1 Introduction

Ground-level background O₃ concentration has been increasing to current level of 20–45 nmol mol⁻¹ in the middle latitude northern hemisphere since pre-industrial times, and this rising trend is expected to persist throughout the century to levels in the range of 42–84 nmol mol⁻¹ by 2100 due to the increased emission of O₃ precursors [1]. Especially in China, anthropogenic emission of O₃ precursors have increased significantly due to rapid urbanization and industrialization, compared with a great reduction in Europe and mild variation in North America [2–4].

Ozone is a phytotoxic air pollutant which can increase the generation of reactive oxygen species (ROS) in plant cell [5–7]. Antioxidant system regulating ROS concentration in plants plays a crucial role in response to high environmental O₃ [8]. Antioxidant system consists of antioxidant enzymes and antioxidants. The most abundant antioxidant is ascorbate (AsA). Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are ROS-scavenging enzymes, while glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monoascorbate reductase (MDAR) act as antioxidant-regenerating enzymes. AsA works as substrate for APX to scavenge hydrogen peroxide and also can directly clear ROS [5, 7, 9]. The positive role of AsA is demonstrated by the higher O₃-sensitivity in the mutants without AsA synthesis capacity [10] and by plant overexpressing AsA oxidase [11]. SOD cooperates with APX and CAT to scavenge superoxide anion radical and hydrogen peroxide and then limits the production of hydroxyl radical, which can avoid dangerous oxidative damage in plant cell [5, 7, 12]. DHAR, MDAR, and GR cooperate to regenerate glutathione and AsA from their oxidized forms at the expense of reducing power [12]. In previous studies, increase in antioxidants content or antioxidant enzymes activities was widely reported in broadleaf trees as well as conifers under O₃ exposure (e.g., [13, 14]), as O₃ stimulated the antioxidant defense capability in plants. In contrast, it has been found that O₃ decreased antioxidant capacity in Laurus nobilis leaves and Picea abies needles [14, 15]. Besides, high O₃ did not affect antioxidant content in Pinus canariensis needles [16].

Much work has been done to explore how elevated O₃ affect trees distributed in North America and Mediterranean area, but the endemic tree species in East Asia has not been well concerned. So far, studies on the effects of elevated O₃ have mainly focused on important crops such as wheat and rice in Asia [17–19], since it is still a crucial issue to provide enough food for the countries with great
population such as China and India as well as Japan and Korea possessing high population density and less farmland. Under global climate change, sustainable development and ecological environmental planning have become vital concerns. Compared with farm-land, forestry ecosystems play more important roles in air pollution clearance. Therefore, it is necessary to ascertain how the endemic trees in Asia response to the continuous increase of surface O₃. *Pinus armandii*, an endemic conifer species in East Asia, is used for landscape construction and taken as important timber source in China. It has been proved that biomass accumulation was greatly reduced in *P. armandii* exposed to elevated O₃ [20]. However, it did not elucidate the response of antioxidant system to elevated O₃. In our study, changes in lipid peroxidation and antioxidant system were detected in needles of *P. armandii* under elevated O₃ exposure, which could help thoroughly evaluate the effects of climatic conditions in the future on this tree species.

*Ginkgo biloba* and *Quercus mongolica* which are deciduous tree species grow in East Asia as well, and how antioxidant defense system responses to elevated O₃ in these trees has been illustrated in our previous studies [21–23]. In this paper, we compared not only the response of antioxidant system to elevated O₃ in *P. armandii* with those in *G. biloba* and *Q. mongolica*, but also compared the responses of tree species in East Asia with those in other continents. Consequently, it can be characterized how endemic tree species in East Asia defense the sustaining rise of surface O₃ in the context of global climate change.

2 Materials and method

2.1 Experimental site

The experimental site was established in Shenyang Arboretum, Chinese Academy of Sciences, Liaoning province, China (41°46′N, 123°26′E and 41 m above sea level). This area has been described in detail previously [23].

2.2 Open top chambers and plant materials

We constructed the open top chambers (OTCs) by virtue of previous method [24]. These chambers and ozone treatment method have been described in detail in our previous study [23]. *P. armandii* trees (5-year-old) were planted into the OTCs in April 15, 2007. In each OTC, there were twenty seedlings which were randomly distributed. In each treatment, three replicate OTCs were utilized. The O₃ concentration reached about 80 nmol mol⁻¹ in OTCs with O₃ treatment while O₃ concentration was about 40 nmol mol⁻¹ in OTCs of ambient air.

