



Ozone-induced impairment of night-time stomatal closure in O₃-sensitive poplar clone is affected by nitrogen but not by phosphorus enrichment

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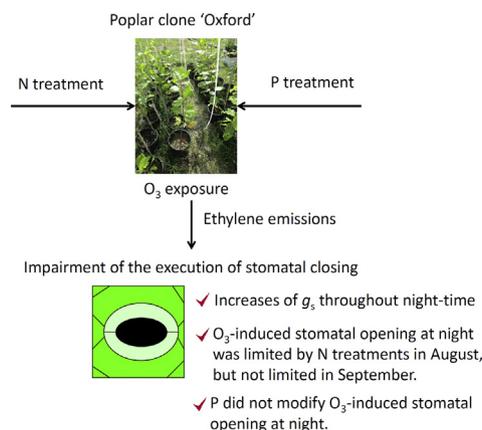
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HIGHLIGHTS

- Effects of O₃ × N × P on night-time g_s were examined in poplar.
- Ozone increased night-time g_s, which was attributed to ethylene emissions.
- The elevated night-time g_s was due to impairment of stomatal closing execution.
- Such stomatal sluggishness was limited by N in August, but not limited in September.
- Phosphorus did not modify the O₃-induced increase in nocturnal g_s.

GRAPHICAL ABSTRACT



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ABSTRACT

Nocturnal transpiration may be a key factor influencing water use in plants. Tropospheric ozone (O₃) and availability of nutrients such as nitrogen (N) and phosphorus (P) in the soil can affect daytime water use through stomata, but the combined effects of O₃, N and P on night-time stomatal conductance (g_s) are not known. We investigated the effects of O₃ and soil availability of N and P on nocturnal g_s and the dynamics of stomatal response after leaf severing in an O₃-sensitive poplar clone (Oxford) subjected to combined treatments over a growing season in an O₃ free air controlled exposure (FACE) facility. The treatments were two soil N levels (0 and 80 kg N ha⁻¹; N0 and N80), three soil P levels (0, 40 and 80 kg P ha⁻¹; P0, P40 and P80) and three O₃ levels

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(ambient concentration, AA [35.0 ppb as hourly mean]; $1.5 \times AA$; $2.0 \times AA$). The analysis of stomatal dynamics after leaf severing suggested that O_3 impaired stomatal closure execution. As a result, nocturnal g_s was increased by $2.0 \times AA$ O_3 in August (+39%) and September (+108%). Night-time g_s was correlated with POD_0 (phytotoxic O_3 dose) and increased exponentially after $40 \text{ mmol m}^{-2} POD_0$. Such increase of nocturnal g_s was attributed to the emission of ethylene due to $2.0 \times AA$ O_3 exposure, while foliar abscisic acid (ABA) or indole-3-acetic acid (IAA) did not affect g_s at night. Interestingly, the O_3 -induced stomatal opening at night was limited by N treatments in August, but not limited in September. Phosphorus decreased nocturnal g_s , although P did not modify the O_3 -induced stomatal dysfunction. The results suggest that the increased nocturnal g_s may be associated with a need to improve N acquisition to cope with O_3 stress.

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1. Introduction

Tropospheric ozone (O_3) pollution can limit the growth of terrestrial plants (Matyssek et al., 2013). The concentration of O_3 has doubled in the northern hemisphere since the 19th century, and currently exceeds the levels known to negatively affect plant physiology in many areas of the world (Mills et al., 2018).

Stomata are pores on the leaf surface through which plants regulate gas exchange with the atmosphere (Larcher, 2003). Technological developments in leaf gas exchange and sap-flow measurements have allowed the detection of low rates of transpiration due to nocturnal stomatal aperture (Caird et al., 2007). Nocturnal control of stomata has been a subject of attention since the days of Darwin and is evolutionarily and ecologically important (Caird et al., 2007; Zeppel et al., 2014; Costa et al., 2015). Nocturnal water loss through stomata is typically 12–23% of daily water loss and is recognized as a significant factor for ecosystem water cycling (de Dios et al., 2015).

Ozone may induce a slower response of stomata to environmental stimuli, which is known as ‘stomatal sluggishness’ (Paoletti, 2005; Hoshika et al., 2013a, 2013b, 2014). Stomatal sluggishness lasts through the night, and may thus result in an increase in stomatal conductance at night (Grulke et al., 2007; Hoshika et al., 2013a, 2013b). The mechanism of stomatal sluggishness is not yet fully understood. Omasa et al. (2002) suggested that O_3 may cause a modulation of the balance in turgor between stomatal guard cells and subsidiary cells, resulting in the impairment of stomatal control. Wilkinson and Davies (2010) suggested that stomatal sluggishness may be triggered by the presence of ethylene and abscisic acid (ABA) under elevated O_3 . There are several reports of an accumulation of those phytohormones induced by O_3 (Tuomainen et al., 1997; Matyssek et al., 2010; McAdam et al., 2017). Blomster et al. (2011) reported that O_3 reduced the production of indole-3-acetic acid (IAA) while increased ABA levels in leaves. The interaction between foliar ABA and IAA may also affect stomatal response (Eamus and Wilson, 1984).

Nitrogen (N) deposition is an additional environmental issue concurrent with O_3 pollution (Reay et al., 2008; Li et al., 2013; Kanakidou et al., 2016). It has been reported that N generally stimulates tree growth (e.g., De Vries, 2014). However, excess N deposition may increase the risk of damage from other abiotic factors (Schulze, 1989). While significant amounts of anthropogenic N are deposited from the atmosphere, the deposition of other macronutrients such as phosphorus (P) is less significant (Peñuelas et al., 2012). Nitrogen deposition can therefore change the nutrient balance in soils and may cause a limitation of key nutrients such as P for plants (Peñuelas et al., 2012). Nitrogen has a major role in production of chlorophyll and nucleic acids, and also stimulates leaf growth as it is involved in cell growth through the synthesis of proteins, while P is an essential structural element in DNA, RNA and phosphorylation as part of adenosine triphosphate (ATP), and thus P is involved in several key plant functions, including photosynthesis, energy storage and transfer (Marschner, 2011). Consistent results have not been reported about the effects of N or P on nighttime g_s (Zeppel et al., 2014). Some studies reported that nocturnal water flux

was increased at low nutrient availability (de Dios et al., 2013; Eller et al., 2017) while the others reported unchanged or even lower night-time transpiration rate at low nutrient condition (Howard and Donovan, 2007; Kupper et al., 2012).

