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Ozone risk assessment is affected by nutrient availability: Evidence from a simulation experiment under free air controlled exposure (FACE)☆

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ABSTRACT

Assessing ozone (O_3) risk to vegetation is crucial for informing policy making. Soil nitrogen (N) and phosphorus (P) availability could change stomatal conductance which is the main driver of O_3 uptake into a leaf. In addition, the availability of N and P could influence photosynthesis and growth. We thus postulated that the sensitivity of plants to O_3 may be changed by the levels of N and P in the soil. In this study, a sensitive poplar clone (Oxford) was subject to two N levels (N0, 0 kg N ha^{-1}; N80, 80 kg N ha^{-1}), three P levels (P0, 0 kg P ha^{-1}; P40, 40 kg P ha^{-1}; P80, 80 kg P ha^{-1}) and three levels of O_3 exposure (ambient concentration, AA; 1.5 × AA; 2.0 × AA) for a whole growing season in an O_3 free air controlled exposure (FACE) facility. Flux-based ($POD_{0\ to\ 6}$) and exposure-based (W126 and AOT40) dose-response relationships were fitted and critical levels (CLs) were estimated for a 5% decrease of total annual biomass. It was found that N and P availability modified the dose-response relationships of biomass responses to O_3. Overall, the N supply decreased the O_3 CLs i.e. increased the sensitivity of poplar to O_3. Phosphorus alleviated the O_3-caused biomass loss and increased the CL. However, such mitigation effects of P were found only in low N and not in high N conditions. In each nutritional treatment, similar performance was found between flux-based and exposure-based indices. However, the flux-based approach was superior, as compared to exposure indices, to explain the biomass reduction when all nutritional treatments were pooled together. The best O_3 metric for risk assessments was POD_4, with 4.6 mmol m^{-2} POD_4 as a suitable CL for Oxford poplars grown under various soil N and P conditions.

1. Introduction

M24 is the mean value of hourly O_3 concentrations during the daily 24 h and its value was about 10 ppb at the pre-industrial era (Voltz and Kley, 1988; Cooper et al., 2014). W126 (the sum of weighted hourly O_3 concentrations, cumulated over the 12-h daylight period from 8:00 a.m. to 8:00 p.m.) is an index designed to reflect the cumulative exposures during three consecutive months in the growing season and is under consideration as secondary standard in the USA (Lefohn et al., 1988; EPA, 2008; EPA, 2015). AOT40 (the accumulated exposure over a hourly threshold of 40 nmol mol^{-1} during the growing season (Fuhrer et al., 1997) was the European legislative standard for plant protection (Directive, 2008/50/EC), and AOT40-based CLs have been proposed for trees (Karlsson et al., 2003) and semi-natural vegetation (Mills et al., 2011). However, such exposure-based metrics lack biological meaning because they disregard the different species- or cultivar-specific sensitivities of plants to O_3 and the effects of environmental and biological variables on O_3 uptake into a leaf (Paoletti and Manning, 2007).

Ground level ozone (O_3) is one of the major air pollutants in many developed or developing countries, and causes serious threat to forest and natural ecosystems (Paoletti, 2007; Ainsworth et al., 2012; Matyssek et al., 2013). A meta-analytic review estimated that the O_3-caused loss in the biomass of trees ranged from 7 to 17% under the current O_3 levels relative to projected levels by 2100 at middle latitude in the northern hemisphere (Wittig et al., 2009; Li et al., 2017). The assessment of O_3 risk to plants including forest

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vegetation is topical in research and policy making nowadays (Mills et al., 2011; Watanabe et al., 2012; De Marco et al., 2015; Sicard et al., 2016b). Several O_3 metrics have been suggested for risk assessments (Paoletti and Manning, 2007). Critical levels (CLs), above which O_3-caused loss would occur, have been developed for identifying areas of CL exceedance (LRTAP Convention, 2010; Mills et al., 2011; Büker et al., 2015).

Ozone enters the leaf through stomata, which are the main regulating apparatus of gas exchange and play key roles in determining the potential O_3 damage (Matyssek et al., 2013). There are many reports that O_3-caused negative effects - such as foliar visible injury, inhibition of photosynthesis and decrease of biomass - are more closely related to the stomatal O_3 flux than to O_3 exposure (Karlsson et al., 2007; Bagard et al., 2015; Sicard et al., 2016b). The accumulated stomatal O_3 flux is called phytotoxic ozone dose (POD) and has a potential to be the best metric for setting future O_3 CLs for forest protection against O_3 pollution (Karlsson et al., 2003; Mills et al., 2011; Sicard et al., 2016a). New CLs are thus suggested as flux although AOT40 still has some fields of application (CLRTAP, 2017).

Nitrogen (N) deposition is increasing mainly due to elevated anthropogenic N emissions such as nitrogen oxides (NOx) emission from combustion of fossil fuels (Galloway et al., 2004). The background N deposition in some areas, such as Sichuan basin in China and California central valley in USA, was even higher than 80 kg N ha^{-1} yr^{-1} (Fenn et al., 2003; Peng et al., 2017). It has been indicated that nutrient availability dominates carbon retention in forests (UNECE, 2011; De Vries, 2014). However, N deposition of more than 26 N ha^{-1} $year^{-1}$ was negatively related with basal area increment in forests of Switzerland (Braun et al., 2017). Since nitrogen oxides (NOx) are the main O_3 precursor, O_3 exposure and N deposition are often interrelated (Ollinger et al., 2002). Nitrogen is considered as an important modifier of plant responses to O_3 (Schulze et al., 1994; Utriainen and Holopainen, 2001a). There are some reports that additional N supply may increase the tree sensitivity to O_3 (e.g. Watanabe et al., 2012). On the other hand, some opposite responses were also found such as in hybrid aspen (Häikiö et al., 2007). In contrast to N, trees are facing phosphorus (P) deficiency by depletion, soil barriers, low-P parent materials, and sink-driven and anthropogenic limitations (Vitousek et al., 2010; Peñuelas et al., 2012). Phosphorus is an essential macronutrient for physiological functions, including photosynthesis (Larcher, 2003). Phosphorus availability in the soil is an important regulator of the aboveground net primary productivity of forests (Domingues et al., 2010). Soil acidification induced by excess N deposition may cause P limitation for tree growth (Huang et al., 2016; Braun et al., 2017). Phosphorus availability may affect stomatal conductance (Fitter, 1988; Tissue and Lewis, 2010; Cernusak et al., 2011; Zimmerli et al., 2012), which modifies stomatal O_3 flux. Therefore, risk assessment of O_3 pollution must not overlook interactions with N and P availability (Matyssek et al., 2013). However, to our knowledge, there are only few reports about the impacts of N and P availability individually or in combination, on the O_3 risk assessment of forest species (Utriainen and Holopainen, 2001b; Wallin et al., 2002). Most of these studies were exposure-based assessment rather than flux-based one. The influences of N or P availability on risk assessment based on stomatal O_3 flux are urgently needed to be investigated.

Among tree species, poplar has been paid particular attention because it is widely used for wood production and as a model system in plant biology (e.g., Christersson, 2010). However, the knowledge of O_3 dose-response relationships for poplar is still limited (Marzuoli et al., 2009; Hu et al., 2015; Hoshika et al., 2018a).

