Original Article

Concentration- and flux-based dose–responses of isoprene emission from poplar leaves and plants exposed to an ozone concentration gradient

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ABSTRACT

Concentration- and flux-based O₃ dose–responses of isoprene emission from single leaves and whole plants were developed. Two poplar clones differing in O₃ sensitivity were exposed to five O₃ levels in open-top chambers for 97 d: charcoal-filtered ambient air (CF), non-filtered ambient air (NF) and NF plus 20 ppb (NF + 20), 40 ppb (NF + 40) and 60 ppb (NF + 60). At both leaf and plant level, isoprene emission was significantly decreased by NF + 40 and NF + 60 for both clones. Although intra-specific variability was found when the emissions were up-scaled to the whole plant, both leaf- and plant-level emissions decreased linearly with increasing concentration-based (AOT40, cumulative exposure to hourly O₃ concentrations >40 ppb) and flux-based indices (POD_y, cumulative stomatal uptake of O₃ > Y nmol O₃ m^{−2} PLA s^{−1}). AOT40- and POD₇-based dose–responses performed equally well. The two clones responded differently to AOT40 and similarly to POD_y (with a slightly higher R² for POD₇) when the emission was expressed as change relative to clean air. We thus recommend POD₇ as a large-scale risk assessment metric to estimate isoprene emission responses to O₃ in poplar.

Key-words: AOT40; O₃ sensitivity; phytotoxic ozone dose; tropospheric O₃.

INTRODUCTION

Biogenic volatile organic compounds (BVOCs) emitted from plants are the most important source of atmospheric non-methane hydrocarbons and almost double the VOCs produced by human activities (Guenther, Nicholas, Erickson, *et al.* 1995). Isoprene (C₅H₈, 2-methyl-1,3-butadiene) is the single most representative BVOCs on the Earth (Atkinson & Arey 2003), plays vital roles in plant defence against abiotic and biotic stresses (Holopainen & Gershenzon 2010; Loreto & Schnitzler 2010) and is involved in chemical processes in the

atmosphere, such as ozone (O₃) and secondary organic aerosol (SOA) formation (Guenther *et al.* 2000; Claeys, Grahlan, Vas, *et al.* 2004; Kulmala, Suni, Lehtinen, *et al.* 2004; Hallquist, Wenger, Baltensperger, *et al.* 2009; Paasonen, Asmi, Petäjä, *et al.* 2013). Improving our understanding of plant isoprene emissions is important for the prediction of atmospheric chemistry and Earth's climate (Monson, Grote, Niinemets, & Schnitzler 2012), especially under the present and future elevated O₃ conditions (Peñuelas & Staudt 2010; Karlsson *et al.* 2017).

Tropospheric O₃ is an important secondary air pollutant, and its background concentrations have risen by approximately 0.5–2% per year since the Industrial Revolution (Hartmann, Klein Tank, & Rusticucci 2013; IPCC 2013; Cooper, Parrish, Ziemke, *et al.* 2014; Karlsson *et al.* 2017). Thanks to control measures, peak O₃ concentrations tend to decline in North America and Europe (Paoletti, De Marco, Beddows, Harrison, & Manning 2014; Sicard *et al.* 2016), while annual averages are still increasing in many other regions, in particular in Asia, due to increasing O₃-precursor emissions (Feng, Liu, & Zhang 2015). For example, high concentrations of O₃ frequently occur in many regions and cities of China, where the daily 8 h average may exceed 50 ppb during the crop growth season (Wang, Wei, Ding, *et al.* 2009; Tang *et al.* 2013; Xin *et al.* 2016). Yuan *et al.* (2015) observed frequent peaks exceeding 170 ppb during summer days in Beijing.

Rising tropospheric O₃ not only causes damage to plants by inducing visible leaf injury, impairing growth and reducing productivity (Ashmore 2005; Paoletti & Manning 2007; Feng, Sun, Wan, Hu, & Calatayud 2014), but also has potential impacts on isoprene biosynthesis and emission (Loreto & Schnitzler 2010). Although the interactions between isoprene and O₃ have been investigated for a couple of decades, the result is controversial as reviewed by Peñuelas & Staudt (2010). Previous studies showed that elevated O₃ can stimulate, inhibit or do not affect isoprene production and emission. Such different results may be due to different O₃ doses (Peñuelas, Llusià, & Gimeno 1999; Pinelli & Tricoli 2008; Fares, Oksanen, Länneppää, Julkunen-Tiitto, & Loreto 2010), plant species (Calfapietra, Fares, & Loreto 2009; Laohawornkitkul, Taylor, Paul, & Hewitt 2009) or genotypes (O₃ sensitivity) (Heiden,

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Hoffmann, Kahl, *et al.* 1999; Vuorinen, Nerg, Vapaavuori, & Holopainen 2005; Calfapietra, Scarascia, Karnosky, Loreto, & Sharkey 2008; Ryan, Cojocariu, Possell, Davies, & Hewitt 2009), and even different developmental leaf stages (Fares *et al.* 2010). These variable responses of isoprene emission to O₃ are a challenge to a proper prediction of atmospheric chemistry. Furthermore, several studies reported that the effects of O₃ on isoprene emission from either individual leaves or entire plants were inconsistent due to leaf area adjustments (Calfapietra, Wiberley, Falbel, *et al.* 2007; Fares, Loreto, Kleist, & Wildt 2008; Carriero, Brunetti, Fares, *et al.* 2016). Recent work by Yuan *et al.* (2016) showed that the interaction of O₃ (80 ppb) and mild drought on hybrid poplar clone '546' (*Populus deltoides* cv. 55/56 × *P. deltoides* cv. Imperial) was significant at plant level, but not at leaf level. To improve existing regional or global isoprene emission models, increased understanding of isoprene emission under O₃ at both leaf and plant level is required.