Braun et al. (2017) postulated that the nutrient imbalance between N and P may change the physiological responses of trees to O_3 . In fact, N and P may change the contents of secondary metabolites related to antioxidant systems against the oxidative stress caused by O_3 (Koricheva, 2002; Fares et al., 2008). The question is therefore raised whether N and P may exacerbate or mitigate O_3 -induced stomatal sluggishness. However, the effects of the interaction between O_3 , N and P on nocturnal stomatal response are unknown.

Our main objective was to investigate whether combinations of O_3 , N and P may affect stomatal dynamics and change nocturnal stomatal conductance in a poplar clone (Oxford: *Populus maximoviczii* Henry \times *Populus berolinensis* Dippel) classified as O_3 -sensitive (Zhang et al., 2018a, 2018b). Specifically, we asked the following questions: (1) Can N and P mitigate O_3 -induced stomatal sluggishness at night? (2) Is stomatal sluggishness explained by the hypothesis of an interaction of ABA and ethylene after O_3 exposure (Wilkinson and Davies, 2010) or their interaction with IAA (Eamus and Wilson, 1984)? (3) Is stomatal sluggishness caused by the modulation of guard cell osmoregulation? To quantify stomatal dynamics, we employed the analysis of stomatal response after leaf severing according to Powles et al. (2006).

2. Materials and methods

2.1. Experimental site and plant material

The experimental site was located at Sesto Fiorentino, Florence, central Italy ($43^\circ 48' 59'' \text{ N}$, $11^\circ 12' 01'' \text{ E}$, 55 m a.s.l., mean daily temperature: 22.9° C , total precipitation: 226.6 mm during May to September in 2016). Cuttings of the Oxford poplar clone were prepared in December 2015. After rooting, they were transplanted into 10 L plastic pots filled with a mixture of sand:peat:soil = 1:1:1 (v:v:v) on 1st April 2016 (Zhang et al., 2018a). The mean height (mean \pm S.E.) and number of leaves (mean \pm S.E.) in August 2016 were 57.4 ± 1.1 cm and 23.8 ± 0.4 . The plants were irrigated to field capacity ($29.5 \text{ m}^3 \text{ m}^{-3}$, Paoletti et al., 2017) every 2–3 days to avoid water stress. We set three replicated plots within each of three O_3 levels: ambient air concentration (AA), $1.5 \times AA$ and $2.0 \times AA$, in a Free Air Controlled Exposure (FACE). Ozone was generated from pure oxygen and delivered through a three-dimensional network of teflon tubes (Paoletti et al., 2017). The enhanced O_3 treatments were applied from 1 May to 30 September 2016. Ozone concentrations above plants (approximately 1 m) were continuously recorded by an O_3 monitor (Mod. 202, 2B Technologies, Boulder CO, USA). It can be assumed that leaves (from top to bottom) were equally exposed to O_3 because a $< 20\%$ spatial gradient of O_3 concentration within the plots was detected (Paoletti et al., 2017). Averaged values of 24-h hourly O_3 concentrations (mean \pm S.E.) were 35.0 ± 0.3 ppb in AA, 51.6 ± 0.5 ppb in $1.5 \times AA$ and 66.7 ± 0.6 ppb in $2.0 \times AA$ during the O_3 enrichment period. Maximum and minimum hourly

Table 1

Measured stomatal conductance (g_s) parameters, and target O_3 and nutrient treatments in Oxford poplar clone. Plants were grown under different O_3 exposure (AA, ambient O_3 concentration; $1.5 \times AA$ and $2.0 \times AA$ in 2016; AA and $1.4 \times AA$ in 2015) and subjected to six combinations of soil nutrient treatments [two levels of N (0 and 80 kg N ha^{-1} ; N0 and N80) and three levels of P (0, 40 and 80 kg P ha^{-1} ; P0, P40 and P80)].

Parameter	Year	Measurement month	Target O_3 treatment	Target nutrient treatment
Time course of night-time g_s	2015	August–September	AA, $1.4 \times AA$	N0P0
Instantaneous night-time g_s	2016	June, August, September	AA, $1.5 \times AA$, $2.0 \times AA$	N0P0, N0P40, N0P80, N80P0, N80P40, N80P80
Dynamic g_s after leaf severing	2016	June, August, September	AA, $2.0 \times AA$	N0P0, N0P80, N80P0, N80P80
Ethylene emission	2016	September	AA, $2.0 \times AA$	N0P0, N0P80, N80P0, N80P80
IAA and ABA contents	2016	September	AA, $1.5 \times AA$, $2.0 \times AA$	N0P0, N0P40, N0P80, N80P0, N80P40, N80P80