The objectives of the present study were: (1) to derive exposure based and flux based dose-response relationships for poplar biomass responses to O_3 and nutritional availability; and (2) to define O_3 CLs for protecting poplar from O_3 stress under different nutritional conditions.

2. Materials and methods

2.1. Plant material

Rooted cuttings of Oxford poplar clone (*Populus maximoviczii* Henry × *Populus berolinensis* Dippel) were propagated in December 2015, and then transplanted in 10 l plastic pots filled with a mixture of sand:peat:soil = 1:1:1 (v:v:v) in April 2016. Plants were irrigated sufficiently to field capacity every 2–3 days to avoid water stress.

2.2. Ozone and nutrient treatments

Ozone treatments were applied at three levels: ambient air concentration (AA), 1.5 × AA and 2.0 × AA, from 1st May to 1st October, 2016, 24 h per day, in a free air controlled exposure (FACE) system located in Sesto Fiorentino, Florence, Italy (43° 48′ 59″ N, 11° 12′ 01″ E, 55 m a.s.l.). Details on this FACE facility can be found in Paoletti et al. (2017). Three replicated 25 m^2 blocks were assigned to each O_3 concentration, with 18 plants in each block. Three of the plants in each block were randomly assigned to each of six nutritional treatments as explained below. The pot position was changed every two weeks within each block to eliminate possible positional effects due to irrigation or light. Ozone was generated by a generator (TGOC13X, Triogen ltd., Glasgow, UK). A mixture of O_3 and ambient air was distributed into the O_3 FACE by a system of 25 teflon tubes in each block hanging down from a fixed grid above the seedlings (Paoletti et al., 2017). Ozone concentration at plant height was continuously monitored by O_3 analyzers (Model 202, 2B Technologies Inc., Boulder, Colorado, USA).

Two levels of N concentration (N0: 0 kg N ha^{-1}, i.e. 0 mg N $seedling^{-1}$; N80: 80 kg N ha^{-1}, i.e. 392.5 mg N $seedling^{-1}$) were supplied as NH_4NO_3 (0 and 5 mM solution) according to Thomas et al. (1994). Three levels of P concentration (P0: 0 kg P ha^{-1}, i.e. 0 mg P $seedling^{-1}$; P40, 40 kg P ha^{-1}, i.e. 196.3 mg P $seedling^{-1}$; P80: 80 kg P ha^{-1}, i.e. 392.5 mg P $seedling^{-1}$) were added using KH_2PO_4 (0, 0.5 and 1.0 mM solution) according to Lewis and Strain (1996). Based on P affinity constant and adsorption maxima, these levels of P were selected to simulate a realistic range in soil available P (Yu et al., 2017). Therefore, there were 6 combinations of nutrient treatment, i.e. N0P0, N0P40, N0P80, N80P0, N80P40, N80P80. In detail, 200 ml solution of NH_4NO_3 or KH_2PO_4 with different concentrations as described above were added into the soil twice a week during the whole treatment period. At the same time, KCl was supplied into the soil that did not receive KH_2PO_4 to keep an equal amount of K among all treatments (Tissue and Lewis, 2010; Mao et al., 2014). In total, 162 plants (3 levels of O_3 × 2 levels of N × 3 levels of P × 3 replicated blocks × 3 plants in each block) were used. At the end of the experimental period, soil was sampled for measuring the pH of the soil aqueous solution using a pH meter (Model MP220, Mettler Toledo, Switzerland). In addition, contents of N and P in the soil were measured. Total N content was determined using modified Kjeldahl method. In this method, titanium dioxide was used as catalyst instead of selenium (classic Kjeldahl method), distillation apparatus being Gerthardt (Cools and De Vos, 2010; ISO 11261). Content of total P was determined by inductively coupled plasma–optical emission spectroscopy (ICP–OES, iCAP7000, Thermo Fisher Scientific, Waltham MA, USA). During the whole treatment period, the hourly mean, maximum, and minimum temperature were 22.9, 37.4, and 9.3 °C, respectively, and the precipitation was 226.6 mm. The volumetric soil water content (SWC) was measured in the root layer (5 cm depth in pots) by EC-5

soil moisture sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). The averaged SWC during the experiment was 28.5 ± 0.0 $m^3 m^{-3}$, which was nearly equal to the field capacity (29.5 $m^3 m^{-3}$).

2.3. Stomatal conductance measurement

Fully expanded sun leaves (4th to 6th from the shoot tip) of plants in each combination of O_3, N, and P treatment were selected for daily measurement of stomatal conductance using a portable gas exchange system (CIRAS-2 PP Systems, Herts, UK). The measurements were carried out in 1–3 plants per plot every 3 h (8:00–16:00 h) in two days for each month (23rd and 27th June, 26th and 27th July, 24th and 25th August, 26th and 27th September) and at night (23:00 h in 24th and 25th June, 17th and 18th August, 28th and 29th September). Natural illumination projected on the upper leaf surface was used for the measurement and the leaf chamber was oriented so that the leaves were fully and directly exposed to sunlight. The CO_2 concentration in the chamber (Ca) was set to 380 ppm. Temperature and relative humidity in the chamber were set to the ambient conditions. Data from all 826 measurements were pooled for estimating the parameters of the stomatal conductance model.

2.4. Nitrogen and phosphorus content of leaves

At the end of the experiment (October 2016), leaf samples (4th to 6th order leaves from the tip of shoots) were taken for determination of N and P content of leaves. These leaf samples were dried in an oven at 70 °C for 1 week. The dried leaves were then ground into fine powder. Total N content was determined using the modified Kjeldahl method referred to above. For P measurements, the leaf powder samples were digested by HNO_3, HCl, and H_2O_2. Total P was then measured with an inductively coupled plasma–optical emission spectroscopy (ICP–OES, iCAP7000, Thermo Fisher Scientific, Waltham MA, USA).

2.5. Parameterization of stomatal conductance model

The stomatal conductance model was based on the multiplicative algorithm described by Jarvis (1976) and CLRTAP (2017). The model was parameterized according to the following equation:

$$g_{sO3} = g_{max} \cdot f_{phen} \cdot f_{O3} \cdot f_{light} \cdot \max\left\{f_{min}, \left(f_{temp} \cdot f_{VPD} \cdot f_{SWC}\right)\right\} \quad (1)$$

where g_{sO3} is stomatal conductance for O_3 ($g_{sO3} = g_{sw} \cdot 0.663$; mol m^{-2} PLA s^{-1}); g_{sw} is stomatal conductance for water vapor, and the factor of 0.663 is the ratio of diffusivities between O_3 and water vapor (CLRTAP, 2017). g_{max} represents the maximum stomatal conductance (mol O_3 m^{-2} Projected Leaf Area (PLA) s^{-1}). The f functions (scaled from 0 to 1) are limiting factors of g_{max}. f_{phen}, f_{light}, f_{temp}, f_{VPD}, f_{swc}, and f_{O3} are the variation in stomatal conductance with phenology, photosynthetic photon flux density at the leaf surface (PPFD, μmol photons m^{-2} s^{-1}), air temperature (T, °C), vapor pressure deficit (VPD, kPa), volumetric soil water content (SWC, m^3 m^{-3}), and cumulative O_3 uptake (mmol m^{-2}), respectively. f_{min} is a fraction of g_{max} denoting the minimum stomatal conductance. Each response function is described in the supplementary material.