Over the past decades, policy makers and scientists put forward many methods/standards in order to assess the potential adverse O₃ effects on vegetation at regional scale (Emberson, Büker, & Ashmore 2007; Mills *et al.* 2007, 2011a, 2011b; Anav *et al.* 2016). Among them, AOT40 (Accumulated Ozone over a Threshold of hourly concentration of 40 ppb) is the simplest and most widespread one, which is a numerical index that describes the cumulative exposure to O₃ hourly concentrations exceeding 40 ppb over the daylight hours during the growing season (Paoletti & Manning 2007; CLRTAP 2015). However, the effects of O₃ on plants rely not only on the atmospheric O₃ concentrations but also on stomatal O₃ uptake (Matyssek *et al.* 2007). Recently, an index based on stomatal O₃ uptake (Phytotoxic Ozone Dose, POD_Y, accumulated stomatal O₃ flux above a threshold Y nmol O₃ m⁻² s⁻¹ of projected leaf area (PLA) over the growing season) was developed to take into account the environmental conditions affecting the O₃ uptake process (such as temperature, light, water vapour pressure deficit (VPD), etc.) (Emberson, Ashmore, Cambridge, Simpson, & Tuovinen 2000; Büker, Feng, Uddling, *et al.* 2015; CLRTAP 2015). To date, however, most experiments have used biomass loss or yield reduction as response indicator (Karlsson, Uddling, Braun, *et al.* 2004; Feng, Tang, Uddling, *et al.* 2012; Büker *et al.* 2015; Hu *et al.* 2015), and only a few papers have addressed the response of BVOC emission to O₃ gradients and cumulative metrics. An exponential increased sesquiterpene emission was reported by Bourtsoukidis *et al.* (2012) as a response to increasing ambient O₃ concentrations for spruce. Carriero *et al.* (2016) suggested an enhanced emission of monoterpenes in response to O₃ exposure, expressed as AOT40. Calfapietra *et al.* (2009) and Yuan *et al.* (2016) hypothesized a hormetic O₃ dose-response curve for isoprene emission, which described a low-dose stimulatory and a high-dose inhibitory response. Research on the relationship of POD_Y and BVOC emission, however, has not been carried out, while an experimentally validated dose-response relationship between POD_Y and isoprene emission will help to improve process-based ecosystem models so as to assess total isoprene emission emitted by plants at regional scales. Thus, we used both

AOT40 and POD_Y to build up the relationship between O₃ dose and isoprene emission.

Poplars are widely distributed around the world. They are important isoprene emitters (Kesselmeier & Staudt 1999; International Poplar Commission 2004; Guidolotti, Calfapietra, & Loreto 2011). In China, 19% of the total forest plantation areas are poplar plantations, which cover over 7 million ha (Liang, Hu, Liu, & Zhang 2006). Poplars have been confirmed to be sensitive to O₃ (Yun, Park, & Laurence 2001; Oksanen, Amores, Kokko, Santamaria, & Kärenlampi 2001; Woo & Hinckley 2005; Hu *et al.* 2015), although such sensitivity is clone specific (Calfapietra *et al.* 2008; Häikiö *et al.* 2009). In this study, two poplar clones (clone '107', *Populus euramericana* cv. '74/76' and clone '546', *Populus deltoides* cv. '55/56' × *P. deltoides* cv. 'Imperial') were exposed to five O₃ concentrations with the main aim of obtaining the dose-response relationships for isoprene emission at leaf and plant level versus two O₃ risk assessment metrics (AOT40 and POD_Y). Previous studies showed that clone '546' was more sensitive to O₃ than clone '107' (Hu *et al.* 2015). Two major hypotheses were tested: (i) isoprene emissions from both the O₃-sensitive and the O₃-tolerant clones were affected by elevated O₃, but the O₃ dose-response relationships were different; and (ii) the O₃ dose-response relationships for isoprene emission depended on the O₃ risk assessment index (AOT40 and POD_Y), leaf position or age (upper and middle leaf, i.e. younger and older leaves) and plant organ (leaf or plant level).

MATERIALS AND METHODS

Study area and plant material

The study site was located in Tangjiapu (40°47'N, 116°34'E), northwestern Beijing, with a temperate continental monsoon climate, which is a transitional zone of semi-arid and semi-moist region. The annual average temperature and the total rainfall are about 8 °C and 400–500 mm, respectively.

Rooted cuttings were raised in 20 L pots (sandy loam soil) at a greenhouse since April 10th, 2016, and then transferred to the study site on June 19th, 2016. After 7 d of adaption to open-top chamber (OTC, octagonal shape, 12.5 m² basal area and 3.0 m height, covered with tempered glass), cuttings with similar stem length and diameter were used for this experiment. Six cuttings per clone were assigned to each chamber.

Exposure to ozone

The experiment was conducted in 15 OTCs with five treatments: charcoal-filtered ambient air (CF), non-filtered ambient air (NF) and NF with targeted O₃ addition of 20 ppb (NF + 20), 40 ppb (NF + 40) and 60 ppb (NF + 60). Each O₃ treatment had three OTC replicates. The daily O₃ fumigation period was 10 h (from 08:00 a.m. to 6:00 p.m.), and O₃ fumigation was halted in rainy days (Feng, Pang, Nouchi, Kobayashi, & Yamakawa 2010). Fumigation was carried out for 97 d from June 26th to September 30th, 2016 (for details about the O₃ fumigation, see Hu *et al.* 2015 and Yuan *et al.* 2016). Average O₃

concentrations (08:00 a.m. – 6:00 p.m., 10 h) for all treatments are presented in Table 1.

Relevant meteorological parameters inside and outside the OTCs were collected by using a CR1000 data logger (Campbell Scientific, North Logan, Utah, USA) with sensors for temperature (T), relative air humidity (RH) inside and outside the OTC, and photosynthetic photon flux density ($PPFD$) inside the OTC. Water vapour pressure deficit (VPD) was calculated using Eqn 1:

$$VPD = \left(1 - \frac{RH}{100}\right) \times 0.611 \times \exp\left(\frac{17.502 \times T}{T + 240.97}\right). \quad (1)$$

The range of T , VPD and $PPFD$ was 1.25–43.46 °C, 0.00–7.03 kPa and 0–1539 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. The highest temperature difference between inside and outside the OTCs was 5.8 °C, only in a few hot days at noon. Relative to ambient air, the mean air T and RH inside the OTCs increased by 1.7 °C and decreased by 4.5%, respectively.