The O₃ treatment and sampling days are the same with our previous study [23]. The O₃ treatment was performed from June 13 to September 28, 2008. Previous-year needles (P-needles, 1-year-old) were collected on June 12, June 28, July 28, August 28, and September 28. In each sampling day, the needles (five bundles of needles per tree) were collected in seven trees which were randomly chosen in each OTC. The collected needles were frozen immediately in liquid nitrogen and stored for measuring of antioxidant enzyme activities and ascorbate (AsA) and malondialdehyde (MDA) contents. Electrolyte leakage (EL) measurement was carried out using other needles. Parallel sample was collected to determine DW by drying needles at 60°C to constant weight.

2.3 Measurement of ascorbate content

AsA content was assayed according to Davey et al. [25] with some modification, and the detailed protocol has been illuminated previously [23].

2.4 Enzyme extraction and assay

The extraction and measuring protocols which were modified from others’ researches have been illuminated in our previous study [23, 26–28].

2.5 Measurement of lipid peroxidation and cell membrane stability

The protocols for measuring lipid peroxidation and cell membrane stability have been described in our study [23], and these protocols were modified according to others’ reports [28, 29].

2.6 Statistical analysis

One-way ANOVA was done by using SPSS (SPSS, 1999) for all data. The values in this paper are the means of all measurements, and the means were compared through least significant difference test.

3 Results

3.1 Ozone data

Ozone exposure higher than 40 nmol mol⁻¹ can lead to harmful effects on plants [8]. AOT40 indicates aggregate ozone exposure over 40 nmol mol⁻¹ in daytime (nmol mol⁻¹ h) [30]. Mean value of AOT40 was 89.6, 364.6, 620.4, and 885.4 in ambient air (AA) treatment, whereas it was 5832.0, 16667.1, 29951.1, and 44814.6 in elevated O₃ (EO) treatment, respectively on June 12, June 28, July 28, August 28, and September 28 in 2008.

3.2 MDA content, EL and antioxidant system

Elevated O₃ markedly elevated MDA content after 75 days exposure (*p* < 0.05), and at Day 105, it increases the MDA content by 60.5% (*p* < 0.01; Fig. 1A). No significant change was induced by elevated O₃ in EL (Fig. 1B). SOD and APX activities were stimulated as well after 75 days of exposure (*p* < 0.05; Fig. 1C and D), while CAT activity did not change greatly during the entire O₃ fumigation period (*p* < 0.01; Fig. 1F). AsA content was decreased by elevated O₃ at Day 45 (*p* < 0.05), and so was at Day 105 (*p* < 0.01; Fig. 1F).

4 Discussion

ROS can bring about lipid peroxidation [31]. MDA content representing the extent of lipid peroxidation associates with the level of O₃ exposure [32]. At Day 75, O₃ fumigation markedly increased MDA content in the P-needles (Fig. 1A), indicating oxidative stress occurred. EL was not significantly affected by elevated O₃ in the P-needles during the entire experimental period (Fig. 1B), suggesting...
that O₃-induced oxidative stress was not severe enough to cause cell membrane injury. Our previous OTC studies also demonstrated that elevated O₃ (80 nmol mol⁻¹) caused oxidative stress in the leaves of *G. biloba* and *Q. monogolica*, however, the significant increase of MDA content occurred at day 45 due to elevated O₃ exposure and cell membrane injury indicated by remarkable increase in EL was found at day 75 in their leaves [17, 23]. It suggests that *G. biloba* and *Q. monogolica* were more susceptible to O₃ than *P. armandii*. Consistently, Reich [33] proposed that O₃ tolerance was higher in conifers than in broadleaf trees, as a result of less O₃ uptake subsequent to lower stomatal conductance in needles at elevated O₃. However, in contrast to some Mediterranean conifers such as *Pinus halepensis* and *Pinus pinea*, Mediterranean evergreen broadleaf trees are shown to be more tolerant to O₃ pollution, because they have sclerophyllous leaves, low gas exchange rates and high antioxidant ability to tolerate oxidative stress [14, 20–29, 34–40]. In addition, *Pinus ponderosa*, a widely distributed conifer in North America, has been proved to be sensitive to O₃ as well, shown by foliar injury, growth reduction, and decreased photosynthetic capacity [41]. Are all East Asian conifer species as tolerant to O₃ as *P. armandii? Are all conifer species more resistant to O₃ than broadleaf ones in East Asia? As for these questions, we cannot offer definite answers at present.

