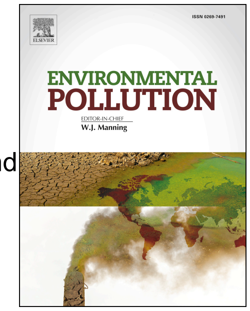


# Unraveling the difference of sensitivity to ozone between non-hybrid native poplar and hybrid poplar clones: a flux-based dose-response analysis

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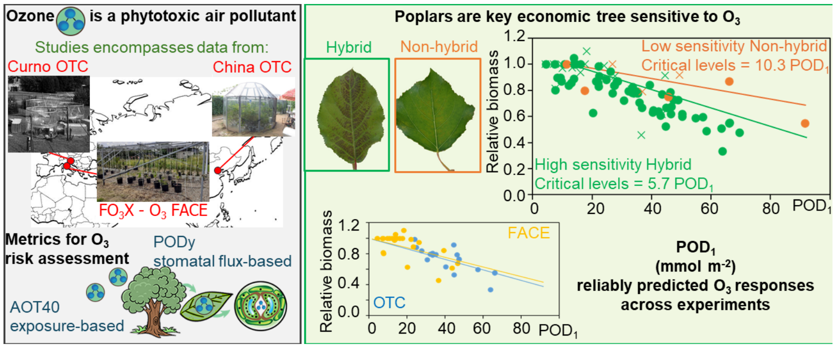
Ozone is a phytotoxic air pollutant
Studies encompasses data from:
Curno OTC
China OTC
FO3X - O3 FACE
Metrics for O3 risk assessment
PODy stomatal flux-based
AOT40 exposure-based
Poplars are key economic tree sensitive to O3
Hybrid
Non-hybrid
Low sensitivity Non-hybrid
Critical levels = 10.3 POD1
High sensitivity Hybrid
Critical levels = 5.7 POD1
Relative biomass
POD1
FACE
OTC
POD1 (mmol m-2) reliably predicted O3 responses across experiments

**Article type:** Original article

# Unraveling the difference of sensitivity to ozone between non-hybrid native poplar and hybrid poplar clones: a flux-based dose-response analysis

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**Abstract**

Poplars are economically important tree crops and biologically important model plants, which are known to be sensitive to ozone ($O_3$). Although surface $O_3$ is considered as a significant global environmental issue because of its phytotoxicity and greenhouse effect, the knowledge of the dose-response (DR) relationships in poplars for the assessment of $O_3$ risk is still limited. Hence, this study aimed at collecting data of studies with manipulative $O_3$ exposures of poplars within FACE (Free Air Concentration Enhancement) and OTC (Open-Top Chamber) facilities. The datasets contain studies on hybrid poplar clones and a non-hybrid native poplar (*Populus nigra* L.) reporting both AOT40 (Accumulated exposure Over a Threshold of 40 ppb) and POD1 (Phytotoxic Ozone Dose above a threshold of 1 nmol $m^{-2}$ Projected Leaf Area [PLA] $s^{-1}$) to compare exposure- and flux-based indices. As a result, linear regression analysis showed that the flux-based POD1 was better than the exposure-based AOT40 to explain the biomass response of poplars to $O_3$. From the DR relationships, a critical level (CL) of 5.7 mmol $m^{-2}$ POD1 has been derived corresponding to 4% biomass growth reduction for hybrid poplar clones, which can be considered very sensitive to $O_3$, while the non-hybrid native poplar was less sensitive to $O_3$ (CL: 10.3 mmol $m^{-2}$ POD1), although the potential risk of $O_3$ for this taxon is still high due to very high stomatal conductance. Moreover, the different experimental settings (OTC *vs.* FACE) have affected the AOT40-based DR relationships but not the POD1-based DR relationships, suggesting that poplar responses to $O_3$ were principally explained by stomatal $O_3$ uptake regardless of the different experimental settings and exposure patterns. These results highlight the importance of the flux-based approach, especially when scaling up from experimental datasets to the $O_3$ risk assessment for poplars at the regional or global scale.

Key words: Ozone risk assessment; Poplars; Dose-response relationships; Phytotoxic Ozone Dose; Ozone FACE

## 1. Introduction

Ground-surface ozone ($O_3$) is a harmful air pollutant for plants, resulting from chemical reactions between nitrogen oxides (NOx) and volatile organic compounds (VOCs) in the presence of sunlight (Agrawal et al., 2021). Tropospheric $O_3$ is a threat to terrestrial ecosystems and biodiversity (Agathokleous et al., 2020; Grulke and Heath, 2020; Cakaj et al., 2023), with increasing background levels even by night (Sicard et al., 2016, 2017; Agathokleous et al., 2023). The $O_3$ risk assessment for terrestrial ecosystems can be considered a pivotal concern for forthcoming policies regarding precursor emission management (Mills et al., 2018; De Marco et al., 2022). The AOT40 metric, which measures Accumulated Ozone Exposure above a Threshold of 40 ppb (Fuhrer et al., 1997), serves as the European legislative standard for plant protection (Directive 2008/50/EC: European Commission, 2008). However, these exposure-based metrics lack biological significance because they ignore the species-specific sensitivities to $O_3$, as well as the influence of environmental and physiological factors on $O_3$ uptake by the leaves (Paoletti and Manning, 2007; Agathokleous et al., 2022). Therefore, the scientific communities have currently emphasized the importance of the stomatal $O_3$ flux metrics, known as the cumulative $O_3$ uptake or Phytotoxic Ozone Dose above a threshold y nmol $m^{-2}$ $s^{-1}$ (PODy), for a quantitative risk assessment of adverse effects of $O_3$ on plants (Mills et al., 2018; Paoletti et al., 2022). The flux-based critical level (CL) for sensitive tree species such as *Fagus sylvatica* L. (European beech) and *Betula pendula* Roth (European silver birch) has been set at 5.2 mmol $m^{-2}$ POD1 (CLRTAP, 2017) for a 4% of growth rate reduction.