AOT40 calculation

The O_3 exposure index AOT40 was calculated using Eqn 2, for daylight hours (08:00 a.m. – 6:00 p.m.) during the experimental period, according to the methodology for O_3 risk assessment (CLRTAP 2015). In order to test the seasonal effects of elevated O_3 , the accumulation period was divided into (i) first sampling stage: from the beginning of the growing season on June 26th to August 15th (Table S1), and (ii) second sampling stage: from June 26th to September 30th (Table 1). Although the isoprene sampling lasted 5 d, we used the end of each sampling period to accumulate the O_3 metrics until the first and the second sampling. As daily mean ambient O_3 concentrations were very high, the average O_3 hourly concentration in CF was occasionally higher than 40 ppb.

$$\text{AOT40} = \int_{i=1}^n \max([[\text{O}_3] - 40], 0) \cdot dt \quad (2)$$

where $[\text{O}_3]$ is hourly O_3 concentration (ppb) measured by an ultraviolet absorption O_3 analyser (Model 49i; Thermo Scientific, Franklin, MA, USA); n is the number of hours to be included in the calculation period, and dt is time step (1 h).

Estimation of stomatal conductance and POD_Y

To assess POD_Y , stomatal conductance (g_{sto}) was calculated according to Eqn 3 (Jarvis 1976; CLRTAP 2015).

$$g_{\text{sto}} = g_{\text{max}} \times \min(f_{\text{phen}}, f_{\text{O}_3}) \times f_{\text{PPED}} \times \max[f \min(f_{\text{temp}} \times f_{\text{VPD}} \times f_{\text{SWP}})] \quad (3)$$

where g_{sto} is the actual stomatal conductance ($\text{mmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$) and g_{max} is the clone-specific maximum stomatal conductance ($\text{mmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$). The parameters f_{phen} , f_{O_3} , f_{PPED} , f_{temp} , f_{VPD} and f_{SWP} are all expressed in relative terms (i.e. they vary between 0 and 1 as a proportion of g_{max}). The clone-specific parameters used in our study were obtained from a previous study (Hu *et al.* 2015).

Following CLRTAP (2015), stomatal O_3 flux (F_{st} , $\text{nmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$) to an unshaded and horizontal leaf was calculated as Eqn 4:

$$F_{\text{st}} = [\text{O}_3] \times \frac{1}{r_b + r_c} \times \frac{g_{\text{sto}}}{g_{\text{sto}} + g_{\text{ext}}} \quad (4)$$

where $[\text{O}_3]$ is the O_3 concentrations at the canopy top, r_b ($r_b = 1 / g_b$, g_b is the leaf boundary layer conductance for O_3) and r_c ($r_c = 1 / (g_{\text{sto}} + g_{\text{ext}})$) are the resistances for O_3 flux over the leaf boundary layer and the leaf surface, respectively. g_{sto} is the stomatal conductance, and g_{ext} is the external leaf, or cuticular, resistance. For details about the calculations, see Hu *et al.* (2015).

Once F_{st} was computed, POD_Y (in $\text{O}_3 \text{mmol m}^{-2} \text{PLA}$) was calculated by using hourly data as Eqn 5:

$$\text{POD}_Y = \int_{i=1}^n (F_{\text{sto}} - Y, 0) \cdot dt \quad (5)$$

where Y is a value ($\text{nmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$) which represents a detoxification threshold, and $dt = 1 \text{ h}$ (CLRTAP 2015). In this paper, POD_Y was estimated for two thresholds Y : 1 $\text{nmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$ as recommended by CLRTAP Mapping Manual (Mills *et al.* 2011a; CLRTAP 2015) and 7 $\text{nmol O}_3 \text{m}^{-2} \text{PLA s}^{-1}$ as suggested by a previous study on Chinese poplar clones (Hu *et al.* 2015).

Sampling and analysing isoprene emission

Isoprene emission was sampled two times, that is, at the middle of August (August 12th to August 16th) and the end of September (September 26th to September 30th), in order to avoid potential effects of leaf senescence. Before sampling, individual leaves were classified as middle and upper leaves according to Yuan *et al.* (2016). In short, we selected the 6th to 8th and 9th to 11th position from the apical bud as upper leaves, and we choose the 13th to 15th and 16th to 18th position

Table 1. Summary of daytime O_3 concentrations, AOT40, POD_1 and POD_7 ($\pm \text{SD}$) for clone ‘546’ and ‘107’, from the beginning (June 26th) to the end of the experimental period (September 30th 2016). Values are mean $\pm$ SD. CF, charcoal filtered air, NF, non-filtered ambient air, NF + 20, NF with addition of 20 ppb, NF + 40, NF with addition of 40 ppb and NF + 60, NF with addition of 60 ppb

	10 h O_3 average 8:00–18:00 (ppb)	AOT40 (ppm h)	POD_1 -Clone ‘546’ ($\text{mmol m}^{-2} \text{PLA}$)	POD_1 -Clone ‘107’ ($\text{mmol m}^{-2} \text{PLA}$)	POD_7 -Clone ‘546’ ($\text{mmol m}^{-2} \text{PLA}$)	POD_7 -Clone ‘107’ ($\text{mmol m}^{-2} \text{PLA}$)
CF	24.85 $\pm$ 1.46	2.48 $\pm$ 0.45	14.36 $\pm$ 0.66	11.02 $\pm$ 0.56	3.15 $\pm$ 0.20	1.37 $\pm$ 0.07
NF	45.17 $\pm$ 0.57	11.65 $\pm$ 0.38	26.77 $\pm$ 0.77	21.65 $\pm$ 0.26	10.20 $\pm$ 0.21	5.85 $\pm$ 0.20
NF + 20	60.37 $\pm$ 1.76	22.77 $\pm$ 1.38	36.23 $\pm$ 0.81	29.73 $\pm$ 0.76	17.95 $\pm$ 0.71	11.96 $\pm$ 0.65
NF + 40	75.71 $\pm$ 1.39	36.90 $\pm$ 1.31	46.05 $\pm$ 0.64	38.00 $\pm$ 0.55	27.25 $\pm$ 0.62	19.63 $\pm$ 0.53
NF + 60	89.89 $\pm$ 0.89	50.83 $\pm$ 0.79	55.35 $\pm$ 0.42	45.93 $\pm$ 0.28	36.35 $\pm$ 0.39	27.39 $\pm$ 0.24