O_3 concentrations during the experiment were 84.9 and 5.0 ppb in AA, 144.1 and 6.4 ppb in $1.5 \times AA$ and 172.3 and 9.4 ppb in $2.0 \times AA$. AOT40 (accumulated exposure over a threshold of 40 ppb) was $14.4 \text{ ppm} \cdot \text{h}$ in AA, $43.8 \text{ ppm} \cdot \text{h}$ in $1.5 \times AA$ and $71.1 \text{ ppm} \cdot \text{h}$ in $2.0 \times AA$. These elevated O_3 levels were similar to the O_3 concentrations observed in high polluted areas of the Northern Hemisphere (Izuta, 2017). Plants were treated with NH_4NO_3 solutions equaling to 0 kg ha^{-1} (N0) or 80 kg ha^{-1} (N80) and KH_2PO_4 solutions equaling to 0 kg ha^{-1} (P0), 40 kg ha^{-1} (P40) or 80 kg ha^{-1} (P80). The total doses of 40 and 80 kg ha^{-1} corresponded to 196.3 and $392.5 \text{ mg plant}^{-1}$ for both N and P. Nitrogen and P were applied by soil drenching twice a week during the whole treatment period. Soil N concentration (mean \pm SE) in N0 was $1.7 \pm 0.1 \text{ g kg}^{-1}$, and that in N80 treatments was $2.7 \pm 0.1 \text{ g kg}^{-1}$ (as reported in our previous paper Zhang et al., 2018a). These values are in agreement with normal soil N ranges (0.2 to 5 g kg^{-1} , Bowen, 1979). Soil P concentrations (mean \pm S.E.) were $0.5 \pm 0.1 \text{ g kg}^{-1}$ in P0 and $1.0 \pm 0.1 \text{ g kg}^{-1}$ in P80, that are within the range of native P in soils (usually 0.5 to 0.8 g kg^{-1} , and the maximum is found as 1.0 to 1.3 g kg^{-1} , Stevenson and Cole, 1999). Three potted plants per each combination of treatments ($O_3 \times N \times P$) were set in each FACE plot. In total, 162 plants were used. More technical details of the fumigation facility were described in previous papers (Paoletti et al., 2017; Zhang et al., 2018a, 2018b).

In addition to the main experiment mentioned above, we conducted a preliminary experiment on the same poplar clone in 2015. Cuttings rooted in Autumn 2014 were exposed to AA and $1.4 \times AA$ in the O_3 FACE from 1 June to 15 October 2015. Hourly mean O_3 concentrations (mean \pm S.E.) were 35.1 ± 1.0 ppb in AA and 49.0 ± 1.3 ppb in $1.4 \times AA$ during the experimental period 2015. AOT40 was $17.8 \text{ ppm} \cdot \text{h}$ in AA and $40.3 \text{ ppm} \cdot \text{h}$ in $1.4 \times AA$. Plants were grown with the same soil as the N0P0 treatment of the experiment in 2016, and were supplied with water to avoid water stress. We summarized the information of measured stomatal conductance (g_s) parameters and target O_3 and nutrient treatments in Table 1.

2.2. Measurements of the number of leaves

We conducted a survey of the number of attached leaves of poplar Oxford clone for all replicated combinations of treatments ($O_3 \times N \times P$, $n = 3$ plots) on 23 September 2016. In addition, the number of shed leaves was assessed by counting the leaf traces. We considered the number of total emerged leaves as the number of attached + shed leaves.

2.3. Measurements of leaf stomatal conductance

Night-time stomatal conductance (g_s) was measured in fully expanded leaves (5–8th leaf from the tip of a shoot, 1 to 3 plants per replicated plot, $n = 3$ plots, per $O_3 \times N \times P$ treatment). Measurements were carried out with a portable infra-red gas-analyzer (CIRAS-2 PP Systems, Herts, UK) on relatively dry days (relative humidity < 70%), starting at three hours after sunset, i.e., between 21:00 to 23:00 solar time on 24–25 June, 17–18 August and 22–23 September 2016. Measurements lasted around two hours.

In the preliminary experiment in 2015, we measured a time course of night-time g_s (5–8th leaf from the tip of a shoot, 1 to 2 plants per replicated plot, $n = 3$ plots, per O_3 treatment). Measurements were carried out from 18 August to 2 September 2015 (from 17:00 to 8:00 h solar time). Stomatal conductance values were recorded every 5 min and mean values were calculated as hourly data.

In all night-time g_s measurements, the CO_2 concentration in the leaf cuvette (C_a) was set to 400 ppm. The temperature in the cuvette was adjusted manually to mimic ambient temperature measured by a thermometer at plant height. Likewise, relative humidity in the cuvette was similar to ambient humidity.

In addition, to examine the underlying mechanisms of stomatal behavior, we assessed dynamic variations of g_s after leaf severing (Paoletti, 2005; Powles et al., 2006). Here we targeted a limited combination of treatment (Table 1: AA and $2.0 \times AA$ plants treated with N0,

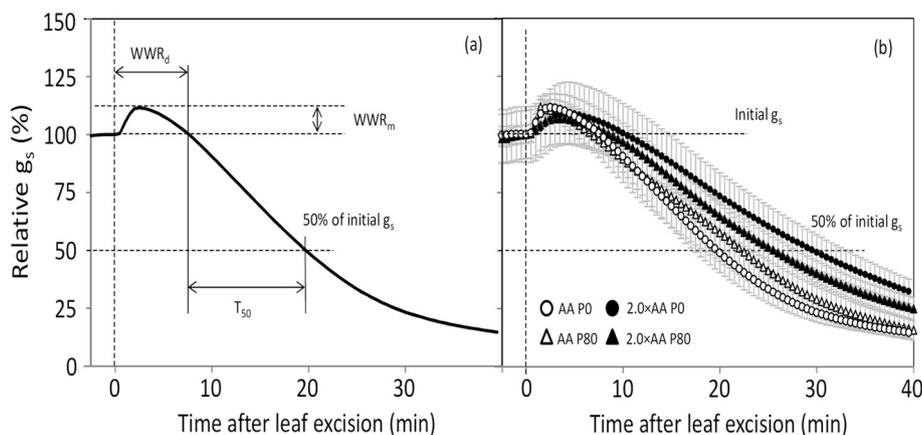


Fig. 1. (a) Time course of stomatal conductance (g_s) after severing a poplar Oxford clone leaf with wrong way response (WWR) duration, WWR_d , and magnitude, WWR_m , and time for 50% decrease in g_s with increasing leaf water stress, T_{50} . (b) Time course of g_s , expressed as 30-sec mean values (\pm S.E.), after severing a leaf in September ($n = 3$ plots, white circle: AA, P0; white triangle: AA, P80; black circle: 2.0AA, P0; black triangle: 2.0AA, P80).