*Populus* species are extensively utilized for timber production and are considered one of the model plants for studying biology and physiology of woody plants (Christersson, 2010). They are widely distributed in the northern hemisphere, and their plantations offer an opportunity to build a green economy worldwide (Christersson, 2010). It is known that poplars are relatively sensitive to $O_3$, in terms of visible foliar injury, photosynthetic capacity, and biomass growth (Marzuoli et al., 2009; Pollastrini et al., 2010; Hoshika et al., 2018a). However, despite of its importance for wood, paper, and energy production, the knowledge of $O_3$ dose-response (DR) relationships for the risk assessment remains somewhat restricted both when investigating exposure-based and flux-based dose-responses (Zhang et al. 2018; Feng et al. 2019).

Poplar breeding started in the early 20th century, developing various hybrid poplar clones for the high timber productivity in plantations (du Cros, 1984). The deleterious effects of $O_3$ on plant physiology and growth (i.e. biomass production) have been therefore intensively studied in hybrid poplar clones, and in native poplars. A recent meta-analysis of $O_3$ effects on poplars reported that hybrid poplars may be more sensitive to $O_3$ than non-hybrid ones (Feng et al., 2019). Therefore, an important question raises: do the sensitivities to $O_3$ differ between native poplars and hybrid clones?

In addition, another important question is whether the difference in experimental systems may affect the DR relationships. To date, data from Open-Top Chamber (OTC) experiments have been frequently used to establish the DR relationships for numerous important tree species (e.g., Büker et al., 2015). However, a difference in meteorological factors (mainly temperature and wind speed) between chambers and natural conditions may affect stomatal conductance, which results in a change in plant response to $O_3$ relative to actual field conditions (Nussbaum and Fuhrer, 2000; Feng et al., 2018). On the other hand, similarly to free-air $CO_2$ enrichment, experiments by Free Air Concentration Enhancement or Free-Air Control Exposure (FACE) of $O_3$ can be considered the best approach to provide a realistic estimate of tree responses under real-world conditions (Paoletti et al., 2017; Montes et al., 2022). However, no comparative study has investigated the different methodologies (OTC vs FACE) for the flux-based DR relationships for $O_3$ impacts on plants.

The objective of this study was to re-analyzed available experimental datasets for the DR relationships to propose flux-based CLs for $O_3$ risk assessment in poplars. The major questions addressed here are the following: 1) Which metric is better to explain the DR relationship for the $O_3$ impacts on poplars, AOT40 or POD? 2) What are the CLs for the biomass development of poplars? 3) Is there any difference in the sensitivity to $O_3$ between hybrid poplars and European native ones? and 4) Is there any difference in the DR relationships derived from OTCs and FACE experiments? As a representative European native poplar, the present study focused on *Populus nigra* L., which is one of the most widely distributed poplars in the world, and in fact, 63% of the poplar cultivars used for plantations descend from this poplar species (Vanden Broeck, 2003).

## 2. Materials and Methods

### *2.1 Data collection*

To derive the DR relationships, data of manipulative $O_3$ exposure studies for poplars were summarized (Table 1). The data were selected based on the criterion that information of both AOT40 and POD1 was reported, to make the comparison between the flux-based and exposure-based indices possible. These data were derived from three experimental sites, including both a FACE facility and OTCs, and involved seven hybrid poplar clones and a non-hybrid European native poplar (*Populus nigra* L.). Cuttings with homogeneous sizes were used for the experiments. Total biomass was used as a response parameter for establishing the DR relationships, although the analysis included one study where above-ground biomass was utilized because root biomass data were not available (Pollastrini et al., 2010). In addition, several experiments contained water-stress treatments to verify if water deficit reduced $O_3$ damage due to limitation of stomatal $O_3$ uptake.

The first dataset was obtained from the $FO_3X$ (Free-air $O_3$ eXposure) experiment carried out at Sesto Fiorentino, Italy (43° 48' 59" N, 11° 12' 01" E). The $FO_3X$ applied three levels of $O_3$, i.e. ambient $O_3$ (AA), 1.5×AA, and 2.0×AA, with three replicated plots (size 5 × 5 × 2 m) for each $O_3$ treatment. Details on experimental set up of the $FO_3X$ system can be found in Paoletti et al. (2017). The mean hourly concentration of $O_3$ in AA was 35 to 40 ppb throughout the experimental years (Table S1, Appendix Method S1). The $FO_3X$ included published data for two hybrid poplar clones (Oxford: *Populus maximoviczii* Henry × *P. berolinensis* Dippel; I-214: *P. deltoides* W. Bartram ex Marshall × *P. nigra* L.) (Oxford clone in the year 2016: Zhang et al., 2018; Oxford and I-214 clones in the year 2020: Pisuttu et al., 2024). In addition, to strengthen the datasets, additional biomass data for Oxford clone and one European native non-hybrid poplar (*P. nigra* L.) were also used. The detailed description of the additional datasets was shown in Appendix Method S2.

