Water stress mitigates the negative effects of ozone on photosynthesis and biomass in poplar plants[☆]

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ABSTRACT

Tropospheric ozone (O₃) pollution frequently overlaps with drought episodes but the combined effects are not yet understood. We investigated the physiological and biomass responses of an O₃ sensitive hybrid poplar clone ('546') under three O₃ levels (charcoal-filtered ambient air, non-filtered ambient air (NF), and NF plus 40 ppb) and two watering regimes (well-watered (WW) and reduced watering (RW), i.e. 40% irrigation) for one growing season. Water stress increased chlorophyll and carotenoid contents, protecting leaves from pigment degradation by O₃. Impairment of photosynthesis by O₃ was also reduced by stomatal closure due to water stress, which preserved light-saturated CO₂ assimilation rate, and the maximum carboxylation efficiency. Water stress increased water use efficiency of the leaves while O₃ decreased it, showing significant interactions. Effects were more evident in older leaves than in younger leaves. Water stress reduced biomass production, but the negative effects of O₃ were less in RW than in WW for total biomass per plant. A stomatal O₃ flux-based dose-response relationship was parameterized considering water stress effects, which explained biomass losses much better than a concentration-based approach. The O₃ critical level of Phytotoxic Ozone Dose over a threshold of 7 nmol O₃.m⁻².s⁻¹ (POD₇) for a 4% biomass loss in this poplar clone under different water regimes was 4.1 mmol m⁻². Our results suggest that current O₃ levels in most parts of China threaten poplar growth and that interaction with water availability is a key factor for O₃ risk assessment.

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1. Introduction

Tropospheric ozone (O₃) is a phytotoxic air pollutant causing negative effects on crops and forests (Avnery et al., 2011; Felzer et al., 2005; Feng et al., 2014, 2015). In China, regional O₃ pollution is assumed of great environmental concern as hourly maximum O₃ concentration may reach values as high as 164–316 ppb (Feng et al., 2015). Furthermore, tropospheric O₃ levels in the summer months are expected to increase by 1–10 ppb over the coming decades in polluted regions of the world (Jacob and

Winner, 2009), while background O₃ levels are estimated to rise up to 80 ppb in 2100 (IPCC, 2013) with peak episodes that will occasionally exceed 200 ppb (Richet et al., 2012). Elevated O₃ levels have detrimental effects on vegetation (The Royal Society, 2008; Wittig et al., 2009), lowering plant resilience, competitiveness and carbon sequestration, and inducing yield and biomass losses (Broadmeadow, 1998; Edwards and Zak, 2011; Karnosky et al., 2005).

During the present and last century, many parts of the world (Dai, 2011; Mishra and Singh, 2010) including China (Qiu et al., 2013; Xiao et al., 2009) have experienced widespread drought episodes that significantly affected global terrestrial net primary production (NPP) (Zhao and Running, 2010) and ecosystem carbon exchange processes (Krishnan et al., 2006; Rambal et al., 2003). Furthermore, climate change is raising temperature and increasing frequency and intensity of drought in many regions, particularly

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during the summer and normally drier months (IPCC, 2013). In many parts of the world, high O₃ levels are concurrent with water stress periods, as it is the case of the Mediterranean area in summer (Alonso et al., 2014; Grulke et al., 2002), southern Appalachian forest (USA) at mid-July (McLaughlin et al., 2007), or Central Europe in the exceptionally dry year 2003 (Matyssek et al., 2006). As these episodes are expected to be exacerbated in the future (IPCC, 2013; Katul et al., 2012; Matyssek et al., 2014), the interactive effect of O₃ and water stress on trees is a matter of current and future concern (Matyssek et al., 2006; Nikolova et al., 2010).

Co-occurrence of two or more stresses may result in additive effects, but frequently plants respond in a non-additive manner, producing effects that could not have been predicted from the study of either stress individually (Atkinson and Urwin, 2012). In the case of O₃ and water stress, stomata play an important role, as O₃ enters the plant through the stomata, while water vapour escapes through them, and it has been proposed that water stress may exert a protective effect against O₃ (Bohler et al., 2013). So far, several studies have been carried out addressing the effects of O₃ in interaction with water stress on trees. However, contrasting responses have been found. For deciduous species, several studies on Japanese beech (*Fagus crenata* Blume), European beech (*F. sylvatica* L.) and pedunculated oak (*Quercus robur* L.), did not show any significant interaction in terms of photosynthesis, growth or biomass (Kuehn et al., 2015; Löw et al., 2006; Pollastrini et al., 2010; Yonekura et al., 2004). Interactive effects on transpiration and stomatal conductance were observed by Shimizu and Feng (2007) on Erman's birch (*Betula ermanii* Cham.), but not on biomass. On the contrary, interactive effects on pigment contents and also on biomass were found in a maple (*Acer truncatum* Bunge) by Li et al. (2015). Results from birch also revealed that experimental conditions may have an effect on the observed responses, as drought protected plants from O₃ injury in a chamber experiment, while an increase of O₃ injury was observed under milder drought conditions in an open-field experiment (Pääkkönen et al., 1998a,b). A comprehensive understanding of the combined processes is still limited, given the variability of responses between species and even clones. Also foliar responses may differ depending on the target leaf position and growth stage, further increasing the heterogeneity of the results, e.g. in terms of activity of antioxidant enzymes, photosynthetic capacity, chlorophyll *a* fluorescence, cell structure, leaf gas exchange and stable isotope ratios (Alonso et al., 2001; Beyers et al., 1992; Desotgiu et al., 2012; Kivimäenpää et al., 2003; Löw et al., 2006; Pollastrini et al., 2014). Although the impacts of current and future tropospheric O₃ on tree biomass, growth, physiology and chemistry were quantified by a meta-analysis, Wittig et al. (2009) failed to provide conclusive insights into the interaction of drought and O₃, as well as its magnitude or significance for current O₃ levels, due to gaps in these types of studies. For these reasons, it is important to test the interactive responses of O₃ and water stress in a large number of species and clones under the current and future climatic conditions of the territory where they grow.