from the apical bud as middle leaves for clone '546' and '107', respectively. Two plants per clone were randomly selected from the six plants in each of the three OTC replications. For each plant, one upper and one middle leaf were measured at each sampling time (240 samples in total). Moreover, lower leaf position leaves (18th–21th and 21th–24th position for clone '546' and '107', respectively) were sampled in August for obtaining a better fitting of the linear regression.

Isoprene samples were collected through Tenax-TA glass tubes (Thermal Desorption Tubes, Mesh 60/80, Gerstel, Germany) as described by Yuan *et al.* (2016). Three gas exchange systems (LI-6400XT, Li-Cor Corp., Lincoln, NE, USA) with 6400-40 leaf chamber fluorometers (2 cm² leaf cuvette) were used to gather samples from three plants simultaneously at the rate of 200 mL min⁻¹ for 20 min after being filtered with an active carbon cartridge to scavenge O₃ or isoprene present in the ambient air. Samplings were carried out during the central hours of the day (10:00 a.m. – 03:30 p.m.). After sampling, tubes were immediately sealed with Teflon-coated caps, transported to the laboratory, stored at 4 °C and analysed within 3 d.

Samples were desorbed by thermally desorbed device, then injected onto an HP-5 ms capillary column and finally analysed by GC-MS with electron impact ionization (EI). Calibrations were performed using liquid standards for isoprene (Fluka, Buchs, Switzerland) (Kim, Kim, & Brown 2013). At last, results were standardized at 30 °C and 1000 µmol m⁻² s⁻¹ PAR according to Guenther, Zimmerman, Harley, Monson, & Fall (1993). Further details on this procedure are described in Yuan *et al.* (2016).

Assessment of plant-level isoprene emission

In order to calibrate the plant-level isoprene emission, leaf discs for chlorophyll analysis were extracted immediately after isoprene sampling with 95% ethanol as explained in Xin *et al.* (2016). Total chlorophyll (*a* + *b*) content was assayed by using the specific absorption coefficients of Lichtenthaler (1989). A linear function was used to describe the relationship between leaf-level isoprene emission and total chlorophyll content.

As it was impossible to measure chlorophyll content and leaf area from all individual leaves and plants, SPAD readings and leaf size (Area = maximum length × maximum width) of all leaves were measured using a SPAD-502 leaf chlorophyll meter (Japan) and a straight ruler, respectively. Then, a correlation between SPAD values and actual chlorophyll content, and a calibration curve between leaf size measured by the ruler and by a scanner coupled to an image analyser (Image J software, National Institutes of Health, USA) (Bagard, Le Thiec, Delacote, *et al.* 2008; Gao, Guo, Guo, Wang, & Du 2011) were built using spare plants. The related equations are as follows:

1 The regression between SPAD and total chlorophyll content was best described as a single linear function: $y = 0.0077x - 0.0754$ ($r^2 = 0.90$) because no significant difference in the slopes of the regression was found between the two clones ($y = 0.008x - 0.0772$, $r^2 = 0.92$ and $y = 0.0071x - 0.0662$, $r^2 = 0.92$ for clone '546' and '107', respectively), where *y* is the total chlorophyll content

(g m⁻²) and *x* is the SPAD value. We used this function to calculate chlorophyll content from the SPAD measurements of all leaves in the plant.

2 The linear regression between leaf size by the ruler and by the image analyser was described as: $y = 0.6331x + 2.9752$ ($r^2 = 0.97$). Again, a single function was used because no significant difference in the slopes of the regression was found between the two clones ($y = 0.6353x + 3.9222$, $r^2 = 0.97$ and $y = 0.6002x + 4.3168$, $r^2 = 0.97$ for clone '546' and '107', respectively), where *y* is leaf size by the image analyser (cm²) and *x* is leaf size by the ruler (cm²).

Eventually, leaf-level isoprene emission and total leaf area from each plant at each sampling time were calculated, summed up and used to calculate the total isoprene emission per plant as Eqn 6.

$$I_{\text{plant}} = \sum_1^n (I_{\text{leaf}} \times I_{\text{size}}) \quad (6)$$

where I_{leaf} is isoprene emission (nmol m⁻² s⁻¹) of all individual leaves of a plant, estimated from the relationship between measurements of foliar chlorophyll content and isoprene emission, I_{size} is leaf area estimated by the relationship between measurements of leaf size by the ruler and leaf area by the image analyser (m²) and *n* represents the total number of leaves in a plant.

Calculation of relative plant-level isoprene emission

The dose-response for the relative isoprene emission per plant (I_{relative}), that is, the percent change of emission relative to clean air, was calculated as follows. Firstly, a linear regression of each poplar clone was built between the actual total isoprene emission per plant and AOT40 or POD_Y to obtain the *y*-intercept as hypothetical maximum isoprene emission per plant at zero O₃ exposure or uptake. Secondly, I_{relative} was obtained as the isoprene emission per plant at each O₃ treatment divided by the *y*-intercept of each clone regression. Then, the values were multiplied by 100 in order to express I_{relative} as a percentage (%), the percent change of emission relative to clean air). The I_{relative} of each O₃ treatment and clone may be thus compared on a common, relative scale. Finally, the I_{relative} for all clones and O₃ treatments were regressed against AOT40 or POD_Y.