The other two sites were equipped with OTC facilities to expose poplar plants to $O_3$. The Curno site is located in northern Italy, near the Po Valley (45° 70' N, 9° 62' E). This OTC experiment also included the same two poplar species, i.e. Oxford poplar clone and *P. nigra*, used in the FO$_3$X experiments (Pollastrini et al., 2010, 2013, 2014). The other $O_3$ experimental site included in this study is Changping site, located in the northern part of China (40° 19' N, 116° 13' E). Here experiments with five hybrid poplar clones were carried out with the aim of examining their biomass response to $O_3$ as well as the response to $O_3$ and water stress (Hu et al., 2015; Gao et al., 2017). Control treatments in the OTC experiments at Curno and Changping consisted of charcoal-filtered (CF) air chambers, while the $O_3$ treatments were non-filtered (NF) air or non-filtered air with additional $O_3$ (NF+).



### *2.2 Calculation of ozone indices*

In each experiment, the estimation of AOT40 (Accumulated exposure Over a Threshold of 40 ppb of $O_3$) was made according to the methodology described in CLRTAP (2017). It is given by:

$$AOT40(ppm \cdot h) = \sum_{i=1}^{n} \max([O_3]i - 40,0)/1000 \cdot \Delta t, \text{ when solar radiation} > 50 \text{ W m}^{-2} \quad (1)$$

where $[O_3]$I is the i$^{th}$ mean hourly $O_3$ concentration (ppb), 1000 is a conversion factor from ppb to ppm, and $\Delta t$ is the time step (1 h).

A promising index based on stomatal $O_3$ flux, POD1 has been calculated, i.e., Phytotoxic $O_3$ Dose above a threshold y = 1 nmol $m^{-2}$ Projected Leaf Area [PLA] $s^{-1}$ ($POD_y$), as recommended for a woody plant species by CLRTAP (2017). POD1 (mmol $m^{-2}$) was calculated by using hourly $O_3$ concentrations and meteorological data as follows:

$$\mathrm{POD1} = \sum_{i=1}^{n} \left(\mathrm{F_{st,i}} - 1\right) \cdot 1000 \cdot \Delta t \quad (2)$$

where Δt is the averaging period (= 1 h), $F_{st,I}$ is the $i^{th}$ hourly stomatal $O_3$ uptake (nmol $m^{-2}$ $s^{-1}$), 1000 is a conversion factor from nmol to mmol, and n is the number of hours considered for the calculation. According to the mapping manual (CLRTAP, 2017), the standard method was applied for estimating $F_{st}$:

$$F_{st} = [O_3] \cdot g_{sO3} \cdot \frac{r_c}{r_{bO3} + r_c} \quad (3)$$

where $g_{sO3}$ represents the stomatal conductance for $O_3$, $r_c$ denotes the leaf surface resistance [$r_c = 1/(g_{sO3}+g_{ext})$, where $g_{ext}$ is the plant cuticular conductance for $O_3$ (= 0.0004 m $s^{-1}$: CLRTAP, 2017)], and $r_{bO3}$ indicates the leaf boundary layer resistance for $O_3$ (s $m^{-1}$). The detailed calculation of $r_{bO3}$ was shown in Appendix Method S3.

Stomatal conductance for $O_3$ was estimated by the standard multiplicative method developed by CLRTAP (2017):

$$g_{sO3} = g_{max} \cdot f_{phen} \cdot f_{PPFD} \cdot max(f_{min}, \quad f_{temp} \cdot f_{VPD} \cdot f_{SWC}) \quad (4)$$

where $g_{max}$ is the maximum stomatal conductance to $O_3$ based on the project leaf area (PLA, mmol $O_3$ $m^{-2}$ PLA $s^{-1}$), and the other functions are relative terms (0 to 1): $f_{min}$ is the minimum relative conductance, $f_{phen}$ denotes the phenological dependency of stomatal conductance, $f_{PPFD}$,

$f_{temp}$, $f_{VPD}$, $f_{SWC}$ indicate the stomatal response functions to photosynthetic photon flux density (PPFD), air temperature (T), vapor pressure deficit (VPD) and soil water content (SWC), respectively. The specific details of each function were described in CLRTAP (2017) and Manzini et al. (2023).

Stomatal conductance model parameters are species-specific (Emberson et al., 2000). The parametrization is already available for Oxford poplar clone (Curno OTC: Marzuoli et al., 2009, $FO_3X$: Hoshika et al., 2018a) and hybrid poplar clones in China (Hu et al., 2015; Gao et al., 2017). For the I-214 poplar clone and *P. nigra*, in the $FO_3X$ experiment, the model was parameterized by stomatal conductance measurements under various meteorological conditions using an open flow-through differential porometer (Li600, Lincoln, NE, USA). Measurements were conducted at least once a month (12 days in I-214 and 28 days in *P. nigra*) throughout the experiment. All measured data (I-214: 216 data, *P. nigra*: 1100 data) were utilized for the model parameterization according to the boundary line approach (Braun et al., 2010; Hoshika et al., 2012). The $g_{max}$ and $f_{min}$ were set to the 95th and 5th percentiles of all stomatal conductance data, respectively, following the approach described in Bičárová et al. (2019) and Hoshika et al. (2020).

### *2.3 Dose-response relationship and derivation of critical levels*

The exposure- and flux-based DR relationships were fitted by linear regressions of poplar biomass against AOT40 or POD1. In OTC experiments, the reference biomass values for calculating the relative effect of $O_3$ were taken from those in CF treatments as “control”. The biomass values for each $O_3$ treatment at the end of the treatments were then divided by the reference biomass to calculate the relative biomass (RB). On the other hand, since the $O_3$ FACE experiments lack CF treatment for determining a reference biomass value, the extrapolation approach was applied according to Paoletti et al. (2017), assuming the biomass loss relative to a pre-industrial $O_3$ level according to a 24-h mean $O_3$ concentration (M24 = 10 ppb). Since one multiyear experiment was also included, the formula suggested by Büker et al. (2015) was utilized to ensure consistency in analyzing biomass response data from experiments with various duration over time:

$$RB_{annual} = 100 \cdot (B_{treat}/B_{control})^{\left(\frac{1}{n}\right)} \quad (5)$$

where $RB_{annual}$ is the annual value of relative biomass compared to the control, $B_{treat}$ is the biomass levels under elevated $O_3$ exposures, and $B_{control}$ is the reference biomass at control across multiple growing seasons (n). This equation allows for a consistent $O_3$-induced percentage reduction in biomass accumulation throughout multi-year experiments. These $RB_{annual}$ values were thus analyzed against the $O_3$ metrics, and then, the CLs were calculated as POD1 and AOT40 values corresponding to a 4% reduction in biomass as indicated by CLRTAP (2017).

