No significant interactions between nitrogen stimulation and ozone inhibition of isoprene emission in Cathay poplar

Xiangyang Yuan^{a,b,c}, Bo Shang^{a,b}, Yansen Xu^{a,b}, Yue Xin^{a,b,d}, Yuan Tian^e, Zhaozhong Feng^{a,b,c,*}, Elena Paoletti^{a,f}

^a State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^b College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China

^c State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China

^d Key Laboratory of Land Surface Pattern and Simulation, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China

^e School of Food, Beijing Technology and Business University, Beijing 100048, China

^f CNR, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy

HIGHLIGHTS

- The combined effect of O₃ and N on isoprene emission was investigated firstly.
- Elevated O₃ decreased and N load increased isoprene emission of poplar.
- Correlation between isoprene emission and A_{sat} did not change with N load.
- N load did not mitigate the negative effects of O₃ on isoprene emission.

GRAPHICAL ABSTRACT

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ABSTRACT

Isoprene emission from plants subject to a combination of ozone (O₃) and nitrogen (N) has never been investigated. Cathay poplar (*Populus cathayana*) saplings were exposed to O₃ (CF, charcoal-filtered air, NF, non-filtered ambient air and E-O₃, non-filtered air + 40 ppb) and N treatments (N0, 0 kg N ha⁻¹ year⁻¹, N50, 50 kg N ha⁻¹ year⁻¹ and N100, 100 kg N ha⁻¹ year⁻¹) for 96 days. Increasing O₃ exposure decreased isoprene emission (11.5% in NF and 57.9% in E-O₃), as well as light-saturated photosynthetic rate (A_{sat}) and chlorophyll content, while N load increased isoprene emission (19.6% in N50 and 33.4% in N100) as well as A_{sat} and chlorophyll content. Although O₃ and N interacted significantly in A_{sat}, N did not mitigate the negative effects of O₃ on isoprene emission, i.e. the combined effects were additive and did not interact. These results warrant more research on the combined effects of co-existing global change factors on future isoprene emission and atmospheric chemical processes.

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* Corresponding author at: State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.
E-mail address: fzz@rcees.ac.cn (Z. Feng).

1. Introduction

Increasing tropospheric ozone (O_3) concentration has become a common concern in northern mid-latitudes after the Industrial Revolution (Cooper et al., 2014; Hartmann et al., 2013). Recent observations and modeling results point towards a decrease in hourly peak O_3 concentrations in many parts of Europe and North America (Cooper et al., 2015; Dentener et al., 2010; Paoletti et al., 2014; Sicard et al., 2016), while the values are increasing in many Asian regions (Ding et al., 2008; Wang et al., 2009; Huang et al., 2015; Revell et al., 2015). Feng et al. (2015) indicated that tropospheric O_3 concentration in large regions of China will likely continue to rise in the immediate future due to the high O_3 -precursor emission rates, e.g. nitrogen oxides (NO_x). Nitrogen (N) deposition is another serious issue of global environmental change. A global transport-chemistry model projects that global N deposition will likely double the existing levels by 2050 (Galloway et al., 2004), especially in China, which is the major manufacturer and emitter of reactive N in the world (Liu et al., 2011). Between 1980 and 2000, the average bulk N deposition of China increased by about 60% per year, reaching an annual deposition to soil of $21.1 \text{ kg N ha}^{-1}$ (Liu et al., 2013). In some areas of southeast China, N deposition rates have reached $30\text{--}50 \text{ kg N ha}^{-1} \text{ year}^{-1}$ (Fan et al., 2007; Liu et al., 2011) and are expected to increase with rising fuel combustion and production and utilization of nitrogenous fertilizer (Liu et al., 2011, 2013).