N80, P0 and P80) since this kind of measurements is time consuming (up to 60 min per each) and must be carried out under similar environmental conditions. Measurements were carried out three times, from 8:00 to 12:00 solar time on 25–27 June, 18–20 August and 23–25 September 2016. For all the measurements, leaf temperature (25 °C), relative humidity (40 to 60%), photosynthetic photon flux density (1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and CO_2 concentration (400 ppm) were kept constant. When g_s reached equilibrium under constant light, the leaf petiole was cut according to the methodology suggested by Paoletti (2005). The variation of g_s was then recorded with 30 s intervals for 40 min. Two phases of g_s response were observed (Fig. 1): (i) a transient increase, called the transient 'wrong-way response' (WWR), due to a difference in turgor pressure between guard cell and epidermal cells (Powles et al., 2006); and (ii) a decrease following increasing leaf water stress. Duration and magnitude of WWR (WWR_d and WWR_m , respectively) and time for 50% decrease of g_s (T_{50}) were recorded. Based on modeling of stomatal water relations, WWR_d and WWR_m relate to induction of the guard cell osmoregulation during WWR while T_{50} is an index for the execution of the guard cell osmoregulation (Powles et al., 2006).

2.4. Assessment of ethylene emission

After the g_s measurements on 23–25 September 2016, three leaves per plant in AA and $2.0 \times \text{AA}$ treatments with four combinations of nutrients (N0, N80, P0 and P80) were collected (5–8th leaf from the tip of a shoot) and placed in a 120 mL glass vial sealed with Teflon septum and Parafilm®. Ethylene accumulation in the vial headspace was measured after 1 h incubation at room temperature by an ethylene detector ETD-300 (Sensor Sense B.V., Nijmegen, The Netherlands <http://www.sensor-sense.nl>). The detector consists of a CO_2 laser and a photoacoustic cell, and is able to detect on-line about 300 ppt by volume of ethylene within a 5-s time scale. The gas handling was performed by a valve control box (type VC-6, Sensor Sense B.V., Nijmegen, The Netherlands), designed for measuring up to six sampling cuvettes. A scrubber with soda lime pellet (Sigma) and CaCl_2 was used to reduce the CO_2 and water content in the gas flow. Control air was provided by a compressed air source and contained $<0.001 \mu\text{l l}^{-1}$ ethylene.

In this experiment, as the ethylene production from the leaves was expected to be low, a stop-and-flow mode was chosen. In this way, ethylene accumulated in the headspace for 1 h in the vial, before it was transported to the ethylene detector with a high flow rate of 5 L h^{-1} for a period of 15 min. This flow rate was considered the best one by preliminary experiments to detect the ethylene peak of the target samples. After the ethylene measurement, leaves were immediately weighted with an analytical balance (Model BP110, Sartorius) and ethylene emission rate was expressed in nanomoles per hour per gram of fresh weight. As a supplementary information, the results as a function of leaf area, dry weight, relative water content (i.e., the ratio of dry to fresh weight), specific leaf weight (leaf dry weight per leaf area) were also shown.

2.5. Measurement of abscisic acid and indole-3-acetic acid contents

Three leaves per plant (5–8th leaf from the tip of a shoot) were collected (23–25 September 2016) from all replicated combinations of treatments, to determine the ABA and indole-3-acetic acid (IAA) contents. The leaves were frozen in liquid N immediately after collection and afterward freeze-dried.

The content of the free forms of IAA and ABA was determined according to Brilli et al. (2019). Approximately 0.02 g DW of lyophilized leaf material were extracted with 1 mL of isopropanol:acetic acid (95:5, v/v), to which 100 ng each of $^{13}\text{C}_6$ -IAA and $^2\text{H}_6$ -ABA (OChemIm Ltd., Olomouc, Czech Republic) were added as internal standards. The extraction was repeated twice, each time adding 500 μl extraction solvent. The total extracts (1.5 mL) were evaporated to dryness with a

rotary evaporator. The residues were re-suspended in 300 μl methanol and methylated with 500 μl diazomethane in the dark for about 30 min, then dried under a gentle N_2 gas stream (Baraldi et al., 1988). The samples were finally re-suspended in 30 μl ethyl acetate. Four μl of the sample were injected into a GC-MS system (7890A-5975C, Agilent Technologies, US) in splitless mode onto a HP1 capillary column (length 60 m, inner diameter 0.25 mm; film thickness 0.25 μm , Agilent Technologies, US). Helium was employed as a carrier gas and provided at a flow rate of 1 mL min^{-1} . For absolute quantification, the endogenous hormone levels were estimated from the corresponding peak area: IAA content was calculated on the ratios between m/z 130/136 and m/z 189/195, while ABA content was calculated on the ratios between m/z 190/194 and 162/166, according to the principles of isotope dilution (Cohen et al., 1986).

2.6. Relationship between night-time stomatal conductance and stomatal ozone uptake (POD)

Phytotoxic O_3 dose above a flux threshold of Y (POD_Y) is currently recommended in the risk assessment of O_3 for forest species (CLRTAP, 2017). The POD_Y above an hourly stomatal uptake threshold of $0 \text{ nmol m}^{-2} \text{ s}^{-1}$ (POD_0) was calculated for the target poplar clone according to our previous work (Zhang et al., 2018a), and used to assess dose-response relationships between the ratio of night-time g_s to day-time value (f_{night}) and stomatal O_3 uptake.