When the regressions were significant, statistical comparisons were conducted by applying analysis of covariance (ANCOVA) to explore differences in biomass response to $O_3$ between hybrid poplar clones and the non-hybrid European native poplar (*P. nigra*), as well as between the experimental settings (OTC vs FACE). Results were considered significant at $p \leq 0.05$. All analyses were performed using R software version 4.3.1 (R Core Team, 2023).

# 3. Results

## *3.1 Parameters of stomatal conductance model for poplars*

Stomatal conductance parameters for hybrid poplar clones taken from literature and new parameters for I-214 and *P. nigra* are listed in Table S2. The values of $g_{max}$ ranged from 240 to 575 mmol $O_3$ $m^{-2}$ PLA $s^{-1}$, while the $f_{min}$ values (fraction) were in between 0.01 and 0.10 depending on species and clones. The limiting function of PPFD to stomatal conductance ($f_{PPFD}$) indicated an exponential increase of $g_{sO3}$ when increasing light intensity with a lightsaturating point of 500 to 900 µmol $m^{-2}$ $s^{-1}$ of PPFD. The optimal temperature for stomatal opening ranged from 25 to 30 °C, although some hybrid clones had a higher $T_{opt}$ (32 to 33 °C for clone 84K and 156 at the OTC facility in China). A high VPD (> 2 to 3 kPa) caused stomatal closure regardless of the species. The $f_{phen}$ values indicated a peak of $g_{sO3}$ in mid-summer (June to August) in all poplar species. The limiting function of SWC ($f_{SWC}$) indicated that $g_{sO3}$ for poplars started to decrease when SWC reached 70 to 80% of field capacity during the water limitation conditions.

***3.2 Dose response relationships for poplars and critical levels***

Fig. 1 shows the biomass response to AOT40 and POD1 for both hybrid poplar clones and the non-hybrid native poplar (*P. nigra*). Although both regressions against the exposure- and flux-based indices had a relatively high determination coefficient ($R^2$) for the hybrid poplar clones, the AOT40-based DR relationship was not statistically significant for the non-hybrid native poplar. As a result, POD1 was better than AOT40 to explain the DR relationship for poplars (hybrid: $R^2$ = 0.59 *vs* 0.71 for AOT40 and POD1, respectively; non-hybrid native: $R^2$ = 0.17 *vs* 0.50 for AOT40 and POD1, respectively). In fact, the reduced water treatment decreased stomatal $O_3$ uptake when AOT40 was still high (Fig. 2). Subsequently, $O_3$-induced negative effect on biomass was limited under the water stress conditions.

Also, Fig. 1 revealed a possible different sensitivity to $O_3$ between hybrid poplars and the non-hybrid native European poplar (*P. nigra*). A statistical difference in the regression lines between hybrid and non-hybrid native poplars was observed using the flux-based index, POD. Based on the regression line, the AOT40-based CL for a 4% reduction in biomass was estimated to be 7.8 ppm·h AOT40 for hybrid poplar clones, whereas the flux-based CLs were 5.7 and 10.3 mmol $m^{-2}$ POD1 for hybrid and non-hybrid native poplars, respectively.

***3.3 Comparison of dose response (DR) relationships between the experimental settings***

The Oxford poplar clone was focused on because this species was studied in OTC and FACE conditions with sufficient data (Table 1). A comparison of DR relationships between OTC and FACE for the Oxford clone is shown in Fig. 3. When using the AOT40, an ANCOVA test confirmed that the regressions were significantly different between OTCs and FACE, implying that $O_3$ sensitivity may be higher in OTC than in $O_3$-FACE experiments. However, no statistical difference in the regression lines between the experimental settings was observed when using the flux-based index POD. This indicates that the response of poplar biomass to stomatal $O_3$ uptake was not different between the two experimental settings.

## 4. Discussion

### *4.1 Dose-response relationships in poplars POD vs AOT40*

Exposure metrics such as AOT40 are still the commonly used indicators for the $O_3$ risk assessment because they require just the hourly $O_3$ concentrations (Ferretti et al., 2018; Lefohn et al., 2018). Model simulations reported that the AOT40 values for forest trees in polluted areas may reach 30 to 50 ppm·h in Europe and North America and 50 to 90 ppm·h in Asia (Anav et al., 2022), which may correspond to 15-26% of the biomass decline in Europe and North America and 26-46% of the biomass decline in Asia according to the AOT40-based DR relationships for the hybrid poplar clones in the present study. However, it is acknowledged that the effects of $O_3$ on plants depend on the $O_3$ dose absorbed through stomata rather than $O_3$ exposure only (Grulke and Heath, 2020). In the present study, the flux-based index, POD1, was better than AOT40 in explaining the DR relationships for both hybrid poplar clones and non-hybrid native poplar. In fact, the flux-based approach significantly improved the correlations with biomass growth reductions compared to AOT40, especially when water stress treatments were included (Alonso et al., 2014; Gao et al., 2017; Hoshika et al., 2018b). A protective function of water deficit against $O_3$ stress has been proposed by reducing $O_3$ uptake caused by stomatal closure (Tingey and Hogsett, 1985; Gao et al., 2017). Anav et al. (2022) suggested that a regional distribution of POD was not directly related to $O_3$ concentrations due to the influence of microclimatic conditions on stomatal conductance, such as during summer drought. In addition, the $f_{SWC}$ functions indicate that poplars have a relatively sensitive response of $g_s$ to SWC compared to the other tree species (Büker et al., 2012). Therefore, the POD should be recommended for a proper risk assessment of $O_3$ for poplar plantations, especially in regions where hot and dry summers are often experienced, such as in the Mediterranean Europe.