Parameters (f_{light}, f_{VPD}, f_{temp}) were estimated by using a boundary line technique (Emberson et al., 2000; Hoshika et al., 2012a; b; Azuchi et al., 2014). First, the stomatal conductance data were divided into classes with the following step-wise increases for each variable (Hoshika et al., 2012a; b): 200 μmol photons m^{-2} s^{-1} for PPFD (when PPFD values were lower than 200 μmol photons m^{-2} s^{-1}, PPFD classes at 50 μmol photons m^{-2} s^{-1} steps were adopted), 2 °C for T, and 0.4 kPa for VPD. A function was fitted against each model variable based on the 95th percentile value per each class of environmental factors. The 95th and 5th percentile values measured in the whole treatment period were used as g_{max} and f_{min}, respectively. Parameters of the fo_3 were derived by a linear regression between relative stomatal conductance to daily g_{max} and cumulative O_3 uptake (POD_0) (Hoshika et al., 2012b). In each measurement day, we defined daily g_{max} as the stomatal conductance measured under the nearly optimal condition (PPFD > 500 μmol m^{-2} s^{-1}, VPD < 1.5 kPa, T = 20–30 °C). To estimate the empirical parameter b of f_{O3}, we used the iterative method (Martin et al., 2001; Kercher and Chambers, 2001). Here b and POD_0 are described as b_i and $POD_0(i)$, respectively, for explanation (i is the number of iteration). The computation started from $b_0 = 0$ (no reduction of g_s by O_3). Then $POD_0(0)$ was calculated in the case of $b_0 = 0$. After calculating the $POD_0(0)$, the new parameter b_1 was obtained as a slope of the regression line between daily g_{max} and the $POD_0(0)$. Then, using the value of b_1, we calculated again $POD_0(1)$ to obtain the values of b_2. On average, less than five iterations were needed to achieve a deviation of 5% in the successive value of b. The phenology function (f_{phen}) was considered as $f_{phen} = 1$ throughout the experiment, because sprouting of new leaves started before the experimental period and plants were harvested before leaf senescence started. Terms describing modification of stomatal conductance by the soil moisture (i.e., f_{swc}) were not used in this study. Soil water availability was sufficient because plants were irrigated. No reductions in stomatal conductance due to soil water content were recorded (data not shown).

2.6. Ozone metrics

Ozone metrics were calculated as follows:

AOT40 (ppm h): the sum of the differences between hourly O_3 concentrations and 40 ppb for each hour when the concentration is above 40 ppb during daylight hours (short wave radiation > 50 W m^{-2}) according to CLRTAP (2017) during the treatment period (from 1st May to 1st October). Solar radiation was measured by a Watchdog station (Mod. 2000; Spectrum Technology, Inc., Aurora, IL, USA). The equation is as follows:

$$AOT40 = \sum_{i=1}^{n} \max([O_3]_i - 40, 0) \quad (2)$$

$[O_3]_i$ is the i th measured hourly O_3 concentrations (ppb), i from 1 to n in the integral where n is the number of hours included in the calculation period and dt is 1h. The equation of W126 is as follows:

$$W126 = \sum_{i=1}^{n} [O_3]_i \cdot 0.001 \cdot \left(\frac{1}{1 + (4403 * \exp(-0.126 * [O_3]_i))}\right) \quad (3)$$

Hourly stomatal O_3 flux (F_{st}; nmol m^{-2} s^{-1}) was calculated as:

$$F_{st} = [O_3] \cdot g_{sO3} \cdot \frac{r_c}{r_b + r_c} \quad (4)$$

where r_b is the leaf boundary layer resistance (s m^{-1}), r_c is the leaf surface resistance (s m^{-1}), and r_c is defined as $r_c = 1/(g_{sO3} + g_{ext})$ where $g_{ext} = 0.0004$ m s^{-1} is the external leaf conductance that

indicates slight absorption of O_3 on leaf surface (CLRTAP, 2017). r_b was calculated from the wind speed (u, m s^{-1}), and the cross-wind leaf dimension ($L_d = 0.04$ m, which was obtained from the ellipse shape assumption by using the leaf length and width data as the mean value of 3–5 leaves per 6 seedlings in each treatment, according to Schuepp, 1993):

$$r_b = 1.3 \cdot 150 \cdot (L_d/u)^{0.5} \quad (5)$$

where the factor 1.3 represents differences in diffusivity between O_3 and heat (CLRTAP, 2017).

POD_Y (mmol m^{-2}): the phytotoxic O_3 dose above a flux threshold of Y was calculated based on the methodology outlined in the Mapping Manual (CLRTAP, 2017). The equation for POD_Y during the experiment is as follows:

$$POD_Y = \sum_{i=1}^{n} \max(F_{st\_i} - Y,\ 0) \quad (6)$$

where F_{st_i} is the i th hourly stomatal O_3 flux (nmol m^{-2} s^{-1}), n is the number of hours included in the calculation period. Y is a species-specific or cultivar-specific stomatal O_3 flux threshold, below which it is assumed that any O_3 molecule absorbed by the plant will be detoxified (nmol m^{-2} s^{-1}). A range of thresholds (0–6 nmol m^{-2} s^{-1}) were tested with an increment of 0.5 nmol m^{-2} s^{-1}.

2.7. Biomass assessment and O_3 critical level calculation

At the end of the experimental period, two plants in each block per combination of O_3, N, and P treatments were immediately harvested. Leaves, current year stem, fine roots (with diameter < 2 mm) and coarse roots were separated. They were dried in the oven (80 °C) until constant values were reached. Then dry mass of each part was measured by an electronic balance (Model Bp110, Sartorius, Germany). As the seedlings were rooted cuttings, total annual biomass was calculated as sum of leaf dry mass, current year stem dry mass, fine root dry mass, and coarse root dry mass. According to Paoletti et al. (2017), linear regression between total annual biomass and M24 was conducted to obtain the hypothetical maximum biomass for each treatment at M24 of 10 ppb, which is considered as the pre-industrial O_3 concentration (e.g., Wittig et al., 2009). Then, the relative biomass (RB) was calculated as the ratio of total annual biomass/hypothetical biomass in low and high N (N0 and N80, respectively) because main effects of N on the biomass were found (see the Result section).

Exposure and flux based dose-response functions were used to estimate CLs with or without nutrients (N or P or together). Exposure based dose-response functions were derived following the methodology described in the Mapping Manual (CLRTAP, 2017). Linear regressions between RB versus W126 or AOT40 were conducted for testing the capability of prediction.

Flux based dose-response functions were derived as a linear regression between RB and POD_Y over a threshold of Y (Y from 0 to 6, with an increment of 0.5) nmol m^{-2} s^{-1}.

Two criteria were used to select the best exposure or flux dose-response functions according to Büker et al. (2015): 1) Y-intercept = 1 must be included in the confidence interval (C.I.) of the function, and 2) the function with the highest R^2 value from the equations meeting the criterion 1 was chosen. CLs were calculated as the level which causes a 5% loss of the total annual biomass according to CLRTAP (2017).