Both the above-mentioned pollutants are known to co-occur in the environment and affect the biodiversity and function of forest ecosystems (Ainsworth et al., 2012; Sutton et al., 2014). Enhanced O_3 pollution reduces photosynthesis, decreases plant growth, and induces common effects such as visible injuries (Feng and Kobayashi, 2009; Feng et al., 2014; Wittig et al., 2009). Enriched N nutrition is usually beneficial under O_3 stress as a result of increased photosynthetic activity (i.e. photosynthetic enzyme activity) and biomass production (Häikiö et al., 2007; Handley and Grulke, 2008). However, N inputs may exceed critical loads, i.e. low levels of N load increase plant production while high levels may render plants more susceptible to pollutants and natural environmental stressors (Cardoso-Vilhena and Barnes, 2001). Moreover, such O_3 and N induced changes in the growth and metabolism of plants may affect the emission of biogenic volatile organic compounds (BVOCs) emitted by plants (Loreto and Schnitzler, 2010) due to a clear link between BVOC and photosynthesis (Sharkey and Yeh, 2001). There is thus a need to further investigate the correlation between BVOC emission and physiological parameters under the effects of elevated O_3 pollution and N load.

Plants collectively produce thousands of BVOCs, of which isoprene is the most represented component of the biosphere-atmosphere interactions (Sharkey and Monson, 2017). This compound accounts for about 50% of the total emission of BVOCs into the atmosphere (Guenther et al., 1995), and plays important roles in plant response to abiotic and biotic stresses (Filella et al., 2013; Holopainen and Gershenzon, 2010) and in the chemistry of the atmosphere. In the atmosphere, isoprene is a precursor of O_3 , peroxyacetyl nitrates and secondary organic aerosols (Claeys et al., 2004; Paasonen et al., 2013). Given the important interaction between phytogenic isoprene emission and global climate change, there is great interest in detecting how realistic combinations of global change factors affect the emission of isoprene from vegetation.

The research on isoprene emission under elevated O_3 or N deposition has been previously conducted separately, and the results are sometimes conflicting (Peñuelas and Staudt, 2010). Previous studies reported that elevated O_3 levels may increase, decrease, or do not change isoprene emission (Calfapietra et al., 2008; Fares et al., 2006, 2010; Llusà et al., 2002; Peñuelas and Staudt, 2010). Compared to O_3 , knowledge on N load effects on isoprene emission is even more limited. Most of previous studies on N effects on BVOCs were focused on monoterpenes or sesquiterpenes, and the results were inconsistent. Blanch et al. (2007) found that the emissions of terpene from *Pinus halepensis* and *Quercus ilex* decreased under high N load, while Kainulainen et al.

(2000) indicated that N availability did not affect monoterpene production in Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). For isoprene, only a couple of old studies reported that increasing N availability stimulated isoprene emission in velvet beans (*Mucuna* sp.) (Harley et al., 1994) and in aspen and white oak trees (Litvak et al., 1996). Research on the combined effect of O_3 and N on BVOC emission is also very limited. Recently, Llusà et al. (2014) reported that individual O_3 and N reduced the emission of terpenes in two Mediterranean leguminous species (*Ornithopus compressus* and *Trifolium striatum*), while the effect of the combination was not significantly interactive in any of the two species. Carriero et al. (2016) observed that O_3 exposure increased BVOC emission from a monoterpene-emitter (*Betula pendula*), the effect of N depended on the individual monoterpene, and the combined effects of O_3 exposure and N load on BVOC emission were additive, i.e. did not show significant interactions. To our knowledge, no previous papers have considered the effects of a combination of O_3 exposure and N load on isoprene emission from plants.

Poplars are widespread deciduous plants in temperate and boreal forests and significant isoprene emitters (Kesselmeier and Staudt, 1999). In China, poplar is a native species, which covers > 10 million ha (Xu et al., 2009). In this study, the combined effect of elevated O_3 and N load on the isoprene emission of Cathay poplar (*Populus cathayana*) was investigated. We tested the hypotheses that: (1) elevated O_3 decreased and N load increased isoprene emission of Cathay poplar, and the effects were additive, i.e. did not show significant interactions; and (2) isoprene emission was affected by stress-driven modifications in photosynthetic parameters (carbon assimilation, stomatal conductance, pigment content and intercellular CO_2 concentration) and this effect did not change under the combination of O_3 and N. In detail, we tested whether N load altered the response of isoprene emission to accumulated O_3 exposure and whether the combination of N load and O_3 exposure uncoupled the usual correlations between photosynthetic parameters and isoprene emission.