2.7. Statistical analysis

The effects of O_3 , N and P on the number of leaves, ethylene emission rate, ABA and IAA contents were tested via three-way analysis of variance (ANOVA). For this ANOVA test, ethylene emission rate was log-transformed. In addition, the effects of O_3 , N, P and measurement month on leaf gas exchange and dynamic stomatal responses after severing a leaf were tested via four-way ANOVA. Differences among means were tested by the Tukey HSD test. Data were tested for normal distribution (Kolmogorov-Smirnov D-test) and homogeneity of variance (Levene's test). To assess the effects of ethylene emissions, ABA and IAA on stomatal parameters in September, a multiple linear regression

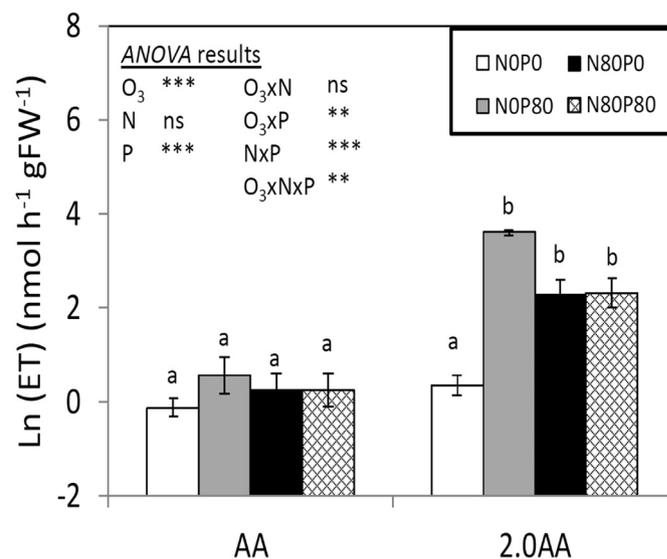


Fig. 2. Log-transformed ethylene emission rate (ET) from leaves of Oxford poplar clone under different O_3 exposure (AA, ambient O_3 concentration; $2.0 \times \text{AA}$) and subjected to four combinations of soil nutrient treatments [two levels of N (0 and 80 kg N ha^{-1} ; N0 and N80) and two levels of P (0 and 80 kg P ha^{-1} ; P0 and P80)]. Bars represent mean \pm S.E. ($n = 3$ plots). Asterisks show the significance of ANOVA tests: *** $p < 0.001$, ** $p < 0.01$, ns: not significant. Different letters show significant differences among treatments ($p < 0.05$, Tukey test).

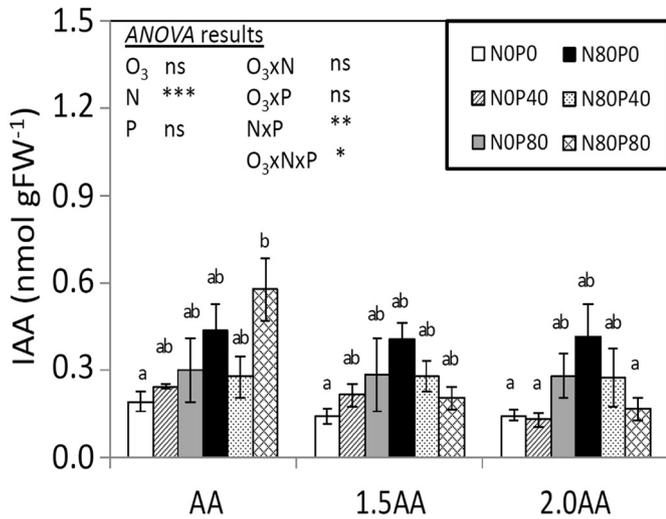


Fig. 3. Indole-3-acetic acid (IAA) contents in leaves of Oxford poplar clone under different O₃ concentrations (AA, ambient O₃ concentration; 1.5 × AA; 2.0 × AA) and six combinations of soil nutrient treatment [two levels of N (0 and 80 kg N ha⁻¹; N0 and N80) and three levels of P (0, 40 and 80 kg P ha⁻¹; P0, P40 and P80)]. Bars represent mean ± S.E. (n = 3 plots). Asterisks show the significance of ANOVA: *** p < 0.001, ** p < 0.01, * p < 0.05, ns: not significant. Different letters show significant differences among treatments (p < 0.05, Tukey test).

analysis was applied considering their interactions, i.e., effects of ethylene × ABA, ethylene × IAA, ABA × IAA and ethylene × ABA × IAA. In the multiple regressions, the variables were log-transformed. Simple linear regressions were applied to examine a relationship between night-time g_s and stomatal parameters during the WWR. A quadratic function was employed to explain a relationship between f_{night} and POD₀. Results were considered significant at p < 0.05. All statistical analyses were performed in R 3.5.1 (R Core Team, 2018).

3. Results

3.1. Number of attached and shed leaves

Ozone decreased the number of attached leaves while N increased it (Fig. S1). The increase of the attached leaves due to N was limited by P treatments (+51% by N80 compared to N0 in P0 treatments; +31% by N80 compared to N0 in P40 treatments; +18% by N80 compared to N0 in P80 treatments), as indicated by the interaction between N and P. The number of shed leaves was increased by O₃ (+56% by 1.5 × AA compared to AA; +142% by 2.0 × AA compared to AA) and N (+16% by N80 compared to N0). A significant interaction of the three factors

(O₃ × N × P) was found for shed leaves, suggesting that the O₃-induced increase of shed leaves was enhanced by the combined N and P treatments. Total leaves (attached+shed leaves) increased by O₃ (+1% by 1.5 × AA compared to AA; +12% by 2.0 × AA compared to AA) and N (+25% by N80 compared to N0). A significant interaction of N and P indicates that the increase of the total leaves due to N was limited by P treatments (+37% by N80 compared to N0 in P0 treatments; +23% by N80 compared to N0 in P40 treatments; +17% by N80 compared to N0 in P80 treatments).