Given their sensitivity to O₃ (Bortier et al., 2000; Hu et al., 2015; Novak et al., 2005) and water stress (Monclus et al., 2006; Zhang et al., 2004), poplar species are of particular interest for studies on interactions between these two factors. However, available information on O₃ response relationships for poplar from experiments considering both factors is still limited (Desotgiu et al., 2013; Pollastrini et al., 2014), and in none of them the stomatal O₃ flux approach has been applied so far. While response relationships have been derived in the past using exposure-based metrics (e.g. AOT40, Fuhrer et al., 1997; Holland et al., 2002), a new generation of response relationships are being currently developed using the stomatal O₃ flux approach (Büker et al., 2015; Hu et al., 2015;

Karlsson et al., 2007) which relies on the O₃ uptake by the plant rather than on the O₃ concentrations in the air (CLRTAP, 2015).

The first objective of the present paper is to assess the physiological and biomass responses to O₃ in combination with water stress at leaf and plant level in a poplar clone widely cultivated in China. We postulate a protective effect of water stress against O₃ and that the interactive effects of both factors may depend on leaf age. The second objective is to compare exposure-based and flux-based metrics for establishing O₃ critical levels for sensitive species in Asia taking into account water stress interactions with O₃. We postulate that a flux-based risk assessment would better account for the effects of water stress on O₃-induced biomass losses than an exposure-based approach.

2. Materials and methods

2.1. Experimental site and plant material

The experiment was conducted in open-top chambers (OTCs) assembled at the Seed Station Field of Changping, Northwest Beijing (40°19'N, 116°130'E), China. The region has a semi-humid continental climate, with an annual mean temperature of 11.8 °C, and a total annual precipitation of 550 mm measured in 2015 (Hu et al., 2015). Rooted cuttings of the O₃-sensitive hybrid poplar clone '546' (*Populus deltoides* cv. '55/56' × *P. deltoides* cv. 'Imperial') were cultivated in a greenhouse in early March 2015, and moved to the Station on 2 May 2015, when they were transplanted into individual 20 l circular plastic pots filled with local light loamy farmland soil. Leaf number, height and stem diameter of all the seedlings were measured on 27 May. Similar-size plants were selected, randomly distributed in nine OTCs (10–12 plants per chamber), and pre-adapted to chamber conditions for 10 days before O₃ fumigation. No additional fertilization was applied and some plants were occasionally treated with a pesticide when insects were detected.

2.2. Ozone and water stress treatments

Plants were exposed to three O₃ treatments: charcoal-filtered ambient air (CF), non-filtered ambient air (NF), and NF with targeted O₃ addition of approximately 40 ppb (E-O₃) in three replicated chambers. The last treatment simulates a future scenario for China (Feng et al., 2015; Wang et al., 2012). Fumigation lasted for 96 days, from 5 June to 8 September 2015. Ozone was generated from pure oxygen by an O₃ generator and simultaneously mixed with ambient air as described in Hu et al. (2015) to achieve the target O₃ concentrations. Daily O₃ fumigation was from 08:00 to 19:00 (5 June–13 July) or 9:00 to 18:00 (14 July–8 September), in order to adapt to the local seasonal daylight period.

Ozone concentrations inside the OTCs were continuously monitored using an UV absorption O₃ analyzer (Model 49i, Thermo Scientific, USA), via a Teflon solenoid valve switch system, which collected air from sampling points at approximately 10 cm above the plant canopy during the whole experiment. The analyzer was calibrated with a 49i-PS calibrator (Thermo Scientific, USA) before the experiment and once a month during the experiment. Mean O₃ concentration during the growing season was 33.5 ± 2.4 ppb (mean ± 95% CI, Confidence Interval) in the CF treatment, 51.1 ± 4.1 ppb in the NF treatment, and 78.2 ± 5.5 ppb in the E-O₃ treatment (Fig. S1). The monthly averages, peak concentrations and AOT40 (accumulated daytime O₃ exposure over an hourly threshold of 40 ppb) values for each treatment are shown in Table S1. The monthly average of daily O₃ concentration in NF was 59.5, 58.3 and 41.6 ppb, and peak daily concentration reached 109, 93 and 72 ppb in June, July and August, respectively. The charcoal filtration

efficiency was 98% at the exit of the fan (1.1 kW, 1080 Pa, $19 \text{ m}^3 \text{ min}^{-1}$, CZR, Fengda, China) but about 40% at canopy level due to the high O_3 concentration of ambient air. During the fumigation period, from June to September, the accumulated O_3 exposures in the CF, NF and E- O_3 treatments expressed as AOT40 were 4.3, 16.0 and 38.7 ppm h , respectively.

Two irrigation treatments were applied during the O_3 fumigation period. Every 1–2 days, half of the plants per OTC were irrigated with reduced water inducing water stress (RW) and the other half were well irrigated so that the soil was kept close to field capacity (WW). Plants from the RW treatment were supplied with ~60% less water than the WW plants. In order to avoid additional water inputs by rain, each pot of both WW and RW treatments was covered with a plastic cover, which was removed when there was no rain. In short, there were six $\text{O}_3 \times \text{Water}$ treatments in this experiment.