### *4.2 Comparison of the sensitivity to ozone between hybrid poplars and a non-hybrid native one*

The present study demonstrates that hybrid poplar clones are sensitive to $O_3$, showing a flux-based CL (5.7 mmol $m^{-2}$ POD1) similar to the other representative sensitive tree species such as beech and birch (5.2 mmol $m^{-2}$ POD1, CLRTAP, 2017). In addition, according to the DR relationships, hybrid poplar clones were more sensitive to $O_3$ than non-hybrid native poplar, consistent with a meta-analytic review by Feng et al. (2019). Similar findings were reported for agricultural crops, in which recent cultivars with high yield are more sensitive to $O_3$ (Biswas et al., 2008; Osborne et al., 2016), suggesting that the higher sensitivity in recently developed cultivars of agricultural crops may have resulted from high stomatal conductance and thus high stomatal $O_3$ uptake. On the other hand, this was not the case for poplars where the values of $g_{max}$ in hybrid clones did not differ from that in the native poplar. However, hybrid poplar clones tend to allocate more photosynthates to leaves to maximize $CO_2$ uptake, resulting in a higher $O_3$ uptake by the whole plant due to their larger foliar area (Feng et al., 2019).

The $g_{max}$ is one of the most important parameters to determine species-specific stomatal $O_3$ uptake (Tuovinen et al., 2007). Poplars are known to be high water-demanding species as their natural habitats are often established along the riverside (Christersson, 2010). According to the data collection, the values of $g_{max}$ varied between 240 and 575 mmol $O_3$ $m^{-2}$ PLA $s^{-1}$, which was much higher than those reported in other tree species, e.g., 125 and 155 mmol $O_3$ $m^{-2}$ PLA

$s^{-1}$ for Norway spruce and beech, respectively (CLRTAP, 2017). Although the present study suggests that a non-hybrid native poplar was less sensitive to $O_3$ based on the flux-based CL, the potential risk of negative impacts for this species should still be high considering its very high stomatal conductance ($g_{max}$ = 435 mmol $O_3$ $m^{-2}$ PLA $s^{-1}$).

It is known that there is also a variation in the sensitivity to $O_3$ among the various hybrid poplar clones (Hu et al., 2015; Pisuttu et al., 2024). For example, at the $FO_3X$ experiments, I-214 clone did not show a significant decline in biomass growth under moderately elevated $O_3$ exposure (1.5×AA), while two elevated $O_3$ treatments (1.5×AA and 2.0×AA) reduced the biomass in Oxford poplar clone, suggesting that I-214 was relatively less sensitive to $O_3$ compared to Oxford clone (Pisuttu et al., 2024). The I-214 clone exhibited reduced damage by oxidative stress due to biochemical characteristics (Pisuttu et al., 2024). Nowadays, selective breeding is moving toward a sustainable cultivation in recognition of climate change, by developing varieties that are better adapted to changing environmental conditions (Chaudhary and Agrawal, 2015; Niemczyk et al., 2019). Since the number of studies is still insufficient, future studies will also be needed to compare the flux-based DR relationships among hybrid clones.

### *4.3 Comparison of the dose-response relationships between OTC and FACE experiments*

There have been discussions about whether the experimental settings may affect the plant response to $O_3$ for decades since chamber methods (e.g., controlled closed chamber, OTCs) can change the microclimate, such as temperature and wind speed, modifying quantitatively the impacts of $O_3$ exposure on plants (Nussbaum and Fuhrer, 2000; Kobayashi, 2022). Although Feng et al. (2018) suggested that the response of crop yield to AOT40 was different between OTC and FACE experiments, there was no comparison in the DR relationships between the experimental settings based on the flux-based approach. According to the present result, while the AOT40-based DR relationships in Oxford poplars differed between OTCs and

FACE, such a difference was not observed in the POD1-based DR relationships. This result indicates that, although the sensitivity of poplar to $O_3$ was apparently higher in OTC than in FACE experiments when using AOT40, poplar responses to $O_3$ was principally explained by stomatal $O_3$ uptake regardless of the different experimental settings.

