described previously (Feng et al., 2011). Briefly, after being cut into 4 cm segments, flag leaves were infiltrated with 100 mM KCl in a 50-ml polyethylene syringe, and then centrifuged at 650 g at 4 °C for the collection of apoplastic fluid. Segment weights before and after infiltration, and following centrifugation were recorded for the calculation of the recovery of the infiltrated solution in order to calculate the apoplast ascorbate content (Burkey et al., 2006). The presence of glucose 6-phosphate (G6P) was used as a marker for cytoplasm contamination. The sample was discarded when a G6P signal was observed in the apoplastic fluid.

2.5. Extraction of leaf tissues

Frozen tissues were ground in liquid nitrogen with a mortar and pestle, and extracted in buffer containing 6% (w/v) metaphosphoric acid, 0.2 mM diethylenetriaminepentaacetic acid. Fresh extraction buffer was prepared at each sampling day and added in a ratio of 10 ml g FW^{-1}. After centrifuging at 6 000 g for 10 min at 4 °C, the supernatants were ready for ascorbate measurements (Burkey et al., 2006).

2.6. Assay of ASC and DHA

The spectrophotometric method described previously by Luwe and Heber (1995) was employed to determine the reduced ascorbate (ASC) and oxidized ascorbate (DHA) in the apoplast and leaf tissue extracts. Specifically, initial absorbance of extract was measured at 265 nm in 100 mM K-phosphate buffer (pH 7.0), and then independently monitored following the addition of ascorbate oxidase or DL-dithiothreitol (DTT) for estimating ASC or DHA, respectively (Feng et al., 2010). For calculation of the ascorbate content, an extinction coefficient of 14 mM^{-1} cm^{-1} for ASC at 265 nm was used (Nakano and Asada, 1981). The redox state of ascorbate was calculated as ASC/(ASC + DHA) × 100%.

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