2.8. Statistical analysis

The study was conducted in a well-replicated split-plot experiment with a full factorial combination of treatment levels where ozone was the whole-plot factor and nutrients were the randomized split-plot factors. Each O_3 treatment had three blocks and each block had three plants per nutrient supply. The biomass values of plants per treatment were averaged in each block for data analysis. The relationships between RB and O_3 indices were fitted using linear regression analysis using SPSS (20.0, SPSS, Chicago, USA). The significance of the regressions was assessed by P value (<0.05) and the goodness of fit was judged by the R^2. The relative biomass loss caused by 1.5 × AA or 2.0 × AA was calculated as (Mean $biomass_{AA}$ - Mean $biomass_{1.5\times AA}$)/Mean $biomass_{AA}$ and (Mean $biomass_{AA}$ - Mean $biomass_{2.0\times AA}$)/Mean $biomass_{AA}$ under each nutrient treatment. Effects of nutritional treatments (i.e., N and P) on g_{max} were tested via two-way analysis of variance (ANOVA). We employed three-way ANOVA to test effects of O_3, N and P on total biomass.

3. Results

3.1. Soil characteristics

The mean pH values of soil in N0P0, N0P40, N0P80, N80P0, N80P40, and N80P80 treatments were 6.92, 6.77, 7.09, 6.97, 6.74, and 6.90, respectively. The total N contents in soils at N0P0, N0P40, N0P80, N80P0, N80P40, and N80P80 treatments were 1.73, 1.71, 1.80, 2.69, 2.54, and 2.84 g kg^{-1}, respectively. The total P contents in soils at N0P0, N0P40, N0P80, N80P0, N80P40, and N80P80 treatments were 0.49, 0.73, 0.98, 0.57, 0.80, and 1.12 g kg^{-1}, respectively (Table S1).

3.2. Leaf N and P content

Leaf N and P content and N:P ratios are shown in Table 1. Nitrogen treatments (N80) increased the N concentration in poplar leaves (+20%). Leaf P concentration was higher in poplar cuttings subjected to P treatments (+18% at P40, +72% at P80). However, such an enhancement of P concentrations was not clearly observed in elevated O_3 (2.0 × AA). Nitrogen and P treatments had a significant effect on the N:P ratio, i.e. the ratio was increased by N treatments and decreased by P treatments.

3.3. Parameterizations of stomatal conductance models

Parameters of the stomatal conductance model were estimated (Table 2). No significant effect of O_3 on g_{max} was found (data not shown). We therefore considered the effects of the different nutritional treatments on g_{max} (Fig. 1). The g_{max} values were from 0.30 to 0.40 mol O_3 m^{-2} s^{-1} depending on the nutrient treatments. In detail, the values were 0.37, 0.30, 0.35, 0.40, 0.35, 0.38 mol O_3 m^{-2} s^{-1} for N0P0, N0P40, N0P80, N80P0, N80P40, N80P80, respectively. Nitrogen increased the g_{max} by 11%, while P decreased the g_{max} by 16% in P40 and 6% in P80 relative to P0.

The response of stomatal conductance (g_s) to PPFD (f_{light}) was fitted with a typical light-response curve, in which the light saturation point was above 500 μmol m^{-2} s^{-1} (Fig. S1). The response of g_s to air temperature (f_{temp}) showed that the T_{opt}, T_{min}, and T_{max} for stomatal opening were 29, 5, and 40 °C, respectively (Fig. S2). Stomatal closure was induced when a VPD value was higher than 1.7 kPa (Fig. S3). The response of g_s to O_3 dose (f_{O3}) indicated that g_s was reduced by 0.88% per unit of O_3 uptake (mmol m^{-2}) (Fig. S4).

Table 1
Mean value (±SE) of each treatment on leaf N content, leaf P content and N: P ratio (n = 3 plots for each treatment) for Oxford poplar clone exposed to ozone (AA, ambient air; 1.5 × AA or 2.0 × AA); nitrogen (N0 or N80 i.e. 0 or 80 kg N ha^{-1}), and phosphorus (P0, P40 or P80, i.e. 0, 40 or 80 kg P ha^{-1}) in 2016. Levels of significance (*p* value) of three-way ANOVA for the effects of O_3, N and P were calculated.

Treatments		Leaf N ($mg\ g^{-1}$)	Leaf P ($mg\ g^{-1}$)	N:P ratio (no dimension)
AA	N0P0	10.2 (±0.6)	1.0 (±0.0)	10.8 (±1.1)
	N0P40	10.8 (±0.6)	0.9 (±0.0)	11.9 (±1.1)
	N0P80	10.7 (±0.5)	1.6 (±0.2)	6.9 (±1.3)
	N80P0	11.6 (±0.3)	0.8 (±0.1)	16.3 (±2.8)
	N80P40	11.7 (±1.5)	0.9 (±0.2)	13.8 (±2.3)
	N80P80	11.9 (±0.6)	1.5 (±0.0)	8.0 (±0.4)
1.5AA	N0P0	10.5 (±1.1)	0.9 (±0.1)	12.4 (±2.1)
	N0P40	10.2 (±0.5)	1.3 (±0.2)	8.1 (±0.6)
	N0P80	10.2 (±0.5)	1.4 (±0.2)	7.5 (±1.1)
	N80P0	12.6 (±0.4)	0.6 (±0.1)	21.6 (±2.7)
	N80P40	12.3 (±0.2)	1.0 (±0.2)	14.3 (±3.1)
	N80P80	15.1 (±1.9)	1.7 (±0.2)	9.4 (±1.4)
2.0AA	N0P0	11.0 (±0.5)	0.9 (±0.1)	12.9 (±2.9)
	N0P40	10.2 (±0.5)	0.9 (±0.1)	11.0 (±1.2)
	N0P80	11.9 (±0.8)	1.2 (±0.2)	10.6 (±2.8)
	N80P0	13.5 (±0.6)	0.7 (±0.1)	18.5 (±1.9)
	N80P40	13.5 (±0.6)	1.0 (±0.2)	13.8 (±2.1)
	N80P80	13.0 (±0.2)	0.9 (±0.1)	15.2 (±2.4)
ANOVA test (*p* values)				
	O_3	0.076	0.125	0.135
	N	<**0.001**	0.098	<**0.001**
	P	0.275	<**0.001**	<**0.001**
	$O_3 \times N$	0.121	0.986	0.452
	$O_3 \times P$	0.759	**0.040**	0.127
	$N \times P$	0.888	0.700	0.191
	$O_3 \times N \times P$	0.222	0.223	0.798

Table 2
Parameters of Jarvis-type stomatal conductance model of Oxford poplar measured in 2016. g_{max}: maximum stomatal conductance. f_{min}, minimum stomatal conductance. f_{phen}, f_{light}, f_{temp}, f_{vpd}, and f_{O3}: the variation in g_{max} with phenology, photosynthetic photon flux density at the leaf surface (PPFD, $\mu mol\ m^{-2}\ s^{-1}$), temperature (T, °C), vapor pressure deficit (VPD, kPa), and cumulative ozone (O_3) uptake ($mmol\ m^{-2}\ s^{-1}$), respectively; A_{start} and A_{end}: the year days for start of O_3 treatments, and the year days for the end of the experiment. f_{phen_a} and f_{phen_b}: the number of days for f_{phen} to reach its maximal value and the number of days during the decline of f_{phen} to reach its minimal value. f_{phen_c} and f_{phen_d}: f_{phen} at A_{start} and A_{end}. *a*: a constant determining the shape of the hyperbolic relationship. T_{opt}, T_{min}, and T_{max}: optimal, minimum and maximum air temperature for stomatal opening. VPD_{max} and VPD_{min}: vapor pressure deficit for attaining full and minimum stomatal aperture. *b*: is the slope parameter of the relationship between stomatal conductance and POD_0 (phytotoxic O_3 dose with a threshold 0).