In fact, when using the AOT40, the regression line showed a higher slope in OTCs at the Curno site than at the $FO_3X$, suggesting a higher stomatal $O_3$ uptake per unit $O_3$ exposure in the OTC. This may be explained by the higher $g_{max}$ for Oxford clone in OTCs at the Curno site than at the $FO_3X$ experiments, probably because the Curno site is located in the northern part of Italy at the foothill of the Alps, with a relatively cool climate, which is favorable for the growth of Oxford poplar clone (Elwes and Henry, 2014). In addition, the microclimate condition inside chambers modifies stomatal $O_3$ uptake. An enclosure system such as OTC tends to increase the sensible heat of the internal air, and thus an increase in the temperature is often found inside the chamber during the daytime (Piikki et al., 2008; Hollister et al., 2023). Piikki et al. (2008) suggested that the eventual elevated temperature within OTCs might lead to a high VPD, which possibly causes a reduced $g_{sO3}$ and, thus, potentially results in an underestimation of $O_3$ impacts. However, this hypothesis was rejected in this study because VPD reached a maximum of just 3.5 kPa during the experiment at Curno due to the relatively cool environment (Marzuoli et al., 2009). Also, in the OTCs, another important environmental artifact can be conceived. To facilitate the gas mixing, there is a continuous airflow into the OTCs, which reduces a thickness of the leaf boundary layer (Jetten, 1992; Uddling et al., 2004) reducing $r_{bO3}$, promoting stomatal $O_3$ uptake. On the other hand, in actual field conditions, the wind is not constantly blowing, and sometimes there is a windless condition, such as during morning and evening when wind is relatively mild. Given the repair and detoxification process involved, it cannot be ruled out that a continuous $O_3$ entry into leaves under the OTC conditions may have intensified the harmful effect of $O_3$ on plants (Moura et al., 2023). Nevertheless, according to a proper species-specific parametrization achieved here, the results proved a tight relationship between $O_3$ damage and POD, suggesting that further emphasis must be placed on the flux-based concept for the $O_3$ risk assessment on poplars.

## 5. Conclusions

According to the data collection of manipulative $O_3$ exposure experiments, flux-based DR relationships were established for poplars. The flux-based POD1 was better than the traditional exposure-based AOT40 to explain the biomass response of poplars to $O_3$. The new DR relationships also revealed that hybrid poplar clones are more sensitive to $O_3$ compared to a non-hybrid native one, although the potential risk of $O_3$ is still high for the native poplar due to its high stomatal conductance. Moreover, the different experimental settings (OTC *vs.* FACE) affected the AOT40-based DR relationships but did not affect the POD1-based DR relationships, confirming the importance of the environmental and plant parameters incorporated in the calculation of stomatal $O_3$ uptake. Further studies will also be needed for the parametrization of $O_3$ uptake modeling for other poplar species to extend the knowledge to characterize $O_3$ risks for poplar species.

**Author contributions**

Conceptualization, Y.H., R.M., V.C. and P.S.; Data curation, Y.H., M.P., R.M., G.G., E.M., E.A., V.C. and Z.F.; Formal analysis, Y.H., M.P., R.M. and B.B.M.; Funding acquisition, Y.H., E.A., P.S. and E.P.; Investigation, Y.H., M.P., R.M. and B.B.M.; Writing-original draft, Y.H.; Writing-review & editing, M.P., R.M., G.G., B.B.M., E.M., E.A., V.C., Z.F., P.S. and E.P.

**Declaration of competing interest**

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data sharing is not applicable to this article as all new created data is already contained within this article.

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**Appendix A.** Supplementary data

Additional Supporting Information to this article can be found in the online version of this article.

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**Figure legends**

**Fig. 1.** Dose-response (DR) relationships for hybrid poplar clones and a non-hybrid native poplar (*P. nigra*) based on AOT40 (upper panel) and POD1 (lower panel). Linear regression analyses were made. When the regression lines were significant, statistical comparisons were conducted by analysis of covariance (ANCOVA). ns denotes not statistically significant.

**Fig. 2.** Scatter diagrams between ozone indices and relative biomass with different water regimes (well-watered vs. reduced-watered) for poplars on the basis of seasonal AOT40 (upper panel) and POD1 (lower panel). The 95% confidence ellipses for the data points were shown in two water regimes (black line: well-watered, dotted line: reduced-watered).

**Fig. 3.** Comparisons of the dose-response (DR) relationships between the experimental settings (OTC vs. FACE) for Oxford poplar clone on the basis of seasonal AOT40 (upper panel) and POD1 (lower panel). Linear regression analyses were made. When the regression lines were significant, statistical comparisons were conducted by analysis of covariance (ANCOVA). ns denotes not statistically significant.

**Table 1.** Details of the $O_3$ exposure studies carried out in Italy and China, between 2005 and 2023. FACE: Free Air Concentration Enhancement; OTC: Open Top Chamber; AA: ambient air; 1.5×AA: 1.5 times ambient $O_3$ treatment; 2.0×AA: twice ambient $O_3$ treatment; CF: charcoal-filtered air; NF: non-filtered air; NF+: non-filtered plus addition of $O_3$. (values after "+" are $O_3$ concentrations in ppb); TB: Total biomass; AB: Above-ground biomass.

| Site | Exposure system | Species/ Clone | Ozone treatments | Experimental year | Exposure duration (yrs) | Water stress treatment | Biomass parameter | Reference |
|---|---|---|---|---|---|---|---|---|
| Sesto Fiorentino (Italy) | FACE | Oxford clone | AA, 1.5×AA, 2.0×AA | 2016, 2017, 2020, 2021 2022, 2023 | 1 (2 yrs in 2017) | yes (in 2021) | TB | Zhang et al. (2018), This study |
| | | I-214 clone | AA, 1.5×AA, 2.0×AA | 2020 | 1 | no | TB | Pisuttu et al. (2024), |
| | | *P. nigra* L. | AA, 1.5×AA, 2.0×AA | 2021, 2022 | 1 | yes (in 2021) | TB | This study |
| Curno (Italy) | OTC | Oxford clone | CF, NF | 2005, 2008, 2009 | 1 | yes | AB (2005) TB (2008, 2009) | Pollastrini et al. (2010, 2013, 2014) |
| | | *P. nigra* L. | CF, NF | 2009 | 1 | no | TB | Pollastrini et al. (2014) |
| Changping (China) | OTC | Five hybrid clones (84K, 107 90, 546, 156) | CF, NF, NF+20, NF+40, NF+60, NF+80 | 2014 | 1 | no | TB | Hu et al. (2015) |
| | | Clone 546 | CF, NF, NF+40 | 2015 | 1 | yes | TB | Gao et al. (2017) |