3.2. Leaf ethylene emission, abscisic acid and indole-3-acetic acid contents

Ethylene emission was increased by 2.0 × AA O₃ exposure compared to AA (+67% in NOP0, +650% in N80P0, +1638% in NOP80, +678% in N80P80) (Fig. 2), even though the significant difference among the O₃ treatments was found only in high nutrient treatments (N80 and/or P80) (Fig. 2, S2). Ozone did not affect leaf ABA content, while N80 increased it by 44% (Fig. S3, calculated as all N80 treatments [N80P0, N80P40, N80P80]/all NO treatments [NOP0, NOP40, NOP80]). There was a significant interactive effect of N and P, because the increase in ABA content caused by N treatment (N80) was limited by P addition (+208% by N80 compared to N0 in P0 treatments; -14% by N80 compared to N0 in P40 treatments; +10% by N80 compared to N0 in P80 treatments). Ozone did not affect leaf IAA content, while N treatments significantly increased it (Fig. 3: +58% in N80 compared to N0 treatments). We found a significant interaction between N and P on IAA contents, indicating that N-induced increase of leaf IAA was lower in P80 than P0 treatments (+160% increase by N80 in P0; +10% increase by N80 in P80). The interaction of the three factors (O₃ × N × P) was significant for IAA, showing that O₃ caused a significant decrease of leaf IAA (-71%) when adding N and P together.

3.3. Night-time stomatal conductance

Oxford poplars showed night-time g_s values in the range of 0.04 to 0.15 mol m⁻² s⁻¹ during the study period (Fig. 4). In June, night-time g_s was increased by N addition (+47%). However, such a difference disappeared in August and September as confirmed by a significant interaction between N and measured month. Phosphorus decreased night-time g_s (-5% in P40 and -21% in P80 compared to P0 across the measurement months). Ozone significantly increased the night-time g_s especially in August (+12% in 1.5 × AA and +39% in 2.0 × AA compared to AA) and in September (+42% in 1.5 × AA and +108% in 2.0 × AA compared to AA). A significant interaction was found between O₃, N and measurement month, indicating that the O₃-induced increase of night-time g_s was limited by N treatments in August (N0: +8% in 1.5 × AA and +67% in 2.0 × AA compared to AA, N80: +16% in 1.5 × AA

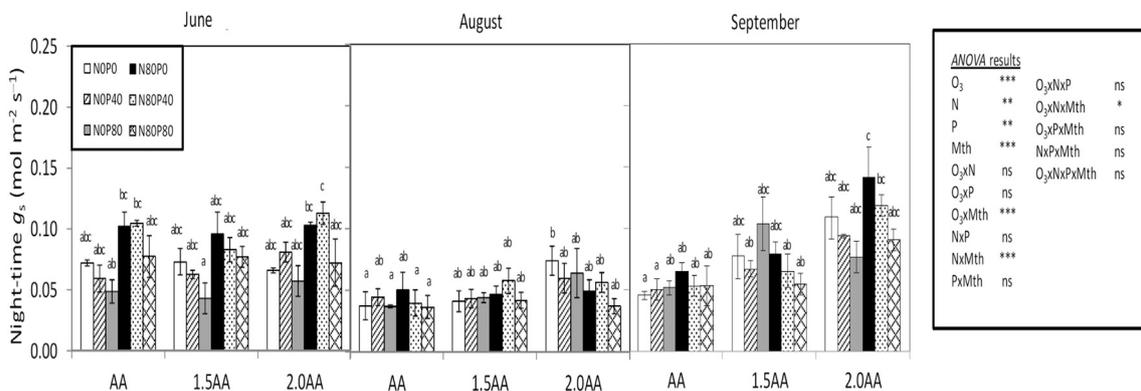


Fig. 4. Seasonal changes of night-time stomatal conductance (g_s) of poplar Oxford clone in 2016 under different O₃ concentrations (AA, ambient O₃ concentration; 1.5 × AA; 2.0 × AA) and subjected to six combinations of nutrient treatment in the soil [two levels of N (0 and 80 kg N ha⁻¹; N0 and N80) and three levels of P (0, 40 and 80 kg P ha⁻¹; P0, P40 and P80)]. Bars represent mean ± S.E. (n = 3 plots). Asterisks show the significance of ANOVA tests: *** p < 0.001, ** p < 0.01, * p < 0.05, ns: not significant. Different letters show significant differences among treatments in each month (p < 0.05, Tukey test).

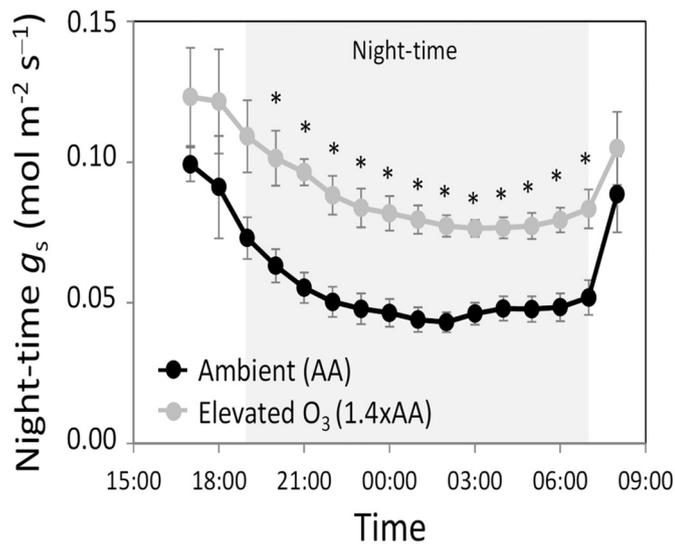


Fig. 5. Time course of stomatal conductance (g_s) during night-time of poplar Oxford clone exposed to two O_3 treatments (AA, ambient O_3 concentration; $1.4 \times AA$) in 2015. Data are shown as mean \pm S.E. ($n = 3$ plots). Asterisks show significant differences among treatments in each measurement time ($p < 0.05$, Student t -test).

and +13% in $2.0 \times AA$ compared to AA), but not limited in September (N0: +68% in $1.5 \times AA$ and +89% in $2.0 \times AA$ compared to AA, N80: +16% in $1.5 \times AA$ and +105% in $2.0 \times AA$ compared to AA).