Soil water content (SWC) was measured continuously using six moisture sensors (EC-5, Decagon Device, UK) at 15 cm depth in the root area since June 18, when probes reached stability, until the end of the experiment. Data were collected every 5 min with a data logger (EM 50, Decagon Device, UK). SWC usually reached saturated water content (~40%) at dusk, when plants were irrigated after fumigation (18:00 or 19:00) (Fig. S2). For the whole experimental period, the daily average SWC of WW and RW treatments was $24.8 \pm 0.38\%$ (95%CI) and $12.8 \pm 0.47\%$ (95%CI), respectively, and the SWC ratio between RW and WW treatments was 51.9%. The other environmental variables required for modelling stomatal O_3 flux, i.e. air temperature, relative humidity and solar radiation, were continuously recorded by a weather station (Campbell Scientific, North Logan, Utah, USA). Wind was not recorded as the leaf boundary layer is assumed to approach zero in OTCs (Ryan et al., 2009).

2.3. Leaf gas exchange, chlorophyll *a* fluorescence and pigment content

For each OTC and water treatment, 2 young leaves (5–6th fully expanded leaves from the top) (YL) from 2 plants were selected for measurements on 4 August; they were labelled for subsequent measurements in September. On 7 September, 3–6 YL, as well as 3–6 older leaves (OL, previously labelled in August) from the middle part of the crown were selected from 2 to 3 plants in each OTC and water treatment. Gas exchange and chlorophyll *a* fluorescence were determined simultaneously from 09:30 to 11:30 each day in both August and September using a LiCor-6400 photosynthesis system (LICOR, Lincoln, NE, USA) fitted with a 6400–40 leaf chamber fluorometer (LCF). During the measurements, photosynthetic active radiation (PAR) was set at $1200 \mu\text{mol m}^{-2}\text{s}^{-1}$, CO_2 at 380 ppm, block temperature at $31 \pm 0.5^\circ\text{C}$ and relative humidity between 50% and 60%. Fluorescence parameters included actual photochemical efficiency of PSII in the saturated light (F_v/F_m'), quenching of photochemical efficiency of PSII (qP), and quantum yield of noncyclic electron transport (Φ_{PSII}). Instantaneous water use efficiency (WUE) was calculated as the ratio between light-saturated photosynthesis rate (A_{sat}) and stomatal conductance (g_s).

The automatic program in the LiCor-6400 photosynthesis system was used to perform CO_2 assimilation rate (A) vs. intercellular CO_2 concentration (A/C_i) curves in 1–2 plants from each treatment per OTC in early September. When stomatal conductance (g_s) reached equilibrium, measurements were made by changing CO_2 concentration in 11 steps: 380, 300, 200, 100, 50, 380, 575, 800, 1000, 1200, 1500 ppm, under PAR of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, block temperature of $31 \pm 0.5^\circ\text{C}$ and relative humidity of 50–60%. Following the procedure in Sharkey et al. (2007), maximum

carboxylation efficiency (V_{cmax}) and maximum rate of electron transport (J_{max}) were deduced by iteratively fitting curves to A/C_i response data. Stomatal limitation (L_s) was calculated as $L_s = 1 - (A_{\text{Ca}380}/A_{\text{Ci}380})$, where $A_{\text{Ca}380}$ and $A_{\text{Ci}380}$ represent net CO_2 assimilation rate at ambient CO_2 concentration (C_a) of 380 ppm and at $C_i = 380$ ppm, respectively.

After measurements of gas exchange and fluorescence, two punches about 0.030–0.037 g of the corresponding leaves were extracted in 95% ethanol in sealed tubes and kept inside the refrigerator until completely faded within 5–7 days. The extract was then assayed for chlorophylls (Chl *a* and Chl *b*) and carotenoid (Car) contents, which were calculated from the absorption coefficients of 663 nm, 646 nm and 470 nm, respectively, according to Lichtenthaler (1987).

2.4. Growth, biomass and senescence

Plants were harvested in mid-September, when growth stopped and before senescence. Leaf area (LA), leaf number (LN), height and basal stem diameter of every single plant were measured. Total leaf area per plant was also calculated from LA and LN. Furthermore, leaf abscission and new leaf formation were calculated (averages of three plants per water treatment and OTC). The different biomass components, i.e. leaves, stems, and roots of each plant were separated and packaged into breathable net pockets. Then they were oven-dried at $70\text{--}80^\circ\text{C}$ until constant weight.

2.5. Stomatal ozone flux

The stomatal flux of O_3 (F_{st} , in $\text{nmol m}^{-2} \text{PLA s}^{-1}$) was estimated according to the following equation (CLRTAP, 2015):

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

where $[\text{O}_3]$ is the ozone concentration at the top of the canopy, r_b is the quasi-laminar resistance and r_c is the leaf surface resistance to O_3 , while g_{sto} is the actual stomatal conductance to O_3 . Considering the high velocity of the airflow through the chambers (Ryan et al., 2009), the value for r_b was negligible in this study, and therefore Eq. (1) can be simplified as:

$$F_{\text{st}} = [\text{O}_3] \cdot g_{\text{sto}} \quad (2)$$

The Jarvis multiplicative model was applied for the calculation of g_{sto} (Emmerson et al., 2000; Jarvis, 1976). g_{sto} was estimated from functions describing the response of stomata to key environmental and species-specific variables. Equation (3) shows the g_{sto} model used in this study (CLRTAP, 2015):

$$g_{\text{sto}} = g_{\text{max}} \cdot f_{\text{phen}} \cdot f_{\text{light}} \cdot \max[f_{\text{min}}, (f_{\text{temp}} \cdot f_{\text{VPD}} \cdot f_{\text{PAW}})] \quad (3)$$

where g_{sto} is the actual stomatal conductance to O_3 and g_{max} is the species-specific maximum stomatal conductance, both expressed on a total leaf surface area. Functions f_{phen} , f_{light} , f_{temp} and f_{PAW} , all expressed in relative terms (i.e., values between 0 and 1), accounted for the variation in g_{max} with leaf age, irradiance, air temperature, vapour pressure deficit and plant available water, respectively. Function f_{min} is the minimum daylight g_{sto} , expressed as a fraction of g_{max} . All functions and calculation methods followed CLRTAP (2015). The parameterization used was based on that proposed by Hu et al. (2015) for poplar clone 546, except f_{PAW} which was not previously available and was parameterized using our own measurements (Table S2).

Finally, the Phytotoxic Ozone Dose (PODY in $\text{mmol m}^{-2} \text{PLA}$), i.e.

the accumulated stomatal O₃ flux above a flux threshold Y, was calculated from hourly data as:

$$\text{PODY} = \sum_{i=1}^n \max[F_{st} - Y, 0] \cdot \Delta t \quad (4)$$

where F_{st} is the stomatal O₃ flux, Y is the threshold, and $\Delta t = 1$ h (CLRTAP, 2015).

2.6. Dose-response relationship

The relationship between O₃ dose and relative biomass (RB) was analyzed in accordance with Bker et al. (2015) and Fuhrer (1994): a linear regression for each water treatment was made between the actual biomass and AOT40 or PODY to obtain the y-intercept which is hypothetically the maximum biomass at zero O₃ exposure or uptake. PODY was calculated with Y thresholds 1 and 7, according to CLRTAP (2015) and Hu et al. (2015), respectively. The relative biomass (RB) was obtained as the biomass at each O₃ treatment divided by the y-intercept of the corresponding water treatment regression. In this way, all RBs were comparable in a common relative scale.

2.7. Statistical analyses

The statistical design was a split-plot, considering the statistical unit as the average across two or three pots for each water treatment in each OTC. After testing for homogeneity of variance, linear mixed models (LMM) were applied using JMP 10 software (SAS Institute, USA), in order to test the effects of O₃, water treatment, leaf age and their interactions. Ozone and water treatment were considered fixed effects and OTC was a random effect. The Tukey's Honestly Significant Difference (HSD) test was applied to identify significant differences. In all analyses, $P \leq 0.05$ was considered as statistically significant.

3. Results

As results from YL in August and September were similar for most of the measured parameters, for the sake of clarity only data from September are presented and discussed. Data from August are provided as supplementary material (Fig. S3–S5; Table S3).

Table 1

Effects on both younger leaves (YL) and older leaves (OL) in September: Analysis of variance (P values) of the effects of O₃, Water stress and Leaf Age, and their interactions on gas exchange, chlorophyll a fluorescence parameters and pigment content. Statistically significant effects ($P < 0.05$) are marked in bold.

	O ₃	Water	Age	O ₃ × Water	O ₃ × Age	Water × Age	O ₃ × Water × Age
Chl a	<0.001	<0.001	0.007	<0.001	<0.001	0.441	<0.001
Chl b	<0.001	<0.001	0.125	0.002	<0.001	0.322	<0.001
Chl a /Chl b	0.168	0.004	<0.001	0.252	0.123	0.751	0.485
Car	<0.001	<0.001	0.132	0.034	0.068	0.264	0.002
A_{sat}	0.003	0.109	<0.001	0.018	0.380	0.002	0.122
g_s	0.285	<0.001	0.021	0.584	0.543	0.731	0.833
C_i	0.905	<0.001	0.004	0.579	0.753	0.499	0.540
WUE	0.975	<0.001	0.009	0.637	0.843	0.509	0.474
F_v/F_m'	0.021	0.245	<0.001	0.230	0.271	0.870	0.552
qP	0.464	0.207	<0.001	0.402	0.775	0.517	0.966
Φ_{PSII}	0.005	0.114	<0.001	0.463	0.088	0.255	0.759
V_{cmax}	0.004	<0.001	<0.001	0.036	0.312	0.996	0.189
J_{max}	0.034	0.093	<0.001	0.138	0.311	0.550	0.701
V_{cmax}/J_{max}	0.134	0.002	0.588	0.608	0.931	0.705	0.409
L_s	0.089	0.227	0.003	0.452	0.191	0.742	0.917

3.1. Leaf pigment content

Both water and O₃ treatments induced significant changes in leaf pigment contents. RW significantly increased chlorophylls a and b and carotenoid contents in both YL and OL (Table 1 and Fig. 1). On the other hand, O₃ significantly reduced chlorophylls a and b and carotenoid contents. The interaction of O₃ × Water × (leaf) Age was significant, suggesting that the response to O₃ was dependent on the combination of leaf age and water stress. Under WW conditions, stronger reductions due to E-O₃ in chlorophyll and carotenoid contents were observed in OL (by 52.7% and 33.3%, respectively, in comparison with CF) than in YL (by 8.2% and 10.1%, respectively), while RW alleviated the O₃-induced decrease in OL (by 20.6% and 7%, respectively) (Table 1 and Fig. 1).

3.2. Leaf gas exchange and chlorophyll fluorescence

Both water treatment and O₃ affected gas exchange and chlorophyll a fluorescence parameters. In both YL and OL, g_s was significantly reduced by the RW treatment with regard to WW, with an associated increase in water use efficiency (WUE) (Table 1 and Fig. 2). Effects of RW treatment on A_{sat} were not significant in both YL and OL (Table 1). High O₃ levels significantly reduced A_{sat} in both types of leaves while g_s showed a serious decline only in YL (Table 1 and Fig. 2). An interactive effect of O₃ and water stress on A_{sat} was found only when both YL and OL were considered (Table 1, $P = 0.018$): a striking reduction in A_{sat} was observed in OL from WW plants (40.4% relative to CF) but not from RW plants (Fig. 2). This reduction in A_{sat} implied a reduction in WUE as it was not paralleled by a similar reduction in g_s (Fig. 2). Both A_{sat} and g_s were significantly reduced with leaf age (Table 1 and Fig. 2). WUE was higher in YL than in OL (Table 1 and Fig. 2). RW also reduced C_i .