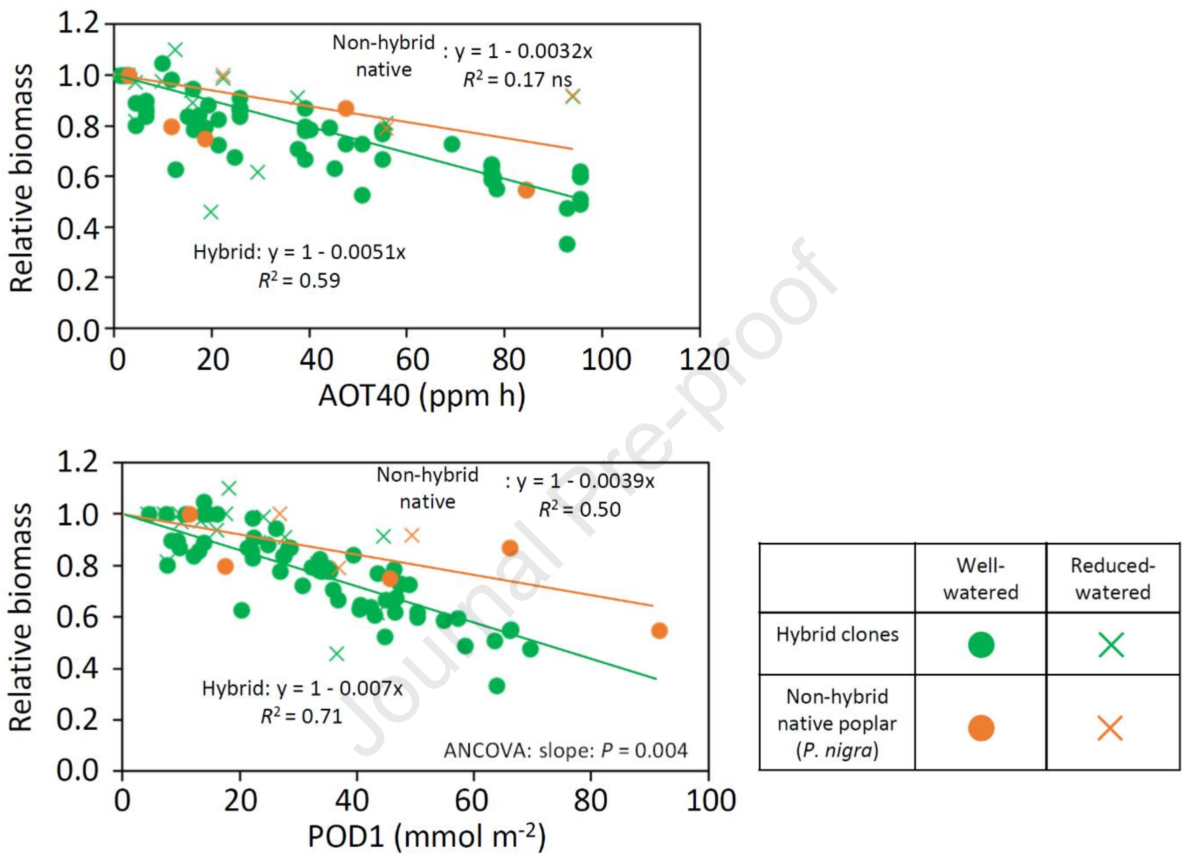


**Fig. 1.** Dose-response (DR) relationships for hybrid poplar clones and a non-hybrid native poplar (*P. nigra*) based on AOT40 (upper panel) and POD1 (lower panel). Linear regression analyses were made. When the regression lines were significant, statistical comparisons were conducted by analysis of covariance (ANCOVA). ns denotes not statistically significant.