In the time course of night-time g_s , significant differences of g_s between AA and elevated O_3 ($1.4 \times AA$) were found during 20:00 h to 7:00 h (Fig. 5). The g_s values during night-time were 0.04 to $0.07 \text{ mol m}^{-2} \text{ s}^{-1}$ in AA and 0.07 to $0.11 \text{ mol m}^{-2} \text{ s}^{-1}$ in $1.4 \times AA$.

The ratio of night-time g_s to daytime value (f_{night}) increased with increasing POD_0 (Fig. 6). The nocturnal g_s increased remarkably when POD_0 values exceeded 40 mmol m^{-2} and reached a peak of 66% increase relative to the daytime value. Multiple linear regression analyses revealed that the increase of night-time g_s in September was significantly related to ethylene emissions (Table 2). No significant effects of ABA and IAA on g_s at night were found.

3.4. Stomatal parameters after cutting the petiole

Steady-state g_s in light-saturated conditions was 0.2 to $0.4 \text{ mol m}^{-2} \text{ s}^{-1}$ (Fig. S4). Ozone exposure induced a decrease of steady-state g_s in September (-21%) although no significant difference

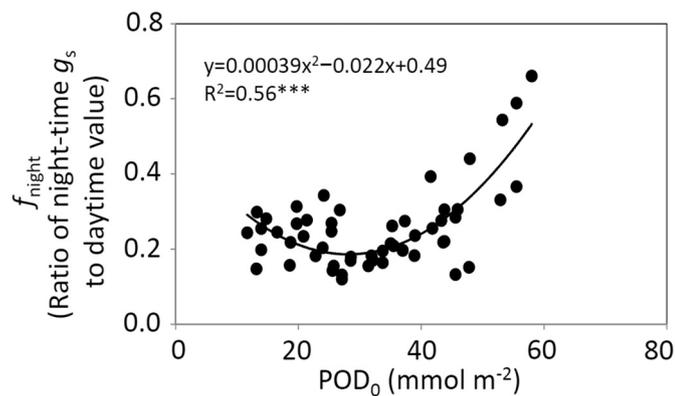


Fig. 6. Quadratic relationship between f_{night} (the ratio of night-time stomatal conductance to daytime value) and phytotoxic O_3 dose (POD_0) in poplar Oxford clone during summer 2016 (August and September) under different O_3 concentrations (AA, ambient O_3 concentration; $1.5 \times AA$; $2.0 \times AA$) and subjected to four combinations of nutrient treatment [two levels of N (0 and 80 kg N ha^{-1} ; N0 and N80) and two levels of P (0 and 80 kg P ha^{-1} ; P0 and P80)]. Asterisks show the significance of regression: *** $p < 0.001$.

between the two O_3 treatments was found in June and August. After severing the leaf, O_3 reduced the degree of stomatal closure over time (Fig. 1). In fact, $2.0 \times AA$ significantly increased T_{50} in P0 from $10.2 \pm 0.8 \text{ min}$ (AA) to $24.7 \pm 2.3 \text{ min}$ in September (Fig. 1, Fig. 7, +142%). On the other hand, both the magnitude and duration of WWR were not affected by O_3 and nutrient availability. Similarly, with regard to night-time g_s , the increase of T_{50} was significantly related to ethylene emission according to multiple linear regression analyses (Table 2). WWR_m decreased with increasing ethylene emission. A significant interaction between ABA and IAA on WWR_m was found, which suggests that ABA decreased WWR_m in the presence of low amount of IAA.

The relationships between night-time g_s and stomatal parameters during the WWR are shown in Fig. 8. A significant positive relationship between night-time g_s and T_{50} was obtained ($R^2 = 0.41$, $p = 0.001$) while no significant relationships were found between the nocturnal g_s and WWR_m or WWR_d .

4. Discussion

4.1. Effects of ozone, nitrogen and phosphorus on leaf ethylene emission, abscisic acid and indole-3-acetic contents

It has been reported that ethylene production rapidly increases in plants exposed to O_3 (Di Baccio et al., 2012). This was confirmed in

Table 2

Results of multiple linear regression analyses for parameters of stomatal sluggishness in an O_3 -exposed Oxford poplar clone in September 2016. Effects of leaf ethylene emission rate, abscisic acid (ABA) contents and indole-3-acetic acid (IAA) contents on those parameters were examined. Night-time g_s ; stomatal conductance during night-time; T_{50} ; time for 50% decrease of g_s after leaf severing; WWR_d ; duration of the transient 'wrong-way response' (WWR) after leaf severing; WWR_m ; magnitude of the WWR after leaf severing. p values < 0.05 are given in bold fonts.

	Coeff.	Std. Err.	t-value	p-value
Night-time g_s				
Intercept	-2.59	0.122	-21.2	<0.001
Ethylene	0.277	0.107	2.60	0.019
ABA	-0.523	0.389	-1.34	0.198
IAA	0.00887	0.219	0.0406	0.968
Ethylene * ABA	-0.451	0.374	-1.20	0.246
Ethylene * IAA	0.149	0.211	0.707	0.490
ABA * IAA	-0.181	0.519	-0.350	0.731
Ethylene * ABA * IAA	-0.527	0.560	-0.940	0.361
T_{50}				
Intercept	2.63	0.108	24.5	<0.001
Ethylene	0.267	0.0938	2.85	0.011
ABA	-0.294	0.343	-0.858	0.404
IAA	-0.0725	0.192	-0.377	0.711
Ethylene * ABA	-0.235	0.330	-0.712	0.487
Ethylene * IAA	0.0453	0.186	0.243	0.811
ABA * IAA	0.425	0.457	0.932	0.365
Ethylene * ABA * IAA	-0.453	0.493	-0.920	0.371
WWR_d				
Intercept	2.33	0.152	15.3	<0.001
Ethylene	0.135	0.133	1.02	0.325
ABA	-0.342	0.485	-0.706	0.491
IAA	-0.179	0.272	-0.660	0.519
Ethylene * ABA	-0.295	0.496	-0.634	0.535
Ethylene * IAA	-0.0656	0.263	-0.250	0.806
ABA * IAA	-0.0554	0.645	-0.0858	0.933
Ethylene * ABA * IAA	-0.147	0.697	-0.210	0.836
WWR_m				
Intercept	-3.40	0.103	-33.1	<0.001
Ethylene	-0.276	0.0895	-3.08	0.007
ABA	-0.113	0.327	-0.347	0.733
IAA	-0.112	0.184	-0.608	0.552
Ethylene * ABA	0.183	0.314	0.582	0.569
Ethylene * IAA	-0.288	0.177	-1.62	0.124
ABA * IAA	-0.931	0.435	-2.14	0.048
Ethylene * ABA * IAA	0.587	0.470	1.25	0.231