Parameter		Units	Oxford clone
g_{max}	($mol\ O_3\ m^{-2}$ PLA s^{-1})		0.30–0.40[a]
f_{min}		(Fraction)	0.08
f_{phen}			1
f_{swc}			1
f_{light}	*a*	(Constant)	−0.0021
f_{temp}	T_{opt}	(°C)	29
	T_{min}	(°C)	5
	T_{max}	(°C)	40
f_{VPD}	VPD_{max}	(kPa)	1.7
	VPD_{min}	(kPa)	5.5
f_{O3}	*b*	(Constant)	0.0088

[a] g_{max} was set separately in each nutrient treatment. Please see Fig. 1 for a detail.

3.4. Ozone metrics

Compared with AA, M24 increased by 48% and 90% in 1.5 × AA and 2.0 × AA, respectively (Table S2). At the end of treatments, AOT40 (+204% and +394%) and W126 (+377% and +734%) were much higher in 1.5 × AA and 2.0 × AA than in AA. POD_Y also increased with increasing O_3 treatment (e.g., in the case of POD_0, +30% and +65% in 1.5 × AA and 2.0 × AA compared to AA, respectively). POD_Y was increased by N treatments (+7–8%, in the case of POD_0). On the other hand, POD_Y decreased with P treatments regardless with or without N treatments (−10% in P40, and -4% in P80 compared to P0, in the case of POD_0). As a result, the combination of N and P treatments (N80P40 and N80P80) did not increase POD_Y compared to N0P0 treatment.

3.5. Biomass responses to treatments

The hypothetical biomass in a clean air (M24 of 10 ppb) was 26.9 g in low N (N0) and 61.4 g in high N (N80) (Fig. 2). Ozone exposure significantly decreased total annual biomass by 19% in 1.5xAA and 26% in 2.0 × AA relative to AA, when all treatments were pooled together (Fig. 3, Table 3). Nitrogen markedly stimulated total annual biomass by 97%, when all N80 treatments were compared to all N0 treatments. The $O_3 \times N$ interaction was significant because the biomass responses to O_3 was changed by N treatments (N0: −19% and −18% at 1.5 × AA and 2.0 × AA, respectively; N80: −18% and −30% at 1.5 × AA and 2.0 × AA, respectively). Phosphorus did not significantly affect the response of total annual biomass to O_3 and thus the interaction was not significant. The

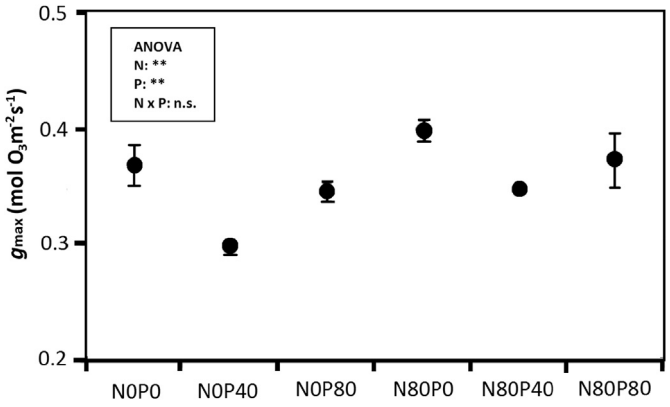

Fig. 1. Maximum stomatal conductance of Oxford poplar clone. The maximum stomatal conductance (g_{max}) of Oxford poplar clone under different nutrient supply. N0P0: no addition of N and P; N0P40, 40 kg P ha^{-1}; N0P80, 80 kg P ha^{-1}; N80P0, 80 kg N ha^{-1}; N80P40, 80 kg N and 40 kg P ha^{-1}; N80P80, 80 kg N and 80 kg P ha^{-1}. No significant effect of O_3 on g_{max} was found. We therefore analyzed only the effects of the nutritional treatments on g_{max}. Data are represented as mean ± SE (n = 3 replicated plots). Two-way ANOVA: **, $p < 0.01$; n.s., $p \geq 0.05$.

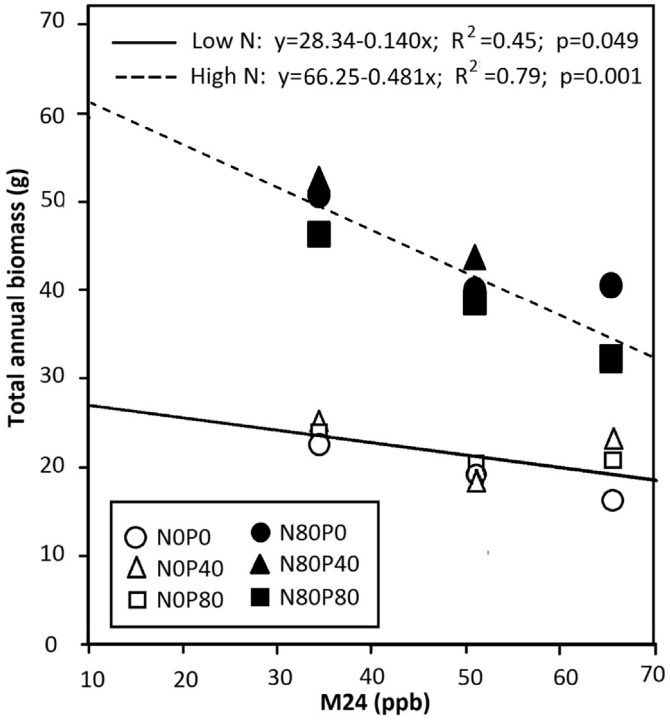

Fig. 2. Regression between total annual biomass of Oxford poplar clone and the 24-h averaged ozone concentration. The regression between total annual biomass of Oxford poplar clone and the 24-h averaged ozone concentration (M24, ppb) during the exposure period (May 1st – October 1st, 2016). N0P0: no addition of N and P; N0P40, 40 kg P ha^{-1}; N0P80, 80 kg P ha^{-1}; N80P0, 80 kg N ha^{-1}; N80P40, 80 kg N and 40 kg P ha^{-1}; N80P80, 80 kg N and 80 kg P ha^{-1}. The equations were used to estimate the control biomass at the theoretical pre-industrial level of ozone concentration (10 ppb M24) for each nutrient treatment (ns: $p \geq 0.05$, *: $p < 0.05$).