2. Materials and methods

2.1. Plant materials and treatments

Rooted cuttings of Cathay poplar were raised in 1-L peat containers in a greenhouse in Spring 2015. After 30 days, they were moved to the experimental research site (Changping, northwest of Beijing, $40^{\circ}19'N$, $116^{\circ}13'E$) and planted to 20-L pots filled with sandy loam soil (0.95 g N kg^{-1}) on May 2nd, 2015. The soil was excavated from a nearby farmland at 0–10 cm depth, sieved out by a 0.3 mm pore mesh, and then carefully mixed for homogeneity. Nine open-top chambers (OTCs, 12.5 m^2) with octagonal shape were used. In order to acclimatize to the environment inside the OTCs, cuttings with similar stem length and diameter were randomly located in the OTCs for 10 days. In total, there were 15 plants in each OTC, i.e. five plants for each of three N levels. Plants were moved weekly within each chamber in order to minimize the effects of microclimate.

Three O_3 treatments were applied: charcoal-filtered ambient air (CF), non-filtered ambient air (NF), and NF with addition of 40 ppb ($E-O_3$). Each O_3 treatment had three OTC replicates. The O_3 fumigation

Table 1

Summary of mean daytime O_3 concentrations (ppb, $\pm$ SD) and AOT40 (ppm h, $\pm$ SD) during the experiment (from June 5th to September 8th, 2015) in charcoal-filtered air (CF), non-filtered air (NF) and NF + 40 ppb ($E-O_3$) treatments.

Target	10-h average O_3 8:00–18:00 (ppb)	AOT40 (ppm h)
CF	34.3 ± 2.30	4.4 ± 0.45
NF	52.1 ± 0.57	16.0 ± 0.38
$E-O_3$	80.3 ± 5.50	38.7 ± 0.61

experiment lasted 96 days from June 5th to September 8th, 2015. The details about the O₃ fumigation procedure were described in Hu et al. (2015) and Yuan et al. (2016). Table 1 shows the mean daytime O₃ concentrations and AOT40 (accumulated O₃ exposure over an hourly threshold of 40 ppb) for the three O₃ treatments. Due to high ambient O₃ concentration during the growing season, the average daytime O₃ concentration was high even in the CF (34.3 ± 2.3 ppb). The 10-h average O₃ concentration was 12.8% lower than the targeted O₃ concentration in E-O₃ (Table 1).

Nitrogen treatments also had three levels: N0, 0 kg N ha⁻¹ year⁻¹, N50, 50 kg N ha⁻¹ year⁻¹ and N100, 100 kg N ha⁻¹ year⁻¹. For each treatment, applications were carried out five times (June 24th, July 12th, July 31st, August 20th, and September 5th, 2015) as 0.089 g and 0.178 g urea dissolved in 100 ml water per time for N50 and N100, respectively. At the end of the experiment, N50 and N100 received 0.208 g and 0.416 g N, compared to the background 0 g N. All plants (135 plants in total) were irrigated regularly at one- or two-day intervals in order to keep the soil moisture.