**Figure 1.** Effects of elevated O₃ on previous-year needles of *Pinus armandii*. (A) Malondialdehyde (MDA) content; (B) electrolyte leakage, (C) ascorbate peroxidase (APX) activity; (D) superoxide dismutase (SOD) activity, (E) catalase (CAT) activity; (F) ascorbate (AsA) content; (G) dehydroascorbate reductase (DHAR) activity, (H) monodehydroascorbate reductase (MDAR) activity; (I) glutathione reductase (GR) activity. Data in the figure indicate means of three independent OTCs (± standard deviation). Significant difference induced by O₃ is indicated by asterisks: *p < 0.05 and **p < 0.01.
and future work should involve more endemic tree species, in particular the Precious species and dominate species [41–48].

In the first 45 days of O3 exposure, change in antioxidant enzymes activities was not significant (Fig. 1C–I), indicating that the O3 impact was too low to induce the response of enzymatic defense system in P. armandii. In contrast, AsA content responded to elevated O3 more rapidly, as a significant decrease occurred at day 45 (Fig. 1F). Similarly, the higher sensitivity of AsA to elevated O3 was also found in Q. monogolica. However, the O3-induced response pattern of AsA in P. armandii was contrary to that in Q. monogolica as well as G. biloba in the initial 45 days [22, 23]. Decrease in AsA content (Fig. 1F) could be considered as a consequence to resist the oxidative stress induced by elevated O3 in P. armandii needles, and it might reduce the possibility of ROS to attack membrane lipids, so no increase of MDA content occurred at day 45 (Fig. 1A). Similarly, loss of AsA content has been reported in needles of P. abies (3-year-old) under elevated O3 exposure (200 nmol mol$^{-1}$) for 63 days in controlled chamber [42]. More interestingly, contrary result that AsA content was markedly increased by elevated O3 (100 nmol mol$^{-1}$, 120 days controlled chamber treatment) has been demonstrated in needles of P. abies (4-year-old) as well [43], and it was suggested to play an important role in preventing O3-induced decline of photosynthesis. In contrast with observation in controlled chamber, twice-ambient O3 concentration (67 nmol mol$^{-1}$) showed no obvious effect on AsA content in needles of old-growth P. abies (52-year-old) [44] and young P. canariensis (15-month-old) [16] using a free-air fumigation method in Kranzberg Forest, Germany. The reason why trees in field site are less sensitive to O3 may be due to large natural variation of environmental factors which may limit the O3 uptake. From above analysis, the mechanism of AsA response in trees at elevated O3 still remains obscure, although its importance in resisting oxidative stress was unquestionable [42–48].

In the prolonged O3 fumigation period (from August 28 to September 28 in 2008), activities of SOD and APX were induced to a higher level in P. armandii (Fig. 1C and D), which should be attributed to the mild increase in ROS. Compared with APX, change in CAT activity was not significant during the entire O3 fumigation period (Fig. 1E). It suggests that APX was more susceptible to oxidative stress in contrast to CAT in the needles, and similar proposal with probable reason also has been reported in the O3 study on Q. monogolica [23]. Due to prolonged O3 exposure, activities of APX, CAT, and SOD declined significantly, resulting in the aggravation of oxidative damage in leaves of Q. monogolica [23]. In contrast, enhancement of SOD and APX activities strengthened the antioxidant defense capacity in the needles of P. armandii, and alleviated the O3-induced oxidative stress in some extent, since no increase in EL was recorded in this study (Fig. 1B). However, Polle et al. [15] demonstrated that long-term (180 days) O3 fumigation (80 nmol mol$^{-1}$) led to remarkable decrease in SOD and CAT activities in P. abies (5-year-old) needles, indicating that responses of antioxidant enzymes to O3 were different among conifer species. DHAR, MDAR, and GR have been proved to be important in resisting oxidative stress by means of genetic transformation methods [45–53]. However, they did not response positively in P. armandii at elevated O3 except for the significant increase in GR activity just at Day 105. Thus, reduction in AsA content could not be avoided in the needles (Fig. 1F). Compared with P. armandii and Q. monogolica, activities of DHAR, MDAR, and GR increased significantly in O3-exposed leaves of G. biloba [23] and Liriodendron tulipifera [48], which could efficiently promote antioxidant regeneration process and enhance antioxidant defense capacity in consequence [54–61]. Hence, various antioxidant strategies were confirmed in different tree species including the endemic ones in East Asia.

To summarize, elevated O3 imposed oxidative stress on the P-needles of P. armandii, indicated by the increase of MDA content. Activities of APX and SOD were enhanced by elevated O3, which probably played an important role in alleviating the oxidative stress and provided further evidence of physiological and biochemical indicators as potential biomarkers responding environmental stress [53–61].

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