N × P interaction was significant because the P treatment resulted in a stimulation of biomass at N0 (+6% and +2% by P40 and P80 relative to P0, respectively) and in an inhibition at N80P80 (−9% relative to N80P0). The O_3 × N × P interaction was significant and is marked by different letters in Fig. 3. Although P alleviated the O_3-caused biomass loss in N0 treatments, such effects of P were not clear in N80 treatments.

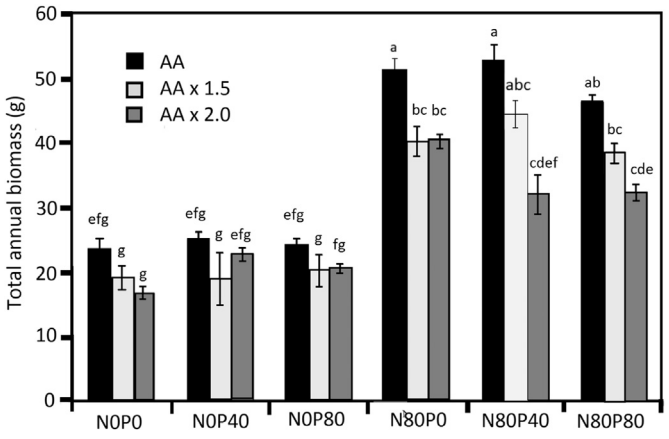

Fig. 3. Total annual biomass of Oxford poplar clone exposed to ozone, nitrogen, and phosphorus. Total annual biomass of Oxford poplar clone exposed to ozone (AA, ambient air; 1.5 × AA or 2.0 × AA); nitrogen (N0 or N80 i.e. 0 or 80 kg N ha^{-1}), and phosphorus (P0, P40 or P80, i.e. 0, 40 or 80 kg P ha^{-1}). Different letters show significant differences as resulting from a three-way ANOVA (Table 3) followed by Tukey test ($p < 0.05$, N = 3 plots with three plants per each plot).

Table 3
Results of a three-way ANOVA for the effects of ozone (O_3), i.e. ambient air (AA), 1.5 × ambient O_3 (1.5 × AA) or 2.0 × ambient O_3 (2.0 × AA); nitrogen (N), i.e. N0, or N80; and phosphorus (P), i.e. P0, P40 or P80, on the total annual dry mass of poplar clone 'Oxford'.

	d.f.	F value	P value
O_3	2	37.763	<0.001
N	1	497.838	<0.001
P	2	1.748	0.189
O_3 × N	2	11.159	<0.001
O_3 × P	4	0.509	0.729
N × P	2	4.253	0.022
O_3 × N × P	4	3.112	0.027

3.6. Exposure- and flux-based dose-response relationships and critical levels

For plants under N0P0 treatment, the first criterion (Y = 1 was included in C.I. of the Y-intercept) was reached in the regressions between RB and AOT40 or W126 or $POD_{0\ to\ 6}$. Among these O_3 indices, POD_4 had the highest R^2 (0.966) (Table S3). The CL based on POD_4 was 3.9 mmol m^{-2} (Table 4). For plants with N0P80 treatment, the first criterion was achieved for all O_3 metrics including AOT40, W126 and $POD_{0\ to\ 6}$. The highest R^2 values were found in AOT40 (0.712) for exposure-based indices, and POD_4 (0.695) for flux-based indices. The CLs for those N0P80 plants were found to be 21.7 ppm h AOT40 and 7.5 mmol m^{-2} POD_4 (Table 4). With N treatment singly (N80P0), the first criterion was achieved for AOT40, W126 and $POD_{0\ to\ 6}$. Among them, AOT40 had the highest R^2 value (0.731). Actually, $POD_{4.5}$ had a similar R^2 (0.720) (Table S3). Under N80P40, $POD_{3.5}$ showed the highest R^2 value (0.994). Under N80P80, the first criterion was achieved for $POD_{0\ to\ 5.5}$. POD_4 had the highest R^2 value (0.997) (Table S3). If the data were divided into the two different N levels (Table 4), $POD_{4.5}$ and POD_3 were selected as a best metric in low and high N treatments, respectively. When all the nutrient treatments were pooled together for analysis, the first criterion was reached for $POD_{0\ to\ 5.5}$, and POD_4 had the highest R^2 value (0.610), followed by AOT40 (0.486).

Table 4
Critical levels (CL) for a 5% decrease in total biomass per the most statistically significant dose-response type (flux-based and exposure-based) and nutrient treatment (N0P0: no addition of N and P; N0P40, 40 kg P ha^{-1}; N0P80, 80 kg P ha^{-1}; N80P0, 80 kg N ha^{-1}; N80P40, 80 kg N and 40 kg P ha^{-1}; N80P80, 80 kg N and 80 kg P ha^{-1}; Low N: all N0 treatments pooled together; High N: all N80 treatments pooled together; Total: all nutrient treatments pooled together). POD_y (phytotoxic O_3 dose above a flux threshold of y nmol m^{-2} s^{-1} of stomatal O_3 flux). CL was not calculated when $R^2 < 0.2$ or Y-intercept of 1 was not included in the confidence interval (C.I.) of the function in the regression line (denoted as "Not available").

Dose-response type	Nutrient treat.	Index	Equation	R^2	CL
Flux-based	N0P0	POD_4	y = 1.027−0.0127x	0.966	3.9 mmol m^{-2}
	N0P40	$POD_{3.5}$	y = 0.912−0.0044x	0.122	Not available
	N0P80	POD_4	y = 0.949−0.0067x	0.695	7.5 mmol m^{-2}
	N80P0	$POD_{4.5}$	y = 0.896−0.0081x	0.720	6.2 mmol m^{-2}
	N80P40	$POD_{3.5}$	y = 1.090−0.0167x	0.994	3.0 mmol m^{-2}
	N80P80	POD_4	y = 0.895−0.0111x	0.997	4.5 mmol m^{-2}
	Low N (all N0)	$POD_{4.5}$	y = 0.955−0.0091x	0.548	5.5 mmol m^{-2}
	High N (all N80)	POD_3	y = 0.988−0.0109x	0.740	4.6 mmol m^{-2}
	Total	POD_4	y = 0.972−0.0109x	0.610	4.6 mmol m^{-2}
Exposure-based	N0P0	AOT40	y = 0.934−0.0046x	0.971	10.9 ppm h
	N0P40	AOT40	y = 0.892−0.0015x	0.139	Not available
	N0P80	AOT40	y = 0.910−0.0023x	0.712	21.7 ppm h
	N80P0	AOT40	y = 0.843−0.0030x	0.714	16.7 ppm h
	N80P40	W126	y = 0.912−0.0032x	0.991	15.6 ppm h
	N80P80	AOT40	y = 0.812−0.0040x	0.999	Not available
	Low N (all N0)	AOT40	y = 0.912−0.0028x	0.437	17.9 ppm h
	High N (all N80)	AOT40	y = 0.869−0.0045x	0.797	Not available
	Total	AOT40	y = 0.890−0.0035x	0.486	Not available

4. Discussion

4.1. Parameterizations of stomatal conductance model

The physiological mechanisms of stomatal response to environment are so complex that a real mechanistic model of g_s is very hard to be developed (Damour et al., 2010). As a compromise, empirical models have been set up, such as the multiplicative algorithm developed by Jarvis (1976) and other similar improved models (e.g. Emberson et al., 2000). In these empirical stomatal conductance models, g_{max} is the key parameter determining the degree of stomatal opening and O_3 uptake (Tuovinen et al., 2007). In the present study, g_{max} of Oxford poplar clone under N0P0 treatment was 0.37 mol O_3 m^{-2} s^{-1}. This value was similar to previous studies on this clone (0.41 mol O_3 m^{-2} s^{-1} as an average: Marzuoli et al., 2009; Hoshika et al., 2012c, 2018a; Desotgiu et al., 2013; Pollastrini et al., 2014), lower than the g_{max} in *Populus*×*euramericana* clones (0.56 mol O_3 m^{-2} s^{-1}: Voltas et al., 2006), and higher than the value in five poplar clones grown in China (0.30 mol O_3 m^{-2} s^{-1}: Hu et al., 2015).