Statistical analyses

Two plants from the same OTC were averaged for each clone and each OTC as a replicate for each O₃ treatment. Before the analysis, Kolmogorov-Smirnov one-sample D test was used to test all data for normal distribution. JMP software (SAS Institute, USA) was applied to test four-way analysis of variance (ANOVA) for each dependent variable with mixed linear model, for example, O₃, clone, leaf position, sampling time and their interactions on leaf-level and plant-level isoprene emission. The significant differences were detected by Tukey's Honestly Significant Difference (HSD) test. The analysis of covariance (ANCOVA) was used to test the difference of the slope in the linear regressions by using

SPSS18.0 (SPSS 18.0, Chicago, IL, USA). In all analyses, results were considered statistically significant at $P \leq 0.05$.

RESULTS

Leaf-level isoprene emission

Ozone, leaf position and sampling time significantly affected leaf-level isoprene emission, while no significant difference was found between clones (Table 2, Fig. 1). Generally, leaf-level isoprene emission decreased with increasing O_3 concentration and extended O_3 fumigation time. Emission was significantly higher in upper leaves than in middle leaves. Emissions in August were 1.42 times higher than in September. Isoprene emission did not show a significant interaction of O_3 and time, but the interactions of $O_3 \times$ Clone and $O_3 \times$ Position were highly significant, as indicated by the fact that elevated O_3 produced more effects in the middle position (-31.1%) than in

the upper position (-14.7%) as well as more effects in clone '546' (-33.4%) than in clone '107' (-8.5%). However, the significant interactions of Clone $\times$ Position and Clone $\times$ Time disappeared when the O_3 effect was included ($O_3 \times$ Clone $\times$ Position, $O_3 \times$ Clone $\times$ Time, $O_3 \times$ Position $\times$ Time or $O_3 \times$ Clone $\times$ Position $\times$ Time).

Because no significant differences were found in the slopes of the regression between isoprene emission and AOT40, POD₁ and POD₇ across two sampling times for different leaf positions as well as different clones (Figs S1 & S2), Fig. 2 shows a single regression line for both leaf positions and both sampling times. Leaf-level isoprene emission significantly decreased with increasing AOT40, POD₁ or POD₇. No significant differences were found between AOT40-based and POD₁-based dose responses (ANCOVA: $P = 0.731$), while POD₇-based regression showed a significantly higher slope than AOT40-based one (ANCOVA: $P = 0.031$). For both AOT40 and POD₇, R^2 and intercept values exhibited a similar

Table 2. Four-way ANOVA results (P values) for the effects of O_3 (CF, charcoal filtered air, NF, non-filtered ambient air, NF + 20, NF with addition of 20 ppb, NF + 40, NF with addition of 40 ppb and NF + 60, NF with addition of 60 ppb), clone ('546' and '107'), leaf position (upper and middle) and sampling time (August and September) on isoprene emission at leaf level, total leaf area and isoprene emission at plant level. Statistically significant effects are marked in bold ($P < 0.05$, $N = 3$ OTCs)

	Isoprene emission at leaf level	Total leaf area	Isoprene emission at plant level
O_3	<0.0001	0.0088	<0.001
Clone	0.2071	<0.0001	<0.0001
Position	<0.0001	—	—
Time	<0.0001	0.4433	0.0266
$O_3 \times$ Clone	<0.0001	0.0036	0.0616
$O_3 \times$ Position	0.0012	—	—
$O_3 \times$ Time	0.8448	0.2347	0.1614
Clone $\times$ Position	0.0431	—	—
Clone $\times$ Time	0.0003	0.6654	0.2728
Position $\times$ Time	0.0147	—	—
$O_3 \times$ Clone $\times$ Position	0.1201	—	—
$O_3 \times$ Position $\times$ Time	0.2869	—	—
$O_3 \times$ Clone $\times$ Time	0.499	0.588	0.9034
Clone $\times$ Position $\times$ Time	0.0333	—	—
$O_3 \times$ Clone $\times$ Position $\times$ Time	0.3901	—	—

Figure 1. Effects of ozone (CF, charcoal filtered air, NF, non-filtered ambient air, NF + 20, NF with additional of 20 ppb; NF + 40, NF with additional of 40 ppb; and NF + 60, NF with additional of 60 ppb), clone ('546' and '107'), leaf position (upper and middle) and sampling time (August and September) on standardized isoprene emission at leaf level. Values are mean $\pm$ SD, significant differences among bars within each variable were labelled by different letters (four-way ANOVA, Tukey test, $P < 0.05$, $N = 3$ OTCs).

Figure 2. Leaf-level isoprene emission ($\pm$ SD) of two poplar clones ('546' and '107') in relation to AOT40 (a), POD₁ (b) and POD₇ (c). ANCOVA shows the significance level of differences in slope between clones. As differences were not significant, one only solid line represents the linear regression of both clones.

pattern, with the maximum values of R^2 (0.63) occurring in the regression with AOT40 (Fig. 2a).

Leaf-level isoprene emission increased with foliar chlorophyll content for both clones across the two sampling times and the three leaf positions, and no significant difference in the slope of the regression between the two clones was found (Fig. 3).

Plant-level isoprene emission

Both clone '546' and clone '107' showed a similar leaf size variation along a vertical profile, with lower leaf size in younger leaves than in mature leaves, along with a moderate decrease as leaves were senescent for both O₃ treatments (Fig. S3).

Compared to CF, increasing O₃ concentrations did not significantly affect total leaf area per plant (Fig. 4a). The maximum increase of total leaf area per plant was observed at NF + 20 (+14.6%), followed by a moderate decline (by 8.0% and 3.7% for NF + 40 and NF + 60, respectively) when averaged across all sampling times and clones. However, the O₃ $\times$ Clone interaction was significant, as total leaf area slightly decreased under NF + 60 in clone '546' (-6.9%), whereas it increased in clone '107' (+15.8%) when averaged across both sampling times. No significant O₃ $\times$ Clone $\times$ Time interaction was observed due to the similar response to O₃ between clones at both sampling times, while clone '546' had a significantly higher total leaf area per plant (+11.8%) than clone '107' across all O₃ treatments and sampling times.