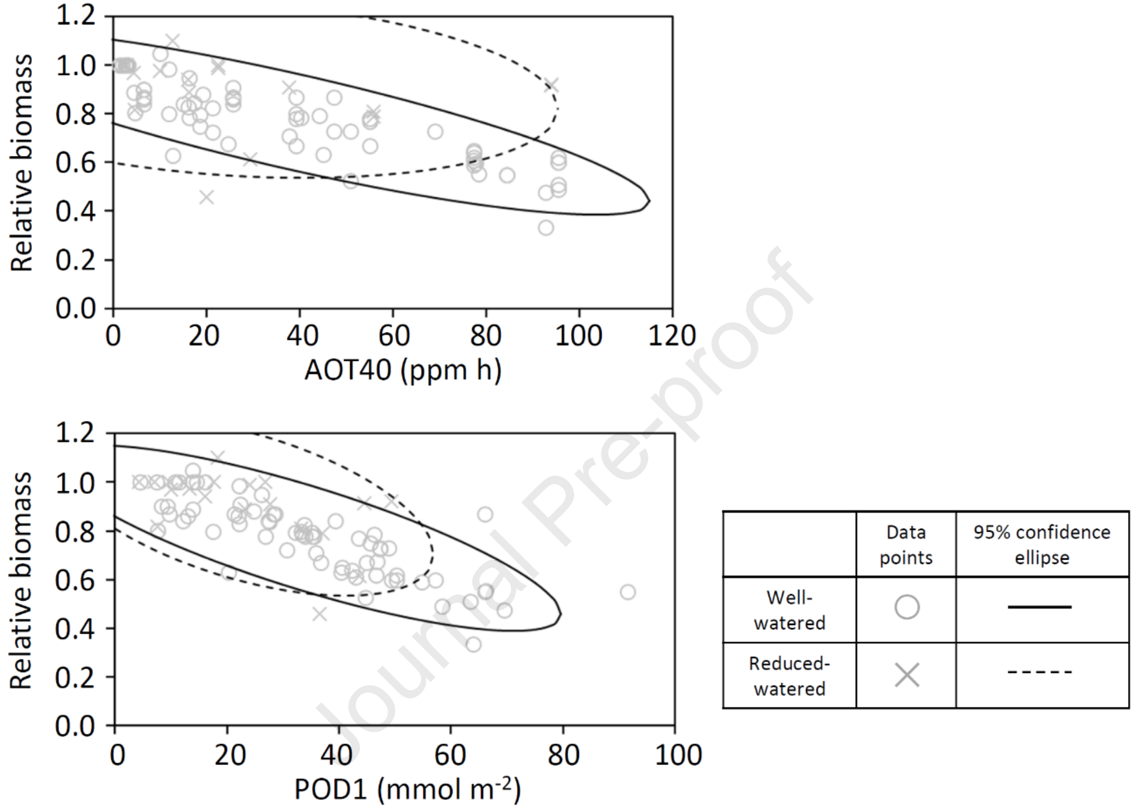




**Fig. 2.** Scatter diagrams between ozone indices and relative biomass with different water regimes (well-watered vs. reduced-watered) for poplars on the basis of seasonal AOT40 (upper panel) and POD1 (lower panel). The 95% confidence ellipses for the data points were shown in two water regimes (black line: well-watered, dotted line: reduced-watered).

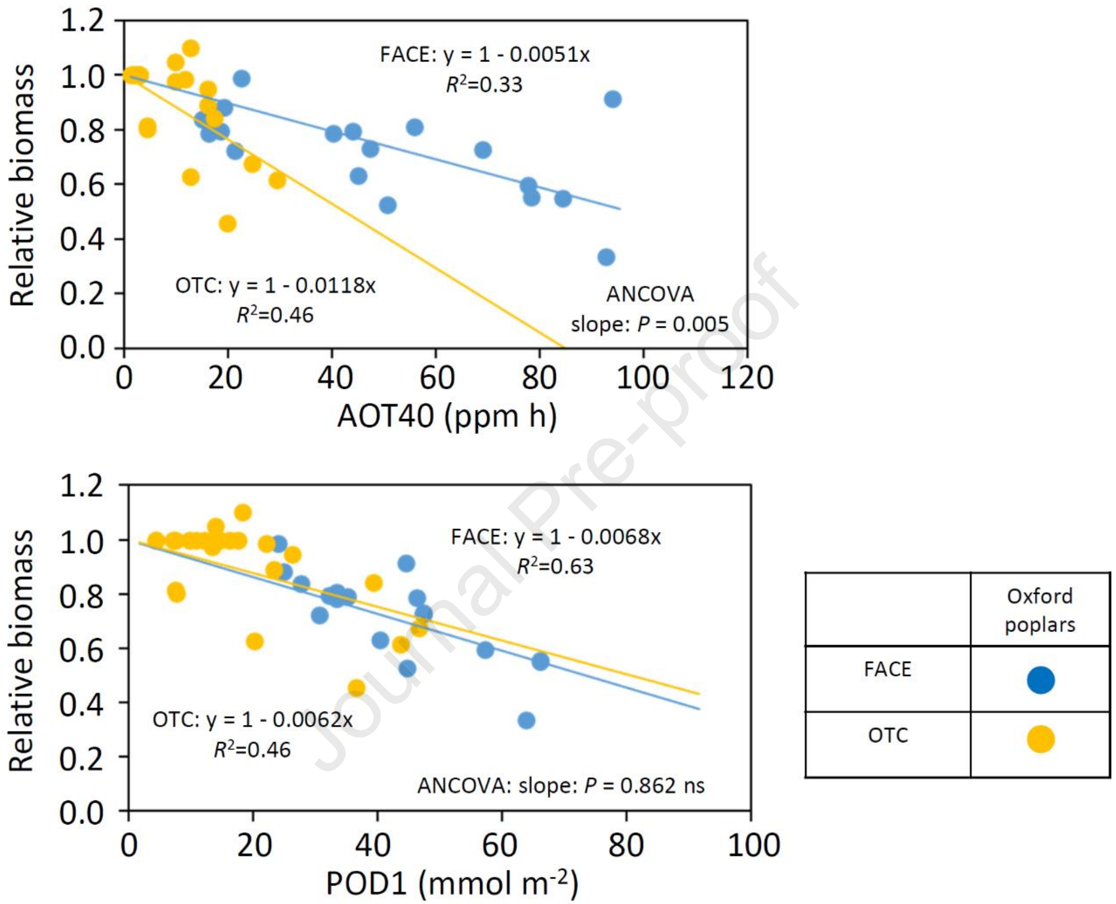




**Fig. 3.** Comparisons of the dose-response (DR) relationships between the experimental settings (OTC vs. FACE) for Oxford poplar clone on the basis of seasonal AOT40 (upper panel) and POD1 (lower panel). Linear regression analyses were made. When the regression lines were significant, statistical comparisons were conducted by analysis of covariance (ANCOVA). ns denotes not statistically significant.

**Highlights**

1. Ozone dose-response function was derived for poplars by data collection.
2. The biomass response to ozone was well explained by the stomatal flux-based index.
3. A non-hybrid native poplar was less sensitive to ozone compared to hybrid clones.
4. Critical levels were 5.7 (hybrid clones) and 10.3 mmol $m^{-2}$ POD1 (native poplar).
5. Different experimental setups didn't affect the flux-based dose-response function.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: