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

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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
during the summer and normally drier months (IPCC, 2013). In many parts of the world, high O$_3$ levels are concurrent with water stress periods, as 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 O$_3$ 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 O$_3$ and water stress, stomata play an important role, as O$_3$ 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 O$_3$ (Bohler et al., 2013). So far, several studies have been carried out addressing the effects of O$_3$ 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 pedunculate oak (Quercus robur L.), did not show any significant interaction in terms of photosynthesis, growth or biomass (Kuehn et al., 2015; Löv 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 content 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 O$_3$ injury in a chamber experiment, while an increase of O$_3$ 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 O$_3$ 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 O$_3$, as well as its magnitude or significance for current O$_3$ levels, due to gaps in these types of studies. For these reasons, it is important to test the interactive responses of O$_3$ 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 O$_3$ (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 O$_3$ 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 O$_3$ 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 O$_3$ flux approach (Büker et al., 2015; Hu et al., 2015; Karlsson et al., 2007) which relies on the O$_3$ uptake by the plant rather than on the O$_3$ concentrations in the air (CLRTAP, 2015).

The first objective of the present paper is to assess the physiological and biomass responses to O$_3$ 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 O$_3$ 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 O$_3$ critical levels for sensitive species in Asia taking into account water stress interactions with O$_3$. We postulate that a flux-based risk assessment would better account for the effects of water stress on O$_3$-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 O$_3$-sensitive hybrid poplar clone ‘546 (Populus deltoides cv. ’55/56′ × P. deltoids 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 O$_3$ 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 O$_3$ treatments: charcoal-filtered ambient air (CF), non-filtered ambient air (NF), and NF with targeted O$_3$ addition of approximately 40 ppb (E-O$_3$) 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 ambient air as described in Hu et al. (2015) to achieve the target O$_3$ concentrations. Daily O$_3$ 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 O$_3$ 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 O$_3$ 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-O$_3$ treatment (Fig. S1). The monthly averages, peak concentrations and AOT40 accumulated daytime O$_3$ exposure over an hourly threshold of 40 ppb) values for each treatment are shown in Table S1. The monthly average of daily O$_3$ 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 m³ min⁻¹, CQR, Fengda, China) but about 40% at canopy level due to the high O₃ concentration of ambient air. During the fumigation period, from June to September, the accumulated O₃ exposures in the CF, NF and E-O₃ treatments expressed as AOT40 were 4.3, 16.0 and 38.7 ppm h, respectively.

Two irrigation treatments were applied during the O₃ 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 O₃ × 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 ± 0.38% (95%CI) and 12.8 ± 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 O₃ 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 µmol m⁻² s⁻¹, CO₂ at 380 ppm, block temperature at 31 ± 0.5 °C and relative humidity between 50% and 60%. Fluorescence parameters included actual photochemical efficiency of PSII in the saturated light (Fv/Fm), quantum of photochemical efficiency of PSII (ΦPSII), and quantum yield of noncyclic electron transport (ΦPSII). Instantaneous water use efficiency (WUE) was calculated as the ratio between light-saturated photosynthesis rate (Amax) and stomatal conductance (gs).

The automatic program in the LiCor-6400 photosynthesis system was used to perform CO₂ assimilation rate (A) vs. intercellular CO₂ concentration (A/C) curves in 1–2 plants from each treatment per OTC in early September. When stomatal conductance (gₛ) reached equilibrium, measurements were made by changing CO₂ concentration in 11 steps: 380, 300, 200, 100, 50, 380, 575, 800, 1000, 1200, 1500 ppm, under PAR of 1200 µmol m⁻² s⁻¹, block temperature of 31 ± 0.5 °C and relative humidity of 50–60%. Following the procedure in Sharkey et al. (2007), maximum carboxylation efficiency (Vc,max) and maximum rate of electron transport (I_max) were deduced by iteratively fitting curves to A/C response data. Stomatal limitation (Lₛ) was calculated as Lₛ = 1 – (AC₃80/A₃80), where AC₃80 and A₃80 represent net CO₂ assimilation rate at ambient CO₂ concentration (Cₐ) 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 70–80 °C until constant weight.

2.5. Stomatal ozone flux

The stomatal flux of O₃ (Fₖ, in nmol m⁻² PLA s⁻¹) was estimated according to the following equation (CLRTAP, 2015):

\[
F_k = \frac{[O_3] \cdot g_{sto} \cdot \frac{rc}{rb} + r_c}{} 
\]

(1)

where [O₃] 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 O₃, while g_sto is the actual stomatal conductance to O₃. 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:

\[
F_k = [O_3] \cdot g_{sto} 
\]

(2)

The Jarvis multiplicative model was applied for the calculation of g_sto (Emerson 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):

\[
g_{sto} = g_{max} \cdot f_{phen} \cdot f_{light} \cdot f_{max} \cdot f_{temp} \cdot f_{VPD} \cdot f_{PAW} 
\]

(3)

where g_sto is the actual stomatal conductance to O₃ 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_{phen} 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 mmol m⁻² PLA), i.e.
the accumulated stomatal O3 flux above a flux threshold Y, was calculated from hourly data as:

\[ \text{PODY} = \sum_{i=1}^{n} \max[\text{Flux}_i - Y, 0] \cdot \Delta t \]  

where \( \text{Flux}_i \) is the stomatal O3 flux, \( Y \) is the threshold, and \( \Delta t = 1 \text{ h} \) (CLRTAP, 2015).

2.6. Dose-response relationship

The relationship between O3 dose and relative biomass (RB) was analyzed in accordance with Büker et al. (2015) and Fuhrer (1994): a linear regression for each water treatment was made between the actual biomass and AOT40 or PO DY to obtain the y-intercept which is hypothetically the maximum biomass at zero O3 exposure or uptake. PO DY 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, \( P < 0.05 \) 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).

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 caroteneind contents in both YL and OL (Table 1 and Fig. 1). On the other hand, O3 significantly reduced chlorophylls \( a \) and \( b \) and caroteneind contents. The interaction of O3 \( \times \) Water \( \times \) (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\(_{\text{sat}}\) were not significant in both YL and OL (Table 1). High O3 levels significantly reduced A\(_{\text{sat}}\) 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\(_{\text{sat}}\) was found only when both YL and OL were considered (Table 1, \( P = 0.018 \)): a striking reduction in A\(_{\text{sat}}\) was observed in OL from WW plants (40.4% relative to CF) but not from RW plants (Fig. 2). This reduction in A\(_{\text{sat}}\) implied a reduction in WUE as it was not paralleled by a similar reduction in \( g_s \) (Fig. 2). Both A\(_{\text{sat}}\) 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 \( g_s \).

Chlorophyll fluorescence parameters were affected by leaf age, with lower values for \( F_{\text{v}}/F_{\text{m}} \), \( qP \) and \( \Phi_{\text{PSII}} \) 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 \( F_{\text{v}}/F_{\text{m}} \) and \( \Phi_{\text{PSII}} \) (Table 1). No interactions were observed for fluorescence parameters.  

3.3. Leaf photosynthetic capacity

The leaf photosynthetic capacity, as indicated by \( V_{\text{cmax}} \), showed a significant interaction between O3 and water treatment (\( P = 0.036 \)), while \( \Omega_0 \) reduced \( J_{\text{max}} \) (\( P = 0.034 \)) (Table 1). When the different types of leaves were considered separately, \( V_{\text{cmax}} \) was

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Table 1

| O3 | Water | Age | O3 × Water | O3 × Age | Water × Age | O3 × Water × Age |
|----|-------|-----|------------|----------|-------------|------------------|
| \( \text{Chi} \_a \) | <0.001 | -0.001 | 0.007 | <0.001 | -0.001 | 0.001 | 0.441 | <0.001 |
| \( \text{Chi} \_b \) | <0.001 | -0.001 | 0.125 | 0.002 | <0.001 | 0.001 | 0.322 | <0.001 |
| \( \text{Chi} / \text{Chi} \_b \) | 0.168 | 0.004 | -0.001 | 0.252 | 0.068 | 0.264 | 0.002 |
| \( \text{Car} \) | <0.001 | -0.001 | 0.132 | 0.034 | <0.001 | 0.018 | 0.380 | 0.002 |
| \( \sigma_{\text{sat}} \) | 0.003 | 0.109 | -0.001 | 0.018 | 0.018 | 0.380 | 0.002 | 0.122 |
| \( \chi \) | 0.285 | -0.001 | 0.021 | 0.584 | 0.543 | 0.731 | 0.833 |
| \( \text{Cl} \) | 0.905 | -0.001 | 0.004 | 0.579 | 0.753 | 0.499 | 0.540 |
| \( \text{WUE} \) | 0.975 | -0.001 | 0.009 | 0.637 | 0.843 | 0.509 | 0.474 |
| \( \text{Fv}/F_{\text{m}}' \) | 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 |
| \( \Phi_{\text{PSII}} \) | 0.005 | 0.114 | -0.001 | 0.463 | 0.088 | 0.255 | 0.759 |
| \( V_{\text{cmax}} \) | 0.004 | -0.001 | 0.001 | 0.036 | 0.312 | 0.996 | 0.189 |
| \( \Omega_0 \) | 0.034 | 0.093 | -0.001 | 0.138 | 0.311 | 0.550 | 0.701 |
| \( V_{\text{cmax}} / \Omega_0 \) | 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 |
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 O3 (E-O3) under well water (WW, irrigated to field capacity) and water stress (RW, 40% irrigation) conditions. Each point represents the mean ± 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 CO2 concentration (C_i), D. Water Use Efficiency (WUE). See caption of Fig. 1 for further details.
significantly reduced in YL by RW by 10.7% across O3 treatments (Fig. 4A, P = 0.036). However, effects on \( V_{\text{cmax}} \) in OL could only be explained by an interaction between the two factors (O3 x Water, P = 0.017), as the RW treatment clearly reduced the impact of E-O3 on OL leaves (33.3% and 14.3% in E-O3 relative to CF under WW and RW conditions, respectively). Consistently with \( V_{\text{cmax}} \) in OL, \( J_{\text{max}} \) significantly declined due to E-O3 (Fig. 4B, P = 0.009) by 17.6% relative to CF. Nevertheless, no interaction between O3 and water treatment was observed. RW significantly reduced the \( V_{\text{cmax}}/J_{\text{max}} \) ratio in OL, but E-O3 significantly increased leaf stomatal limitation (\( L_s \)) only in YL (Fig. 4C–D). No significant interactions were observed in these two parameters. Furthermore, \( V_{\text{cmax}}/J_{\text{max}} \) 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, O3 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-O3 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-WW, respectively), therefore significant interactions were observed between O3 and water treatment. Moreover, both E-O3 and RW reduced the formation of new leaves per plant (Table 2), and the number of abscised leaves per plant strongly increased with increasing O3 levels (Table 2). Total leaf area per plant was significantly lower in the RW treatment relative to the WW treatment (Table 2). Interactions between O3 and water treatment were not significant for any of these parameters (Table 2).

3.5. Dose-response functions

Biomass responses to RW and O3 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 O3 impact 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-O3 levels representing a future scenario, biomass losses under WW and RW were 19.7% and 6.4%, respectively (Fig. 5A).

For O3 dose response relationship, the performance of the model was much higher when POD1 (R\(^2\) = 0.683, P = 0.043) or POD7 (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 PODx. The O3 critical level (CL) for preventing a 4% biomass loss (CLRTAP, 2015) in this poplar clone under different water regimes was POD1 = 5.27 mmol m\(^{-2}\) PLA and POD7 = 4.09 mmol m\(^{-2}\) 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 \( C_a \), and increased WUE in comparison to the WW treatment. Lower \( g_s \) during soil drought in angiosperms may result from effects of leaf turgor (Rodriguez-Dominguez et al., 2016), and higher WUE is an adaptive strategy of plants for living under moisture stress conditions (Oweis, 2012; Rodriguez-Dominguez et al., 2016). Photosynthetic parameters such as \( V_{\text{cmax}} \) 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 O$_3$ (E-O$_3$) under well water (WW) or moderate water stress (RW) conditions. Each point represents the mean ± SD. Statistically significant differences between treatments are noted with different uppercase letters (Tukey test, $P < 0.05$, n = 3).
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 gs 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 (Cvi 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 Fv'/Fm', qP and φPSII (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 Aₘₚ than in gs. E-O₃ accelerated the senescence processes by increasing leaf abscission. The above mentioned

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 CF | WW NF | WW E-O₃ | RW CF | RW NF | RW E-O₃ | O₃ Water | O₃ × Water |
|----------------|-------|-------|---------|-------|-------|---------|---------|-----------|
| Height (cm)    | 96.43 ± 11.16 a | 91.81 ± 3.11 a | 90.07 ± 7.75 a | 66.13 ± 11.04 b | 60.11 ± 7.77 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 ± 6.68 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 (m² 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 ± 2.68 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 (C) and POD (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.
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 Chl, 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 gₛ in water-stressed leaves leading to reduced O₂ 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 nmol m⁻² and POD₂ = 4.1 nmol 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
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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