Chlorophyll fluorescence parameters were affected by leaf age, with lower values for F_v/F_m' , qP , and Φ_{PSII} in OL than in YL (Table 1). RW did not induce any change in fluorescence parameters (Table 1, Fig. 3), while E-O₃ significantly reduced F_v/F_m' and Φ_{PSII} (Table 1). No interactions were observed for fluorescence parameters.

3.3. Leaf photosynthetic capacity

The leaf photosynthetic capacity, as indicated by V_{cmax} , showed a significant interaction between O₃ and water treatment ($P = 0.036$), while O₃ reduced J_{max} ($P = 0.034$) (Table 1). When the different types of leaves were considered separately, V_{cmax} was

Fig. 1. Chlorophyll *a* and *b* contents, chlorophyll *a/b* ratios, and carotenoid contents in leaves sampled from two ages: younger leaves (YL) and older leaves (OL) in September. Plants were grown in charcoal-filtered air (CF), non-filtered air (NF) and elevated O_3 (E- O_3) under well water (WW, irrigated to field capacity) and water stress (RW, 40% irrigation) conditions. Each point represents the mean $\pm$ SD. Statistically significant differences between treatments in each leaf age are noted with different letters, respectively (Tukey test, $P < 0.05$, $n = 3$).

Fig. 2. Photosynthetic and gas exchange parameters in leaves sampled from two ages: younger (YL) and older leaves (OL) in September. A. Light-saturated photosynthetic rate (A_{sat}), B. Stomatal conductance (g_s), C. Intercellular CO_2 concentration (C_i), D. Water Use Efficiency (WUE). See caption of Fig. 1 for further details.

Fig. 3. Electron transport and chlorophyll fluorescence parameters in leaves sampled from two ages: younger (YL) and older leaves (OL) in September. A. Actual photochemical efficiency of PSII in the saturated light (F_v'/F_m'), B. Quenching of photochemical efficiency of PSII (qP), C. Quantum yield of noncyclic electron transport (Φ_{PSII}). See caption of Fig. 1 for further details.

significantly reduced in YL by RW by 10.7% across O_3 treatments (Fig. 4A, $P = 0.036$). However, effects on V_{cmax} in OL could only be explained by an interaction between the two factors ($O_3 \times$ Water, $P = 0.017$), as the RW treatment clearly reduced the impact of $E-O_3$ on OL leaves (33.3% and 14.3% in $E-O_3$ relative to CF under WW and RW conditions, respectively). Consistently with V_{cmax} in OL, J_{max} significantly declined due to $E-O_3$ (Fig. 4B, $P = 0.009$) by 17.6% relative to CF. Nevertheless, no interaction between O_3 and water treatment was observed. RW significantly reduced the V_{cmax}/J_{max} ratio in OL, but $E-O_3$ significantly increased leaf stomatal limitation (L_s) only in YL (Fig. 4C–D). No significant interactions were observed in these two parameters. Furthermore, V_{cmax} , J_{max} and L_s in OL were lower than those in YL (Table 1, Fig. 4A–B and D).

3.4. Growth, biomass and senescence-related parameters

RW induced a significant decline in plant height and weight, stem biomass and diameter, number and biomass of attached leaves and root biomass per plant (Table 2), while significantly increased the root/shoot ratio (Table 2). On the other hand, O_3 significantly reduced total biomass, stem diameter and stem

biomass, as well as number and biomass of attached leaves per plant (Table 2). Total biomass, stem biomass, attached leaf number and leaf biomass were decreased more by $E-O_3$ under WW (by 19.7%, 17.1%, 34.3% and 22.2% relative to CF-WW, respectively) than under RW conditions (by 6.41%, 3.69%, 18.7% and 4.7% relative to CF-RW, respectively), therefore significant interactions were observed between O_3 and water treatment. Moreover, both $E-O_3$ and RW reduced the formation of new leaves per plant (Table 2), and the number of abscised leaves per plant strongly increased with increasing O_3 levels (Table 2). Total leaf area per plant was significantly lower in the RW treatment relative to the WW treatment (Table 2). Interactions between O_3 and water treatment were not significant for any of these parameters (Table 2).

3.5. Dose-response functions

Biomass responses to RW and O_3 exposure (AOT40) showed an important effect of RW on the final biomass, with reductions between 20% and 31% in the RW treatment in comparison with the WW treatment at equivalent AOT40 values (Fig. 5A). From the slope of the regressions between total biomass and AOT40 (Fig. 5A), it can be concluded that RW had an important protective effect on the O_3 impacts on biomass, as the slope of the RW treatment was 4.7 times lower than in the WW treatment. Biomass losses under current ambient levels (NF) were 7.1% for the WW treatment and 3.7% for the RW treatment. For the $E-O_3$ levels representing a future scenario, biomass losses under WW and RW were 19.7% and 6.4%, respectively (Fig. 5A).

For O_3 dose response relationship, the performance of the model was much higher when POD_1 ($R^2 = 0.683$, $P = 0.043$) or POD_7 ($R^2 = 0.829$, $P = 0.012$) were used compared with AOT40 ($R^2 = 0.560$, $P = 0.087$) (Fig. 5B–D). R^2 was higher with higher Y threshold of POD_Y . The O_3 critical level (CL) for preventing a 4% biomass loss (CLRTAP, 2015) in this poplar clone under different water regimes was $POD_1 = 5.27 \text{ mmol m}^{-2} \text{ PLA}$ and $POD_7 = 4.09 \text{ mmol m}^{-2} \text{ PLA}$. As the regression between RB and AOT40 was not significant, this metric was considered inappropriate to derive any CL for clone '546' under conditions with different water regimes.

4. Discussion

4.1. Water stress effects

Plants can acclimate to water stress through a series of mechanisms (Wilkinson and Davies, 2010) including: (1) stomatal closure as a consequence of soil water stress (Díaz-Espejo et al., 2007); (2) structural and morphological changes in the leaves (Anjum et al., 2011); (3) reduced leaf and stem growth rates; (4) maintenance or/increase in root extension rates (Munns and Sharp 1993; and/or (5) increase in root and shoot hydraulic conductance (Hose et al., 2000; Parent et al., 2009). Other drought avoidance or tolerance strategies include shedding of older leaves; assimilate remobilization or diversion from vegetative to reproductive growth; induction of senescence, synthesis of osmotically active solutes involved in the maintenance of cell turgor; and synthesis of antioxidants (Chaves et al., 2003). In the present study, several of the responses listed above were investigated. RW significantly reduced g_s and C_i , and increased WUE in comparison to the WW treatment. Lower g_s during soil drought in angiosperms may result from effects of leaf turgor (Rodríguez-Domínguez et al., 2016), and higher WUE is an adaptive strategy of plants for living under moisture stress conditions (Oweis, 2012; Rodríguez-Domínguez et al., 2016). Photosynthetic parameters such as V_{cmax} declined significantly, which partly explains the observed reduction in

Fig. 4. Leaf photosynthetic capacity and stomatal limitation parameters in leaves sampled from two ages: younger (YL) and older leaves (OL) in September. A. The maximum velocity of carboxylation efficiency ($V_{c_{max}}$), B. the maximum rate of electron transport (J_{max}), C. the ratio of $V_{c_{max}}/J_{max}$, D. stomatal limitation to photosynthesis (L_s). Plants were grown in carbon-filtered air (CF), non-filtered air (NF) and elevated O₃ (E-O₃) under well water (WW) or moderate water stress (RW) conditions. Each point represents the mean $\pm$ SD. Statistically significant differences between treatments are noted with different uppercase letters (Tukey test, $P < 0.05$, $n = 3$).

Table 2

Effects on biomass, growth and senescence-related parameters at the final harvest time, and analysis of variance (*P* values) of O₃, water stress and their interactions. Plants were grown in charcoal-filtered air (CF), non-filtered air (NF) and elevated O₃ (E-O₃) under well water (WW, irrigated to field capacity) and water stress (RW, 40% irrigation) conditions. Each treatment showed the mean ± SD. Statistically significant differences between treatments are noted with different letters (Tukey test, *P* < 0.05, *n* = 3). Statistically significant effects (*P* < 0.05) are marked in bold.

	WW			RW			O ₃	Water	O ₃ × Water
	CF	NF	E-O ₃	CF	NF	E-O ₃			
Height (cm)	96.43 ± 11.16 a	91.81 ± 3.11 a	90.07 ± 7.75 a	66.13 ± 11.04 b	60.11 ± 0.77 b	60.00 ± 4.60 b	0.494	<0.001	0.960
Total biomass (g)	121.16 ± 3.55 a	112.56 ± 4.36 a	97.32 ± 5.32 b	83.54 ± 3.99 c	80.45 ± 5.07 c	78.19 ± 0.68 c	0.003	<0.001	0.049
Stem diameter (mm)	9.51 ± 0.40 a	8.48 ± 0.42 ab	7.63 ± 0.60 b	6.27 ± 0.30 c	5.75 ± 0.40 c	5.56 ± 0.37 c	0.006	<0.001	0.120
Stem biomass (g)	35.99 ± 2.82 a	33.68 ± 1.58 ab	29.84 ± 1.30 b	21.08 ± 1.28 c	20.53 ± 1.24 c	20.30 ± 0.90 c	0.070	<0.001	0.012
Attached leaves (number)	37.22 ± 2.87 a	33.83 ± 2.47 ab	24.44 ± 1.50 cd	28.78 ± 1.26 bc	25.67 ± 0.67 cd	23.39 ± 1.13 d	0.001	<0.001	0.002
Newly formed leaves (number per plant)	24.78 ± 2.14 a	22.11 ± 1.26 ab	17.89 ± 1.9 bc	18.22 ± 2.8 bc	16.31 ± 2.15 c	15.89 ± 1.17 c	0.033	<0.001	0.321
Abscised leaves (number per plant)	1.56 ± 0.19 b	2.33 ± 0.58 b	6.33 ± 0.88 a	1.00 ± 0.33 b	3.56 ± 1.71 ab	5.94 ± 1.58 a	0.001	0.820	0.200
Leaves area (m ² per plant)	0.54 ± 0.05 a	0.53 ± 0.04 a	0.46 ± 0.04 ab	0.36 ± 0.08 b	0.34 ± 0.06 b	0.34 ± 0.05 b	0.370	<0.001	0.312
Leaves biomass (g)	52.81 ± 1.57 a	48.37 ± 2.79 a	41.08 ± 3.76 b	36.42 ± 1.77 b	35.84 ± 0.34 b	34.70 ± 1.90 b	0.006	<0.001	0.025
Root biomass (g)	32.36 ± 0.62 a	30.51 ± 0.18 ab	26.40 ± 3.00 abc	26.05 ± 2.68 abc	24.08 ± 3.68 bc	23.19 ± 1.37 c	0.058	0.002	0.389
Root/Shoot	0.37 ± 0.02 b	0.37 ± 0.02 ab	0.37 ± 0.05 ab	0.45 ± 0.04 a	0.43 ± 0.05 ab	0.42 ± 0.03 ab	0.929	0.001	0.350

Fig. 5. Relationships between total biomass (TB) and AOT40 for the two water treatments (A), and between relative total biomass (RB) and AOT40 (B), POD_1 (C) and POD_7 (D) of the six combined O₃ and drought treatments at the final harvest. Dashed lines denote 95% confidence intervals of the regression. Plants were grown under well water (WW) or water stress (RW) conditions.

growth and biomass parameters (height, total biomass, stem biomass and diameter, leaf number and biomass), and the increase in the root/shoot ratio, i.e. a stronger reduction in the above-ground parts of the plants than in the roots. A reduction in the total foliar surface of the plant in order to limit transpiration and water needs of the whole plant is another typical adaptation to drought (Bosabalidis and Kofidis, 2002; Hernandez-Santana et al., 2017), in agreement with our results. Chlorophyll and carotenoid contents increased, as a result of denser tissues reported under drought conditions (Pääkkönen et al., 1998a,b).

4.2. Ozone effects

The effects of O₃ have been well documented and are consistent

with the results of the present study. Ozone impairs photosynthesis, further affecting different processes. Typically, Rubisco carboxylation efficiency declines, CO₂ assimilation and *g_s* are generally reduced, and chlorophylls are finally degraded when plants are exposed to high O₃ (Renaut et al., 2009; Wittig et al., 2009). Impairment of Rubisco activity leads to an accumulation of leaf internal CO₂ (*C_i* increases), and photochemical processes are frequently down-regulated as an adaptation towards a lower energy demand for photochemistry; this is consistent with the observed reduction in fluorescence parameters such as *F_v'*/*F_m'*, *qP* and Φ_{PSII} (Calatayud et al., 2007). Contrary to the case of plants affected by water stress, WUE was decreased by E-O₃, mainly due to a stronger decline in *A_{sat}* than in *g_s*. E-O₃ accelerated the senescence processes by increasing leaf abscission. The above mentioned

reductions in CO₂ assimilation and total foliar area per plant, in combination with a higher demand in resources for defense and repair, resulted in lower above-ground and below-ground biomass. It is interesting to underline the differences between YL and OL, with much higher O₃ impacts on OL than on YL, which was also found in *Betula pendula*, as indicated by higher accumulation of H₂O₂ in the old leaves (Oksanen et al., 2005).

4.3. Interactive effects of ozone and water stress

Results of the present study showed a protective effect of water stress against O₃ for several of the measured parameters at both leaf and biomass levels, with some significant interactive effects between both factors. Water stress protected leaves from deleterious O₃ effects on important photosynthetic parameters such as Chla, Chlb, Car contents, A_{sat} and V_{Cmax} that were significantly less affected by E-O₃ in the RW treatment (O₃ × Water, $P < 0.001$, 0.002, 0.034, 0.018 and 0.036, respectively). A reduction in g_s in water-stressed leaves leading to reduced O₃ uptake and thus less damage to photosynthetic processes is the obvious mechanism explaining such interactions. Results of biomass were fully consistent with those at leaf level, as interactions between O₃ and water stress were significant for leaf ($P = 0.025$), stem ($P = 0.012$) and total biomass ($P = 0.049$) of the plants, with lower relative biomass reductions in plants of the RW treatment than in WW plants. Leaf senescence, a process typically accelerated by O₃, was also reduced in RW plants in comparison to WW plants (O₃ × Water, $P = 0.02$), also outlining the protective role of drought against O₃.

Despite the mentioned protective role of water stress against O₃ observed in the present study, existing studies on this topic yielded contrasting results. The different responses observed at leaf level may be species-specific, in particular how the different species react against O₃, how tolerant they are to drought and how stomata react against water stress, as these features will affect the resulting interactions. In addition, responses will also depend on the O₃ levels applied, and on the level, timing and duration of the water stress, which further complicates the picture (Pollastrini et al., 2014). The moment at which water stress is applied is also relevant to explain the different responses. In the present case, water stress was applied in parallel with O₃ exposure, favoring stomatal closure and O₃ avoidance. However, if severe water stress episodes are applied before exposure to high O₃ levels, they may impair stomatal function (e.g. increasing stomatal sluggishness, Hoshika et al., 2013) making the plants more vulnerable to subsequent O₃ episodes (Alonso et al., 2014). On the other hand, it is also important to underline that while RW reduced the impact of O₃ as in the case of chlorophyll and carotenoid contents (Fig. 1), the combination of RW and E-O₃ may result in higher detrimental effects for the plants than under WW and E-O₃ conditions, as shown for total biomass (Table 1, Fig. 5A). The fact that both stress factors were not additive but exhibited some clear interactions highlights the importance of taking into account these interactions for risk assessment in order to prevent an overestimation of the effects. In summary, given the complexity of water stress and O₃ interactions, still more studies considering all these factors in different species must be carried out in order to better understand and predict the responses of different species and vegetation types. This is even truer and challenging if we consider that even closely related plants such as different poplar species and clones may show contrasting responses (Noormets et al., 2001).

4.4. Dose-response functions and O₃ critical levels

Results of the present study stress the importance of considering the water stress factor in plant O₃ risk assessment. Water

availability to the plants is currently a key factor for explaining plant responses to O₃ in arid or dry areas of the world as is for example the case of Mediterranean areas (De Marco et al., 2016), where despite the high O₃ levels measured, impacts of this pollutant are lower than in other areas subjected to similar or lower O₃ concentrations but with a higher water availability (Paoletti, 2006; Retzlaff et al., 2000). While CL for plant protection have mostly been developed based on exposure metrics (e.g. the AOT40 index in Europe), they have been also proposed based on stomatal O₃ flux (Hu et al., 2015; Mills et al., 2011; Sicard et al., 2016). This metric is able to incorporate the influence of environmental variables such as irradiance, temperature, leaf-to-air Vapour Pressure Deficit (VPD) or soil moisture for calculating accumulated O₃ uptake over an accumulation period (usually the plant growing period). Our study showed a very good performance of AOT40 index under WW conditions, so that this index, given its simplicity, could be used for risk assessment in regions where poplar is not subjected to water stress. However, this is not frequently the case and, when contrasting soil moisture conditions are considered, our study indicated that the performance of the POD approach was clearly better than the AOT40, and should be preferred. Furthermore, the hypothesis that water stress reduces the impact of O₃ on biomass of poplar due to a reduction in the accumulated O₃ flux is also supported by our results. A similar conclusion on the superior performance of POD against AOT40 was obtained by Karlsson et al. (2007) when data from several studies on European sensitive trees (some of them including water stress treatments) were pooled together. Also Alonso et al. (2014) showed that the O₃ induced effects on biomass in a pooled dataset of two subspecies of Holm oak (*Quercus ilex* L.) subjected to well-watered and drought treatments were better related to POD than to AOT40 in terms of R². However, Karlsson et al. (2007) found that the impact of drought was not large enough to make the POD approach superior to AOT40 when a single species, or a category including similar species, were analyzed. This was explained by the fact that the soil water potential had little impact on modelled stomatal conductance even in the drought treatments (Karlsson et al., 2004, 2007). Also, in contrast with our results, drought did not protect Holm oak from O₃ effects when biomass responses were considered (Alonso et al., 2014). This could be explained by a small limitation of O₃ flux due to stomatal closure under drought stress in Holm oak in comparison with our poplar, as well as by differences in O₃ and drought sensitivity in the two species. Holm oak, in fact, is considered O₃ tolerant (Paoletti, 2006), while poplars are among the most O₃ sensitive tree species (Ainsworth et al., 2012; Wittig et al., 2009). Therefore, among the scarce literature on O₃ fluxes and water stress interactions on tree biomass, the present study is representative of the case of fast-growing species with high water requirements, which are considered more susceptible to O₃ and drought (Alonso et al., 2014; Pääkkönen et al., 1998a,b). The use of the flux-based approach including a function accounting for soil moisture is an important recommendation for an accurate risk assessment (De Marco et al., 2016).

Given the relevance of poplar plantations in China, experiments including different watering regimes in combination with modelled O₃ fluxes are needed for proposing suitable CL for the protection of poplar clones under present and future water-limited conditions. In our study, the O₃ CL for preventing a 4% reduction in biomass based on CLRTAP (2015) were estimated for the thresholds of 1 nmol O₃ m⁻² s⁻¹ (CLRTAP, 2015) and 7 nmol O₃ m⁻² s⁻¹ (Hu et al., 2015). The CL of POD₁ = 5.3 mmol m⁻² and POD₇ = 4.1 mmol m⁻² were proposed for poplar clone '546'. When CL for a 5% reduction were calculated for comparison with the CL estimated by Hu et al. (2015) using data from 5 poplar clones grown under well-watered conditions, the results for POD₁ were rather similar but not those for

POD₇. The CL estimated by Hu et al. (2015) were POD₁ = 6.1 mmol m⁻² and POD₇ = 3.8 mmol m⁻², against 6.6 mmol m⁻² and 5.1 mmol m⁻² in our study, respectively. This suggests that the CL estimated by Hu et al. (2015) using POD₁ could be suitable for clone '546' even under variable water availability conditions. It has to be mentioned that harmonization of the methods for calculating the flux-based CL is urgently needed as resulting values may be very sensitive to the calculation approaches. In particular, relevant differences may be observed depending on 1) how the relative effect was calculated (i.e., taking the y-intercept of the regression in the present paper or the CF treatment as reference), 2) the target reduction percentage (e.g., 4% or 5%), and 3) the selection of the Y threshold for POD_y. Ongoing discussions within the CLRTAP are currently considering a low O₃ (10 ppb as 24 h average) reference level representative of clean air in the calculations (Paoletti et al., 2017), which would result in an increase in the CL, especially for low Y thresholds. In the current analysis, the CL would increase from POD₁ = 5.27 mmol m⁻² PLA to POD₁ = 7.04 mmol m⁻² PLA when the control O₃ concentration rises from 0 to 10 ppb. A full harmonization of the methods is fundamental in order to make studies comparable.

5. Conclusions

The negative effects of O₃ on older leaves were more severe than on younger leaves, as shown by the significant decline in A_{sat} , F_v/F_m , ETR and Φ_{PSII} , as well as V_{Cmax} , but they were alleviated by RW stress. The present study clearly underlines the strong protective effect of RW on O₃ impacts at leaf and plant level on a species of great economic importance such as poplar. Knowledge of the interaction between both factors is fundamental in order to propose reliable critical levels for plant protection under present and future high O₃ and drought scenarios. In this context, the predictive power of O₃ flux-based metrics is much better than exposure-based metrics (e.g. AOT40) for risk assessment as the former metric can account for the reduction of O₃ uptake caused by water stress. Furthermore, critical O₃ levels for the poplar under varying water stress conditions were proposed for the first time. Our results also suggest that irrigation management might be a useful tool to reduce O₃ impacts on poplar in high O₃ polluted areas, although this strategy should be appraised very carefully, as depending on the RW regimes, reductions of photosynthesis and plant productivity may counterbalance the possible benefits against O₃. Interactive effects of water stress and O₃ should be taken into account when developing policies for O₃ control and when evaluating the carbon sink capacity of poplar plantations under multifactorial natural conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.06.044>.

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