2.2. Measurement of gas exchange parameters and isoprene emission

Over 7 consecutive days (i.e. from August 13th to August 19th, 2015), a portable photosynthetic system fitting with circular 2-cm² fluorescence leaf cuvette (LI-6400-40, LI-COR Corp., USA) was used to measure gas exchange and then sample isoprene emission from fully-expanded leaves from the middle layer of the canopy (one leaf per plant, two plants per treatment in each OTC, 54 samples in total) during the central hours of the day (10:00 a.m.–03:00 p.m.). Photosynthetically active radiation (PAR) was kept constant at 1200 μmol m⁻² s⁻¹; the block temperature was set at environmental temperature (31–35 °C). Relative humidity was maintained in the range of 40–60%, and ambient CO₂ was set at 390 ppm. The parameters obtained were the light-saturated rate of CO₂ assimilation (*A*_{sat}), stomatal conductance (*g*_s) and intercellular CO₂ concentration (*C*_i). When gas exchanges reached equilibrium, isoprene samples were collected through 100-mg Tenax-TA glass tubes (Thermal Desorption Tubes, Mesh 60/80, Gerstel, Germany) by means of a vacuum pump (GS1, Gerstel, Germany) at the rate of 200 mL min⁻¹ for 20 min, as explained in Yuan et al. (2016). Using a Li-Cor device for isoprene sampling scrubs O₃ from the sample air, so that no reactions between O₃ and Tenax may occur. At morning and afternoon of each sampling day, blank samples were also collected from ambient air. After sampling, tubes were immediately sealed with Teflon-coated caps, transported to the laboratory and stored in the refrigerator until analysis (less than three days from sampling to analysis).

Sample were analyzed by a thermal desorption system (TDS3; Gerstel, Germany) and GC/MS (GC 5890, MSD 5975; Agilent Technologies, Santa Clara, CA, USA). Samples were pre-focused by Cooled Injection System (CIS, Gerstel, Germany) by liquid nitrogen, and then desorbed and transferred to GC/MS by specific transfer line. The analysis was carried out by rising the column temperature from 40 °C to 250 °C, at a rate of 5 °C min⁻¹ with helium as carrier gas. Isoprene was qualitative based on retention times and the NIST2.0 mass spectral database library (Agilent Technologies, Santa Clara, CA, USA), and then quantified using the pure liquid standards. Blank samples were analyzed synchronously and eventually subtracted from the samples emission results. Further details on those procedures are described in Yuan et al. (2016). Since the environmental conditions varied during sampling, the basic emission rates were standardized at 30 °C and 1000 μmol m⁻² s⁻¹ PAR according to Guenther et al. (1993). Immediately, after isoprene sampling, leaf discs for chlorophyll analysis were sampled from the same leaves and treated with 2 ml 95% ethanol solution in the dark for about 72 h at 4 °C until completely fading. Chlorophyll (*a* + *b*) content was measured by using the specific absorption coefficients (664, 649 and 470 nm) following Lichtenthaler (1989).

2.3. Statistical analyses

Normal distribution and homogeneity of variance were tested by the Kolmogorov-Smirnov one-sample D test and Levene's test, respectively. The single OTC was defined as statistical unit after averaging the two plants for each N level in each OTC. In order to test the effects of O₃, N and their interactions, the data of each dependent variable was subjected to analysis of variance by Tukey's Honestly Significant Difference (HSD) test with Mixed Linear Model using JMP software (SAS Institute, USA). We applied the analysis of covariance (ANCOVA) to check the difference in the slope of regression lines between isoprene emission and photosynthetic parameters by using SPSS18.0 (SPSS 18.0, Chicago, IL, USA). Results were considered significant when *P* < 0.05. Data shown in figures are means ± SD (*n* = 3 OTCs).

3. Results

3.1. Photosynthetic parameters

Both O₃ and N significantly affected *A*_{sat} (Table 2). Relative to CF, *A*_{sat} significantly decreased by 29.2% in NF and 67.5% in E-O₃, when the effects were averaged across all the N treatments (Fig. 1a). Effects of O₃ were also significant in the individual N treatments. Relative to N0, *A*_{sat} significantly increased by 16.2% in N50 and 19.3% in N100, when the effects were averaged across all the O₃ treatments (Fig. 1a). When the N effect was analyzed for any O₃ treatment, the effect was statistically significant only in NF. This is why the O₃ × N interaction was significant, i.e. N did not affect *A*_{sat} in both CF and E-O₃ relative to N0, but significantly increased *A*_{sat} in NF by 24.1% and 43.8% in N50 and N100, respectively.

Ozone significantly reduced *g*_s while N did not affect it (Table 2). Compared to CF, NF slightly decreased *g*_s by 12.4% and E-O₃ significantly reduced *g*_s by 18.3% when averaged across all N treatments (Fig. 1b), while N did not induce any significant difference when averaged across all the O₃ treatments. Also the O₃ and N interaction was not significant.

Both O₃ and N had significant effects on *C*_i (Table 2). Relative to CF, *C*_i was slightly increased by 4.1% in NF and significantly increased by 12.4% in E-O₃, when averaged across all N treatments (Fig. 1c). Relative to N0, *C*_i increased by 1.5% in N50 and significantly decreased by 4.8% in N100 when averaged across all O₃ treatments.

The responses of chlorophyll content (Chl) to O₃ and N were similar to those of *A*_{sat} (Table 2, Fig. 1a, d). Relative to CF, Chl slightly decreased by 3.1% in NF and significantly decreased by 32.4% in E-O₃ (Fig. 1d), when averaged across all the N treatments. Relative to N0, Chl significantly increased by 29.6% in N50 and 43.9% in N100, when averaged across all the O₃ treatments (Fig. 1d). Different from *A*_{sat}, however, Chl did not show a significant interaction between O₃ and N.

3.2. Isoprene emission

Both O₃ and N significantly affected isoprene emission (Table 2, Fig. 2). Relative to CF, isoprene emission significantly decreased by

Table 2

Results of ANOVA (*P* values) for the individual effects and interactions of O₃ (CF, charcoal-filtered ambient air, NF, non-filtered ambient air and E-O₃, elevated O₃) and Nitrogen (N0, 0 kg N ha⁻¹ year⁻¹, N50, 50 kg N ha⁻¹ year⁻¹ and N100, 100 kg N ha⁻¹ year⁻¹) on light-saturated photosynthesis (*A*_{sat}), stomatal conductance (*g*_s), intercellular CO₂ concentration (*C*_i), chlorophyll (*a* + *b*) content (Chl), and isoprene emission. Statistically significant effects (*P* < 0.05) are marked in bold.

	Ozone (O ₃)	Nitrogen (N)	O ₃ × N
<i>A</i> _{sat}	<0.0001	0.0003	0.0260
<i>g</i> _s	0.0174	0.4612	0.1577
<i>C</i> _i	<0.0001	0.0086	0.6847
Chl	<0.0001	<0.0001	0.2390
Isoprene emission	<0.0001	0.0002	0.1200

Fig. 1. Effects of ozone (CF, charcoal-filtered ambient air, NF, non-filtered ambient air and E-O₃, elevated O₃) and Nitrogen (N0, 0 kg N ha⁻¹ year⁻¹, N50, 50 kg N ha⁻¹ year⁻¹ and N100, 100 kg N ha⁻¹ year⁻¹) on light-saturated photosynthesis (A_{sat}), stomatal conductance (g_s), intercellular CO₂ concentration (C_i) and chlorophyll content of Cathay poplar (*Populus cathayana*). Values are mean $\pm$ SD. Different lowercase and capital letters indicate significant differences among bars within each variable and the averaged values across all O₃ or N treatments, respectively (Tukey test, $P < 0.05$, $n = 3$ OTCs).

11.5% in NF and 57.9% in E-O₃ when averaged across all the N treatments. Relative to N0, isoprene emission significantly increased by 19.6% in N50 and 33.4% in N100, when averaged across all the O₃ treatments (Fig. 2). No significant interaction was observed.

3.3. Correlation between isoprene emission and photosynthetic parameters

A significant positive linear correlation was found between isoprene emission and A_{sat} or Chl (Fig. 3a, d), while isoprene emission did not vary significantly with g_s (Fig. 3b) and decreased with increasing C_i (Fig. 3c). ANCOVA results did not show significant differences in the slope of the regression lines for the individual O₃ or N treatments (not shown).

Fig. 2. Effects of ozone (CF, charcoal-filtered ambient air, NF, non-filtered ambient air and E-O₃, elevated O₃) and Nitrogen (N0, 0 kg N ha⁻¹ year⁻¹, N50, 50 kg N ha⁻¹ year⁻¹ and N100, 100 kg N ha⁻¹ year⁻¹) on standardized isoprene emission from Cathay poplar (*Populus cathayana*) leaves. Values are mean $\pm$ SD. Different lowercase and capital letters indicate significant differences among bars within each variable and the averaged values across all O₃ or N treatments, respectively (Tukey test, $P < 0.05$, $n = 3$ OTCs).

Isoprene emission significantly decreased with increasing AOT40 for all N treatments with no significant difference among the slopes of the relationships in any N level (Fig. 4).

Consistent with isoprene, A_{sat} , g_s and Chl significantly decreased with increasing AOT40 for all N treatments with no significant difference among the slopes of these relationships (Fig. 5a, b, d), while C_i significantly increased with increasing AOT40 for all N treatments (Fig. 5c).

4. Discussion

The results presented here extend our understanding of the interactions between O₃, N and isoprene emission from plants. So far, the combined effects of varying O₃ and N levels have been investigated only on monoterpene or sesquiterpene emission (Blanch et al., 2007; Carriero et al., 2016; Llusà et al., 2014; Wang et al., 2012). Similar to *P. alba* (Fares et al., 2006), *P. nigra* (Fares et al., 2010) and hybrid poplar (*P. deltoides* cv. 55/56 $\times$ *P. deltoides* cv. Imperial) (Yuan et al., 2016), Cathay poplar is a significant isoprene emitter.

4.1. Effect of Ozone

Relative to other O₃ fumigation experiments (Bhatia et al., 2013; Booker et al., 2007; Tomer et al., 2015), the average O₃ concentration in NF was very high in our study (Table 1), thus indicating that the suburban areas of Beijing had a very high ambient O₃ concentration during the daytime of summer season. With the rapid industrialization and urbanization, high daily mean ambient O₃ concentrations (> 70 ppb) have been frequently observed during summer days in Beijing in recent years (Xin et al., 2016; Yuan et al., 2015). Therefore, an average daytime O₃ concentration of 80.3 ppb in E-O₃ is a realistic level. AOT40 values in NF and E-O₃ exceeded the O₃ critical level for forest protection (5 ppm h across six months, CLRTAP, 2015) and for Chinese poplar protection (12 ppm h, Hu et al., 2015). As expected, O₃ in NF caused typical symptoms on Cathay poplar leaves especially under no N fertilization,

Fig. 3. Correlation between isoprene emission and light-saturated photosynthesis (A_{sat}), stomatal conductance (g_s), intercellular CO₂ concentration (C_i) and chlorophyll content across all ozone and nitrogen treatments. Circles, squares and triangles stand for charcoal-filtered ambient air (CF), non-filtered ambient air (NF) and elevated O₃ (E-O₃), respectively. White, red and green symbols stand for N0 (0 kg N ha⁻¹ year⁻¹), N50 (50 kg N ha⁻¹ year⁻¹) and N100 (100 kg N ha⁻¹ year⁻¹), respectively. As ANCOVA did not show significant differences in the slope of the regression lines for the individual O₃ or N treatments, one single line is shown.

and significant O₃ visible injuries in E-O₃ treatment in spite of N fertilization (data not show), thus confirming that *P. cathayana* is a very sensitive species to O₃ (Xin et al., 2016).

An O₃-induced reduction in g_s was observed in our study (Table 2, Fig. 1b), consistently with a previous review by Wittig et al. (2007), who indicated that decreased g_s by O₃ is a universal phenomenon in

trees, suggesting that stomatal closure was linked with the safeguard mechanism of plants against negative O₃ stress (Reich, 1987). On the other hand, stomatal closure is considered to be indirect effect of O₃, caused by increased C_i as photosynthesis is impaired (Yamaguchi et al., 2007). Our result was consistent with this theory (Table 2, Fig. 1b, c), then resulting in a reduced photosynthetic rate (Table 2, Fig. 1a).

Although the effects of O₃ on isoprenoid emissions of plants have been suggested to vary with O₃ concentrations, duration of exposure, plant species and environmental conditions (Loreto and Schnitzler, 2010; Pinto et al., 2010), this study indicated that an average daytime O₃ concentration higher than 80 ppb (Table 1, Figs 1, 2) significantly decreased isoprene emission, together with a significant decrease in A_{sat} and Chl. The inhibition of isoprene emission at elevated O₃ occurred in several studies, with long term and high dose of O₃ exposure (Calfapietra et al., 2008; Cojocariu et al., 2005; Ryan et al., 2009; Yuan et al., 2016). When high O₃ concentration impairs the photosynthetic process, the biosynthetic formation of isoprene is decreased (Sharkey and Loreto, 1993).

4.2. Effect of nitrogen

The medium and high N load used in our study (50 kg N ha⁻¹ year⁻¹ and 100 kg N ha⁻¹ year⁻¹, respectively) was higher than the peak value of N deposition in Europe (20.0 to 28.1 kg N ha⁻¹ year⁻¹), South Asia (20.0 to 30.6 kg N ha⁻¹ year⁻¹), USA and Canada (10.0 to 20 kg N ha⁻¹ year⁻¹) (Vet et al., 2014; Zhu et al., 2015). However, these N deposition levels are realistic in China, especially in North

Fig. 4. Isoprene emission as a function of AOT40 (ppm h). Circles, triangles and squares stand for N0 (0 kg N ha⁻¹ year⁻¹), N50 (50 kg N ha⁻¹ year⁻¹) and N100 (100 kg N ha⁻¹ year⁻¹), respectively. As ANCOVA did not show significant differences in the slope of the regression lines for the individual N treatments, one single dotted line is shown.

Fig. 5. Light-saturated photosynthesis (A_{sat}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and chlorophyll content as a function of AOT40 (ppm h). Circles, triangles and squares stand for N0 (0 kg N ha^{-1} year $^{-1}$), N50 (50 kg N ha^{-1} year $^{-1}$) and N100 (100 kg N ha^{-1} year $^{-1}$), respectively. When ANCOVA did not show significant differences in the slope of the regression lines for the individual N treatments, one single dotted line is shown.

China Plain with over-fertilization of agricultural production (Liu et al., 2011). Liu et al. (2006) reported yearly bulk N deposition (i.e. mostly wet) up to 30 kg N ha^{-1} year $^{-1}$ (only inorganic N) in the Beijing area of China, reaching 83.3 kg N ha^{-1} year $^{-1}$ if both inorganic and organic N were included. Such high N depositions are expected to further rise in the future due to rising combustion of fossil fuel and to increasing production and application of nitrogenous fertilizers (Liu et al., 2011, 2013).

Unlike O_3 stress, enhanced N availability significantly increased isoprene emission, A_{sat} , and Chl (Table 2, Figs. 1, 2). Although the current result is consistent with studies, in which elevated N in the soil is highly correlated with A_{sat} and Chl, e.g. in *Larix* (Li et al., 2016) and *Brassica* species (Seepaul et al., 2016), other studies reported that modest N load (40 kg N ha^{-1}) did not affect the photosynthetic rates and reduced the emission of terpenes in *O. compressus* and *T. striatum*, compared to no N load (Llusià et al., 2014). Blanch et al. (2007) also found that a high N load (250 kg N ha^{-1}) reduced the emission of terpenoids from *P. halepensis* by 38%. Those reductions may be explained by the carbon/nutrient balance hypotheses (CNBH), i.e. higher nutrient availability would stimulate more carbon allocation to plant growth rather than to carbon-based secondary compounds such as terpenoids (Peñuelas and Estiarte, 1998). However, in our study, the expected decline did not occur, even at high N load. Nitrogen could promote isoprene emission by promoting leaf photosynthesis and thus improving energy and carbon availability for isoprene synthesis (Ormeño and Fernandez, 2012). Consistent with this theory, isoprene emission increased with increasing A_{sat} in this study (Fig. 3a). Peñuelas and Staudt (2010) reviewed the limited knowledge on the effects of N on isoprenoids and found that positive effects were more frequent for isoprene than for monoterpenes (Harley et al., 1994; Litvak et al., 1996). For monoterpenes, mostly negative (Blanch et al., 2007; Carriero et al., 2016; Llusià et al., 2014) or no effects (Kainulainen et al., 2000) were reported.

4.3. Interaction of ozone and nitrogen

Only A_{sat} showed a significant interaction between O_3 and N as the adverse effect of elevated O_3 was alleviated by higher soil N availability (Table 2, Fig. 1a). A counteracting effect of N and O_3 on A_{sat} has been already reported for *Picea abies* Karst. (Lippert et al., 1996), *Fagus crenata* (Yamaguchi et al., 2007) and *Cinnamomum camphora* seedlings (Feng et al., 2011), although this effect depended on plant type, exposure levels and plant growth stage. However, the interaction of O_3 exposure and N load on isoprene emission was not statistically significant in *Cathay poplar* (Table 2, Fig. 2). This is consistent with Llusià et al. (2014), who reported evident effects of individual N load or O_3 exposure on terpene emission from *O. compressus* and *T. striatum*, while the combined effect was not significant. Such inconsistent response of A_{sat} and isoprene emission suggests that isoprene emission is less sensitive to stressors than photosynthesis (Brilli et al., 2007; Seco et al., 2015).

Increase of isoprene emission was accompanied by an increase of A_{sat} and Chl (Fig. 3a, d) instead of g_s (Fig. 3b), confirming previous findings (Yuan et al., 2016). In contrast, isoprene emission decreased with increasing C_i (Fig. 3c), as expected under O_3 stress (Yuan et al., 2016). Such change may have an indirect effect on isoprene emission by adjusting the allocation proportion of cytosolic phosphoenolpyruvate (PEP) between respiration and the process of synthesizing dimethylallyl diphosphate (DMADP) in chloroplast, which is the immediate precursor of isoprene formation (Rosenstiel et al., 2002; Brilli et al., 2007). When C_i increased under O_3 stress, the chloroplastic import of PEP from respiratory processes towards DMADP formation is decreased, further limiting isoprene emission.

Interestingly, the regression lines between any photosynthetic parameter (but A_{sat}) and isoprene emission were not affected by either

O₃ or N, and thus only one equation was provided. This suggests that O₃ and N did not interact synergistically or antagonistically. An additive effect of the combined O₃ exposure and N load on BVOC emission was also reported in a monoterpene-emitter tree species (*B. pendula*) (Carriero et al., 2016).

In order to test the long-term effects of O₃ on isoprene emission at different N levels, we used AOT40 as a metric. Consistent with A_{sat}, g_s and Chl, isoprene decreased significantly with increasing AOT40 for all N treatments, with no significant effect of N on the slopes of these responses (Figs. 4, 5). However, Carriero et al. (2016) reported an opposite response, which described an increase of monoterpene emissions in *Betula pendula* seedlings with increasing AOT40. Such controversial responses to long-term O₃ accumulation may result from differences in duration and intensity of O₃ exposure, extent of O₃ visible foliar injury, and BVOC type (Loreto and Schnitzler, 2010; Peñuelas and Staudt, 2010).

5. Conclusions

This is the first study that quantifies the combined effects of O₃ exposure and N load on isoprene emission from plants. Our results pointed out that increasing O₃ exposure decreased isoprene emission, in tandem with A_{sat} and Chl, while N load increased isoprene emission still in tandem with A_{sat} and Chl. The combined effects of O₃ exposure and N load on isoprene emission were additive and did not interact significantly. These results will help a proper modeling of the interactive effects of these co-existing global change factors on plant physiology, future isoprene emission and atmospheric chemical processes.

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