Our results showed that N supply increased the g_{max} while P inhibited the g_{max} (Fig. 1). There was no significant interaction between N and P on the g_{max}. This result suggests an urgent need to upgrade the Jarvis model by adding a function of nutrient availability for risk assessment modeling. In general, an increase of g_s by N application is coupled with increasing photosynthetic rates (e.g, Evans, 1989). An increase of g_s by N supply was also reported in Siebold's beech (Yamaguchi et al., 2007), larch (Mao et al., 2014), wheat (Wall et al., 2000) and grass species (Chen et al., 2005), although some studies reported no enhancement by N applications (Nakaji et al., 2001; Ripullone et al., 2003). Phosphorus is essential to metabolisms and energy transfer in plants (Rao, 1997). An adequate supply of inorganic P (Pi) is required for photosynthesis (Ellsworth et al., 2015; Norby et al., 2017). Although P supply might increase g_s by increasing the photosynthetic rate (Tissue and Lewis, 2010), no change or decrease of g_s have also been reported (Cernusak et al., 2011; Mao et al., 2014).

The parameter "*a*" of f_{light} function was lower than that listed in the mapping manual (CLRTAP, 2017: for deciduous trees). However, the "0.006" for the deciduous tree types in the manual was basically obtained from the data of beech, which is known as relatively shade tolerant species. As we know, in beeches, a relatively high initial slope of the light response curve is observed (i.e., with a relatively high "*a*"). On the other hand, our target was a poplar species, which is a light-demanding pioneer species (with a relatively low initial slope of the light response curve). Actually the PPFD when g_s reaches 50% of g_{max} (denoted as $PPFD_{50} = Ln[2]/a$) was 330 μmol m^{-2} s^{-1}, which is within the range reported in the review papers by Körner (1995) and Hoshika et al. (2018).

It has been reported that g_s may be reduced by exposure to O_3 and the reduction should be considered in the model for estimating g_s (Emberson et al., 2000; Hoshika et al., 2012a; b). In the present study, we used an empirical coefficient ($b = 0.88\%$) generated by the linear regression between relative g_s versus POD_0 to estimate the responses of g_s to O_3 uptake. The reduction of g_s may be a secondary response to O_3-induced inhibition of carbon assimilation by chloroplasts (e.g., Heath and Taylor, 1997), because g_s is generally correlated with photosynthesis (Lambers et al., 2008). In previous studies, the coefficient *b* was calculated as 2.6% in *Populus alba* or 2.8% in *Fagus crenata* (Fares et al., 2006; Hoshika et al., 2012a), which was in the same order of magnitude as our value. The f_{O3} parameter may be species-specific and it is necessary to take this function into account when estimating the stomatal conductance model under O_3 treatments.

4.2. Biomass response to ozone, alone and in combination with the nutrients

Soil N and P are among the most important limiting determinants of forest productivity (Aerts and Chapin, 1999) and have different metabolic functions in plants (Conroy, 1992). In the present study, soil N concentrations in N0 treatments were 1.71–1.80 g kg^{-1}, and those in N80 treatments were 2.54–2.84 g kg^{-1}. Normally soil N ranges between 0.2 and 5 g kg^{-1} (Bowen, 1979). Soil P concentrations in P0, P40 and P80 treatments were 0.49–0.57, 0.73 to 0.80 and 0.98–1.12 g kg^{-1}, respectively. These values are within the range of native P in soils (usually 0.5–0.8 g kg^{-1}, and the maximum is found as 1.0–1.3 g kg^{-1}, Stevenson and Cole, 1999). Therefore, we simulated the poplar growth under realistic soil N and P conditions.

The realistic N and P treatments increased the concentration of each nutrient in poplar leaves as reported before (e.g., Mao et al.,

2014). Ozone did not change the leaf N concentration for Oxford poplar clone. However, O_3 suppressed the positive effects of P treatments on leaf P content (Table 1). Ozone might affect P uptake through changes of ectomycorrhizal activities of trees (Grebenc and Kraigher, 2007). Ectomycorrhizal fungi in roots usually help for absorbing water and essential nutrients such as P, and sometimes N, to host plants (e.g., Cairney, 2011). Previous studies reported that O_3 impaired ectomycorrhizal colonization and species richness in European beech (Zeleznik et al., 2007) and Japanese hybrid larch F_1 (Wang et al., 2015). Further investigations of O_3 effects on P acquisition and ectomycorrhizal activity for Oxford poplar clone will be necessary. In the present study, poplar plants showed leaf N:P ratios in a range of 6.9–21.6. Koerselman and Meuleman (1996) suggested that N:P ratios <14 indicate limitation by N and those >16 indicate limitation by P for plant biomass productions, although the optimal N:P ratio for plant growth may depend on geography, temperature, and other climatic factors (Reich and Oleksyn, 2004). Nitrogen addition is generally known to stimulate forest growth (UNECE, 2011; Wooliver et al., 2017). Our realistic N conditions resulted in a marked stimulation of biomass. However, high P loading (P80) partly counteracted the positive effects of N on poplar biomass (Fig. 3), suggesting a N limitation for the biomass production. Such a N limitation for poplar biomass was also found in N80P80 at 2.0 × AA, even though relatively high N:P ratios (>14) were observed (Table 1). This suggests an increased demand for N for poplar growth under elevated O_3, probably because O_3 often decreases N use efficiency (e.g., Watanabe et al., 2013).

It is well known that Oxford poplar clone is sensitive to O_3 (Marzuoli et al., 2009). Obvious losses of biomass were found by elevated O_3 (Fig. 2, Table 3). In the species with indeterminate growth such as poplars, high nutrition may allow high leaf turnover, which could partly compensate the O_3-induced decline of photosynthesis by new leaf growth (Pell et al., 1995). Overall, however, surplus N did not mitigate the O_3-caused biomass loss of Oxford clone. In previous studies, the negative effects of elevated O_3 on biomass growth were more evident under high N supply for Scots pine (Utriainen and Holopainen, 2001a) and Siebold's beech (Watanabe et al., 2012). The additional supply of N increased the g_{max} by 5–10% leading to an increase of stomatal O_3 uptake (Table S2). Also, the nutrient-rich conditions may lead to a lower antioxidant capacity (Kandlbiner et al., 2004; Desai et al., 2014). As compared to low N-fertilized birch plants, the reduced ascorbate level was lower in the leaves of high N-fertilized plants under O_3 exposure, suggesting that high N reduced the detoxification capacity against O_3 stress (Maurer and Matyssek, 1997). On the other hand, P alleviated apparently the O_3-caused biomass loss in N0 treatments due to a lower uptake of O_3. However, such mitigation effects of P were not clear in N80 treatments even though P treatments decreased stomatal O_3 uptake. We conclude that plant biomass responses to O_3 stress are altered by N and P availability, although the general behaviour of increasing biomass losses with increasing O_3 exposure cannot be significantly ameliorated by improved nutrient availability.

4.3. Dose-response relationships for ozone risk assessment

To protect forests against O_3 pollution, appropriate standards, thresholds and CLs are still under discussion (Mills et al., 2011; Sicard et al., 2016a). Recently, metrics based on stomatal O_3 flux such as POD_Y or $\Sigma U/P_n$ (cumulative value of the ratio of hourly stomatal O_3 uptake to net photosynthesis) have been proposed (Emberson et al., 2000; Mills et al., 2011; Hoshika et al., 2018a). POD_Y is recognized as an important standard for protecting forest species in O_3 polluted areas (Mills et al., 2011; Anav et al., 2016). In the present study, the linear regressions between metrics of exposure (AOT40 and W126) or stomatal flux (POD_Y with different Y thresholds) and RB indicated that the best metric was POD_4 for Oxford poplar clone when all the nutritional treatments were pooled together. Ozone-induced decline in poplar biomass under different nutritional treatments depends on stomatal O_3 uptake rather than O_3 exposure. Stomatal flux-based indices are phytomedically relevant and mechanistic, and may provide a more accurate assessment of O_3 risk than exposure indices (Matyssek et al., 2013). Nitrogen and P supply changed POD_Y through the variation of g_{max}, even though the level of O_3 exposure was the same. Such changes of g_{max} with nutritional conditions may be an important factor to be considered for explaining the variation in biomass reduction by elevated O_3.

The Y of POD_Y means an assumed threshold below which O_3 absorbed by the plant will be detoxified (Pleijel et al., 2007; Mills et al., 2011). Although the mapping manual suggested 1 nmol O_3 m^{-2} PLA s^{-1} for all forests (CLRTAP, 2017), there is no unified value of Y which is accepted widely by the scientific community (Assis et al., 2015). Karlsson et al. (2007) used 1.6 nmol O_3 m^{-2} PLA s^{-1} while other researchers recommended using 0–7 nmol O_3 m^{-2} PLA s^{-1} as flux-based threshold Y for forest tree species on the basis of different parameters, e.g. canopy moisture content (De Marco et al., 2015), biomass (Hu et al., 2015; Hoshika et al., 2018b) and visible foliar injury (Sicard et al., 2016b). With regard to forest protection, the productivity is the most important parameter. Our results suggest that 4 nmol O_3 m^{-2} PLA s^{-1} was an appropriate threshold for this sensitive poplar clone to detoxify the O_3-induced injury. The only exceptions were Y = 3 nmol O_3 m^{-2} PLA s^{-1} for high N (all N80 treatments together), suggesting that the higher the nutrient availability, the lower the detoxification threshold denoted by the value of Y. This result is in agreement with studies reporting a lower antioxidant capacity with nutrient-rich conditions (Kandlbiner et al., 2004; Desai et al., 2014). Our Y values were lower than a previous report for poplar clones in China (7 nmol O_3 m^{-2} PLA s^{-1} Hu et al., 2015), suggesting that our clone was more O_3 sensitive than those clones.

In previous studies, CLs of 4 and 8 mmol POD_1 m^{-2} were recommended for O_3 sensitive broad-leaved deciduous species (birch and beech) and for needle-leaved species (Norway spruce), respectively (Mills et al., 2011). In our experiment, a POD_1 value corresponding to 5% biomass reduction was 5.1 mmol POD_1 m^{-2} (Table S3). This indicates that the sensitivity of Oxford poplar clone to O_3 is similar to the sensitivity of beech and birch. Hu et al. (2015) found that CLs of POD_7 for 5% reduction in total biomass for poplar was 3.8 mmol m^{-2}. In the present study, a CL of 4.6 mmol POD_4 m^{-2} is recommended when all nutritional treatments are pooled together.

The N addition decreased the value of the CLs for O_3 risk assessment (on a POD_4 basis, 4.4 and 5.6 mmol m^{-2} in high N and low N, respectively). This indicates that biomass losses per unit of O_3 uptake were increased by the N treatment. Realistic N depositions may lower the O_3 tolerance of Oxford poplar clone.

For risk assessment, plant biomass should be estimated in a clean air as control, since biomass reductions are generally shown on a relative biomass basis (CLRTAP, 2017). In controlled chambers, we can set the control treatment without O_3 effects using charcoal filtered air. However, in O_3 FACEs, we do not have such a charcoal filtered air treatment. In the present study, we set 10 ppb of O_3 concentration (M24) to estimate the control biomass. Previously Pleijel et al. (2002) suggested that O_3 concentrations less than 20 ppb may not affect the yield of potato. The response of plants to O_3 in the low concentration range is still not clear (Sugai et al., 2018). Plants may show an hormetic response in the low concentration range (Sugai et al., 2018). Improvements of the response function in the low concentration range may be useful for

estimation of the control biomass. However, we should note that our approach obtained a CL that is similar to those obtained in open-top chambers, indicating that both approaches are valid and confirming that Oxford poplars can be classified as sensitive to O_3 (e.g., Marzuoli et al., 2009). Our results suggest that nutrient availability should be considered when assessing O_3 risk to vegetation. Our previous study on the O_3 risk assessment of oak species indicated that also soil water availability affects the assessment (Hoshika et al., 2018b). In the future risk assessment, we should consider multi-factorial experimental designs because they are more realistic than single O_3 treatment.

5. Conclusions

Poplars responded differently to O_3 under different nutrient availabilities, which suggests that nutrients can significantly affect O_3 risk assessment. Overall, the addition of N decreased the O_3 CLs i.e. increased the sensitivity of poplar to O_3. We postulate that N supply was of no advantage for counteracting O_3 stress. Phosphorus treatments alleviated the O_3-caused biomass loss and increased the CL. However, such mitigation effects of P were found only in the low N treatment and not in the high N conditions. Mechanistic investigations are needed to clarify the reasons of such nutritional impact on O_3 sensitivity in this poplar clone. POD_4 was the best metric to reconcile the effects of O_3 and plant nutrients on the biomass of well-irrigated poplar. The best flux based dose-response relationships were obtained for POD_4, rather than for POD_1 or POD_7 as recommended by CLRTAP (2017) for the protection of European forests or by Hu et al. (2015) for the protection of poplar in China, respectively. A CL of 4.6 mmol POD_4 m^{-2} is recommended for Oxford poplars when all N and P treatments were pooled together. Our results provide fundamental hints to inform the policies for protecting forests from O_3 pollution with changing a nutritional balance in soils due to an enhancement of N deposition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.03.102.

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