Leaf chlorophyll content was higher in upper leaves, and declined in NF + 40 and NF + 60 relative to CF plants as leaves became older (Fig. S4). Plant-level isoprene emission was scaled up by using Eqn 6. At plant level, NF + 40 and NF + 60 significantly decreased isoprene emission by 18.4% and 32.3%, respectively, on average across sampling times and clones. Although the effects of O₃ on the two clones were not statistically significant ($P = 0.0616$, Table 2, Fig. 4b), NF + 40 and NF + 60 decreased isoprene emission per plant by 23.4 and 41.6% for clone '546', and by 11.8% and 19.7% for clone '107', respectively, across the two sampling times. Overall, clone '546' showed higher plant-level emission than clone '107'. Similarly to leaf-level emission, plants in August emitted significantly more isoprene (+8.1%) than in September across all O₃ treatments and clones. No significant interaction

Figure 3. Isoprene emission from individual leaves as a function of foliar total chlorophyll content across two poplar clones ('546' and '107'), two sampling times (August and September) and three leaf positions (upper, middle and lower leaves). The solid line represents the linear regression of both clones, as ANCOVA showed no differences in slope between the clones. Lower leaf position in August was measured for improving the linear regression.

Figure 4. Effects of ozone (CF, charcoal filtered air; NF, non-filtered ambient air; NF + 20, NF with additional of 20 ppb; NF + 40, NF with additional of 40 ppb; and NF + 60, NF with additional of 60 ppb), clone ('546' and '107') and sampling time (August and September) on total leaf area per plant (a) and standardized plant-level isoprene emission (b). Values are mean $\pm$ SD, significant differences among bars within each variable were labelled by different letters (four-way ANOVA, Tukey test, $P < 0.05$, $N = 3$ OTCs).

between O₃ and time was observed when averaged across the clones, but a significant decrease in NF + 60 was found in September in clone '546' when compared to CF, NF and NF + 20 (Fig. 4b).

The responses of plant-level isoprene emission to AOT40, POD₁ and POD₇ did not differ at different sampling times (Fig. S5), and the correlations were significant when values from the two sampling times were analysed together (Fig. 5).

Consistent with leaf-level emission, isoprene emission per plant decreased with increasing AOT40, POD₁ and POD₇ for both clones (Table 2, Fig. 5). For both AOT40 and POD_Y, clone '546' displayed higher slope, intercept and R^2 values than '107', although slopes did not differ significantly for POD₁. R^2 values were similar for AOT40 and POD₇ and slightly higher than for POD₁, for both clones.

Because the slopes of the regression between Iso_{relative} and the O₃ metrics did not differ significantly when averaged across the two sampling times (Fig. S6), Fig. 6 shows the regression lines across both sampling times. Iso_{relative} decreased with increasing AOT40 and POD_Y in both clones. ANCOVA showed significant differences between the clones when Iso_{relative} was calculated for AOT40, while no clone-specific differences occurred in the case of POD_Y. The regression relationships of Iso_{relative} with POD₁ and POD₇ were similar, although the R^2 value was slightly higher for POD₇ than for POD₁ (Fig. 6b,c). The highest R^2 values were obtained for AOT40 (Fig. 6a). The significance of the regressions was always better in clone '546' than in clone '107'.

DISCUSSION

Effects of O₃ on isoprene emission at leaf level

Ozone induced a significant decrease in isoprene emission from both poplar clones. Compared with CF, NF + 40 and NF + 60 reduced leaf-level isoprene emission by 38 and 46% in clone '546' and by 4 and 20% in clone '107', respectively, when averaged across sampling times and leaf positions (Fig. 1). The inhibition of isoprene emission at elevated O₃ has been described also in aspen clones (*Populus tremuloides*) and hybrid poplar (*Populus deltoides* $\times$ *Populus trichocarpa*)

(Calfapietra *et al.* 2008; Ryan *et al.* 2009) as well as in Mediterranean oak (*Quercus pubescens* L.) (Velikova, Tsonev, Pinelli, Alessio, & Loreto 2005). Our leaf-level results did not differ between clones across O₃, leaf position and sampling time, which was in contrast with a previous study on aspen showing higher isoprene emission in an O₃-tolerant clone (271) than in an O₃-sensitive clone (42E) under combined O₃ and CO₂ exposure (Calfapietra *et al.* 2008). Emission of isoprene, in fact, is considered as a protective mechanism against O₃-induced lipid peroxidation of the cell membranes (Loreto & Velikova 2001) and ROS accumulation in the leaves (Loreto & Fares 2007). We thus expected higher isoprene emission as a protective response to elevated O₃ in the O₃-tolerant clone '107' than in the O₃-sensitive '546', while the present results were different from what we expected. Actually, both our clones were sensitive to O₃, as shown by a significant decline of foliar chlorophyll content (Fig. S3), although clone '546' was 'more' sensitive due to a stimulation of leaf emission as a mechanism of replacing the photosynthetic machinery damaged by O₃ (Fares, Barta, Brilli, *et al.* 2006).

Interestingly, the interactions of O₃ $\times$ Position were highly significant (Table 2), as elevated O₃ induced more effects on the middle leaves (−31.1%) than on the upper leaves (−14.7%) for both clones (Fig. 1). An evident decrease of isoprene emission with increasing leaf position (top position = 1) was previously shown for *Populus nigra* (Fares *et al.* 2010) as well as for clone '546' under the combined effects of O₃ and drought (Yuan *et al.* 2016). As the poplar cuttings were indeterminate growth and continuously emitted new leaves during the whole growing season, the upper and middle leaf position corresponded to younger and older leaves, respectively. Thus, the findings confirm that isoprene emission is under a strong developmental regulation and an inhibitory effect is stronger in old leaves than in young leaves (Wiberley, Linskey, Falbel, & Sharkey 2005; Fares *et al.* 2006).

Effects of O₃ on isoprene emission at plant level

In this study, we developed a correlation between isoprene emission and total chlorophyll content of individual leaves (Fig. 3), following previous studies in poplar (Yuan *et al.*

Figure 5. Plant-level isoprene emission ($\pm$ SD) of two poplar clones ('546' and '107') in relation to AOT40 (a), POD₁ (b) and POD₇ (c). The black and grey dotted lines represent linear regressions of clone '546' and '107', respectively, and are shown only when there was significant difference in the slope between the clones as tested by ANCOVA. Otherwise, a solid line represents the linear regression of both clones.

2016) and in marine phytoplankton (Shaw, Chisholm, & Prinn 2003; Extón *et al.* 2013). Chlorophyll content has been successfully used to model the annual variations of isoprene

Figure 6. Relative isoprene emission (Iso_{relative}) of two poplar clones ('546' and '107') in relation to AOT40 (a), POD₁ (b) and POD₇ (c). The black and grey dotted lines represent linear regressions of clone '546' and '107', respectively, and are shown only when there was significant difference in the slope between the clones as tested by ANCOVA. Otherwise, a solid line represents the linear regression of both clones.

emission from *Quercus robur* in combination with isoprene synthase activity and phenological data (Lehning, Zimmer, & Schnitzler 2001). This is the first correlative study, however, where total chlorophyll content is used as a proxy to estimate isoprene emission from individual poplar leaves.

Results were then scaled up by using the total leaf area to calculate the plant-level isoprene emission.

In a previous study, we found that the combined response of isoprene emission to O_3 and drought was different between leaf level and plant level, as a significant interaction was only found at plant level rather than leaf level, suggesting an important role for changes in total leaf area (Yuan *et al.* 2016). In this study, the total leaf area significantly decreased only in the O_3 -sensitive clone '546' under NF + 60 (−6.9%), while a slight increase occurred for the other O_3 treatments (Fig. 4a). A slight stimulation of plant metabolism under the initial phase of stress followed by a metabolic impairment at higher levels of stress is a common hormetic response (Calabrese 2005). Low-dose O_3 stimulation of plant performances is well known (Agathokleous, Mouzaki-Paxinou, Saitanis, Paoletti, & Manning 2016). However, the hormetic increase of total leaf area per plant was not able to offset a decline of isoprene emission induced by O_3 in individual plants. The decline of leaf chlorophyll content with increasing leaf position can explain the reduced isoprene emission at plant level. Elevated O_3 is known to decrease leaf chlorophyll content by causing oxidative stress (Wittig, Ainsworth, & Long 2007). Such oxidative stress in turn results in lower isoprene emission, because of the impairment of isoprene biosynthesis in the chloroplastic methylerythritol 4-phosphate (MEP) pathway (Laule, Furholz, Chang, *et al.* 2003; Loreto & Schnitzler 2010). Therefore, the decline of O_3 -induced effects on emission of isoprene at plant level was still unclear when we up-scaled from the emission at leaf level.

Dose–response relationships between O_3 and isoprene emission

To assess isoprene emission from plants at a regional and global scale, especially for predicting emission scenarios in an O_3 polluted atmosphere, appropriate functions representative of actual isoprene responses are really needed. We compared the performance of two O_3 indices (AOT40 and POD_Y), which have been widely used for assessing O_3 risk on many plant responses (e.g. Ashmore, Bker, Emberson, Terry, & Toet 2007; Mills *et al.* 2011a; Danielsson, Karlsson, & Pleije 2013; Sicard *et al.* 2016; Anav *et al.* 2016), while little information is available for assessing O_3 impact on isoprene emission.

The regressions between isoprene emission and concentration-based AOT40 or flux-based POD_Y performed equally well, with similar R^2 for all relationships at leaf and plant level (Figs 2 & 5), suggesting that responses of isoprene emission to different O_3 metrics (AOT40 versus POD_Y) were similar at leaf and plant level across the two clones. These results were consistent with previous studies on yield reduction or biomass loss in UK and China for winter wheat (*Triticum aestivum* L.) (Gonzalez-Fernandez *et al.* 2010; Feng *et al.* 2012), and poplar in China as well (Hu *et al.* 2015). In our study, the similar performance of flux- and concentration-based O_3 metrics could be attributed to the fact that the experiment was carried out under well-watered conditions, which is common agricultural practice for poplar cultivation in China.

However, irrigation favours high g_{sto} throughout the growing season (in the present paper no soil water limitation was assumed, i.e. $f_{SWP} = 1$) (Feng *et al.* 2012; Hu *et al.* 2015; Calatayud, Diguez, Sicard, Schaub, & De Marco 2016). Rather, drought stress is known to uncouple AOT40 and POD_Y (De Marco *et al.* 2015) and affect the robustness of POD_Y calculations (De Marco *et al.* 2016). Our conclusion that isoprene emission decreases linearly with increasing O_3 exposure or uptake is in contrast with Bourtsoukidis *et al.* (2012), who observed an exponential increase of sesquiterpene emissions from spruce with increasing ambient O_3 concentrations, and Carriero *et al.* (2016), who found an increase of monoterpene emissions in *Betula pendula* seedlings with increasing AOT40. Such contrasting responses might result from hormetic effects and discrepancies in the BVOCs type (Calfapietra *et al.* 2009), O_3 dose (Pinelli & Tricoli 2008; Fares *et al.* 2010) and/or plant species (Calfapietra *et al.* 2009; Laothawornkitkul *et al.* 2009).

Notably, the responses of isoprene emission in the two clones exhibited a similar pattern at leaf level. When the response was up-scaled to the plant level, however, the more O_3 -sensitive clone '546' displayed significantly higher slope, intercept and R^2 values than the less O_3 -sensitive clone '107', suggesting that effects of leaf area must be taken into account when up-scaling. Therefore, leaf-level dose–response relationships are not well suited to estimate the effect of increasing O_3 levels on isoprene emission at regional or global scale. Similarly, Sun, Niinemets, Huve, Rasulov, & Noe (2013) reported that canopy-scale isoprene emission may largely compensate for possible instantaneous CO_2 inhibition at the leaf scale by the stimulation of leaf area growth.

POD_Y is a plant-specific trait, which depends not only on the O_3 concentrations but also on the environmental effects on the stomata (Matyssek *et al.* 2007). This metric takes into account the varying influences of experimental conditions, including PPFD, T , VPD, soil water, O_3 concentrations and phenology (Mills *et al.* 2011a; CLRTAP 2015). Although POD_1 is recommended and widely used by CLRTAP (2015) for the protection of trees against biomass losses, a previous study indicated that POD_7 was better correlated with the biomass loss of Chinese poplar clones (Hu *et al.* 2015). In the O_3 dose–response relationship for isoprene emission, POD_1 showed similar performances to AOT40 and POD_7 at leaf level (Fig. 2), while the performances were lower at plant level (Fig. 5) and for $Iso_{relative}$ (Fig. 6). These non-uniform responses support the finding that POD_1 may be not suitable for assessing species with high stomatal conductance, like poplar.

Relative reduction of biomass is used by UNECE in assessing O_3 effects on forests (CLRTAP 2015). The use of relative isoprene emission to evaluate the dose–response relationships of the different clones or species was similar to the approach with the reduction of biomass. We used $Iso_{relative}$ to test whether the O_3 dose–response relationships for isoprene emission differed depending on the genotype ('sensitive' or 'tolerant'). The response of $Iso_{relative}$ to AOT40 differed between the clones, while the response to POD_Y did not (Fig. 6). This result suggests that AOT40 may not be as good as POD_Y in parameterizing the responses of isoprene emissions to varied O_3 levels across clones, because POD_Y is able to

reconcile the clone-specific stomatal responses that regulate the actual O₃ uptake and injury. These findings supported our hypothesis that O₃ risk assessment for isoprene depended on the O₃ metric and on the plant organ. Concerning the genotype, the uncertainties of an AOT40-based regional risk assessment would be significantly affected by the clone type. Similarly to Gonzalez Fernandez *et al.* (2010), intra-specific variation reduced the predictive capability of the stomatal flux approach, resulting in better performances for AOT40 than for POD_Y.

CONCLUSIONS

This is the first O₃ dose–response study of isoprene emission. The responses of two poplar clones were investigated at leaf and plant level at two sampling times across a realistic gradient of O₃ concentrations in OTCs. Isoprene emission at both levels was significantly impaired by elevated O₃ and extended O₃ fumigation time for both clones. At leaf level, the reduction was more severe on middle leaves than on upper leaves for both clones, although the O₃-sensitive clone ‘546’ showed much higher decline than the O₃-tolerant clone ‘107’ at the same O₃ level.

The O₃ dose–response relationship showed similar pattern at both leaf and plant level, and isoprene emission was always negatively correlated with the O₃ metrics. While our clones showed little variations in their dose–response relationships at leaf level, the O₃ dose–response relationship at plant level was sharper in the O₃-sensitive clone ‘546’ than in the O₃-tolerant clone ‘107’. Overall, AOT40-based and POD-based dose–responses performed equally well. For a regional or global scale assessment, however, POD_Y is able to address the large variation in response to O₃ among cultivars/clones for any species. To realistically address clone-specific O₃ effects on isoprene emission, we thus recommend considering POD₇ as an O₃ metric for poplar and developing POD_Y dose–response relationships for other species.

While most of isoprenoid production algorithms have included the influences of CO₂ (Arneth *et al.* 2007), few take into account the impacts of O₃. For example, the most widely used isoprene emission scheme, that is, the Model of Emissions of Gases and Aerosols from Nature (MEGAN) proposed by Guenther *et al.* (1993), fails to include the responses to O₃ stress. Our derived responses of isoprene emissions to O₃ doses provide useful validations for modelling isoprene emissions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Summary of mean AOT40, POD₁ and POD₇ values (±SD) for clone ‘546’ and ‘107’ at the first sampling (i.e. accumulated from the start of the growing season on June 26th to August 15th, 2016). The results of the second sampling (i.e. accumulated from June 26th to September 30th 2016) are in Table 1. Values are mean ± SD. Plants were raised in charcoal-filtered air (CF), not filtered air (NF) and NF plus 20, 40 and 60 ppb (NF + 20, NF + 40, NF + 60) open-top chambers (*n* = 3).

Figure S1. Mean leaf-level isoprene emission (±SD) of two poplar clones (‘546’ and ‘107’) as affected by AOT40, POD₁ and POD₇. Black, grey and red lines represent linear regressions of upper, middle and all leaves, respectively. ANCOVA shows the significance level of differences in slope between upper leaves and middle leaves.

Figure S2. Mean leaf-level isoprene emission (±SD) of two poplar clones (‘546’ and ‘107’) as affected by AOT40, POD₁ and POD₇. Black, grey and red lines represent linear regressions of ‘546’, ‘107’ and both clones, respectively. ANCOVA shows the significance level of differences in slope between the two clones.

Figure S3. Effects of ozone (CF, charcoal filtered air; NF, non-filtered ambient air; NF + 20, NF with addition of 20 ppb; NF + 40, NF with addition of 40 ppb; and NF + 60, NF with addition of 60 ppb) on leaf size (±SD) along a vertical profile of two poplar clones (‘546’ and ‘107’). Leaf 1 is top position.

Figure S4. Effects of ozone (CF, charcoal filtered air; NF, non-filtered ambient air; NF + 20, NF with addition of 20 ppb; NF + 40, NF with addition of 40 ppb; and NF + 60, NF with addition of 60 ppb) on foliar chlorophyll content (±SD) along a vertical profile of two poplar clones (‘546’ and ‘107’). Leaf 1 is top position.

Figure S5. Mean plant-level isoprene emission (±SD) of two poplar clones (‘546’ and ‘107’) as affected by AOT40, POD₁ and POD₇. ANCOVA showed the significance level of differences in slope between August and September. As differences were not significant, one only solid line represents the linear regression of both months.

Figure S6. Relative isoprene emission (Iso_{relative}) (±SD) of two poplar clones (‘546’ and ‘107’) in relation to AOT40, POD₁ and POD₇. ANCOVA shows the significance level of differences in slope between August and September. As differences were not significant, one only solid line represents the linear regression of both months.