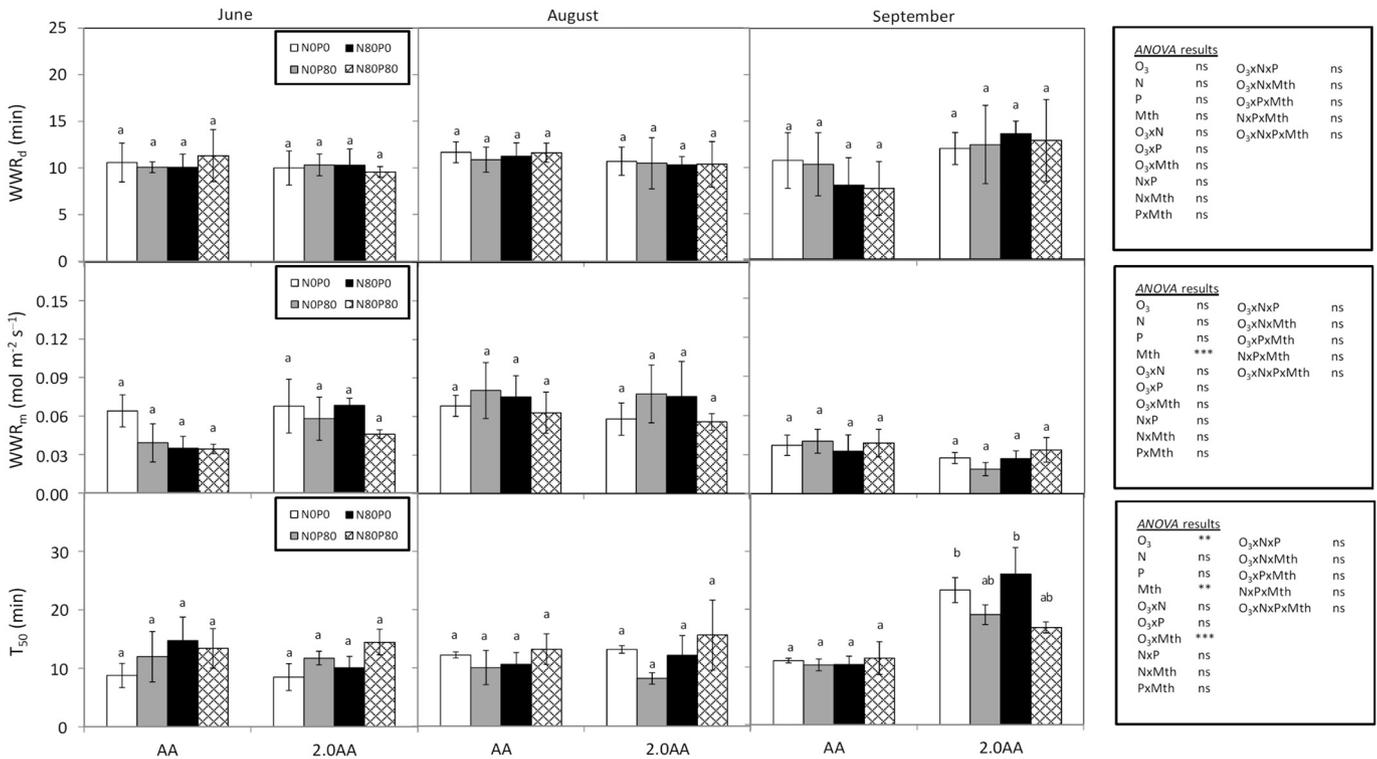


Fig. 7. Seasonal changes of stomatal parameters during the ‘wrong-way response’ (WWR) after leaf excision (WWR_d: duration of WWR, WWR_m: magnitude of WWR, T₅₀: time for 50% decrease of stomatal conductance after the WWR) of poplar Oxford clone in 2016 under different O₃ concentrations (AA, ambient O₃ concentration; 1.5 × AA; 2.0 × AA) and subjected to four combinations of nutrient treatment in the soil [two levels of N (0 and 80 kg N ha⁻¹; N0 and N80) and two levels of P (0 and 80 kg P ha⁻¹; P0 and P80)]. Bars represent mean ± S.E. (n = 3 plots). Asterisks show the significance of ANOVA tests: *** p < 0.001, * p < 0.05, ns: not significant. Different letters show significant differences among treatments in each month (p < 0.05, Tukey test).

the present study. High nutrient availability such as N80 and P80 increased the O₃-induced ethylene emission in poplar leaves. Ethylene is known as senescence hormone (e.g., Iqbal et al., 2017). Poplars may often show a new leaf development to replace the leaves injured by O₃-induced premature senescence especially in high soil nutrient availability (Pell et al., 1995). In fact, in the Oxford poplar clone, O₃-induced

leaf shedding was accelerated by combined N and P treatments (Fig. S1). This result is also confirmed by an O₃-induced increase of proline and ABA/IAA ratio especially in N80 and P80 treatments as reported in a previous paper (Podda et al., 2019), which results in a signaling response that might be part of a premature leaf senescence process. Previous studies reported that O₃ may increase foliar ABA content (Bianco

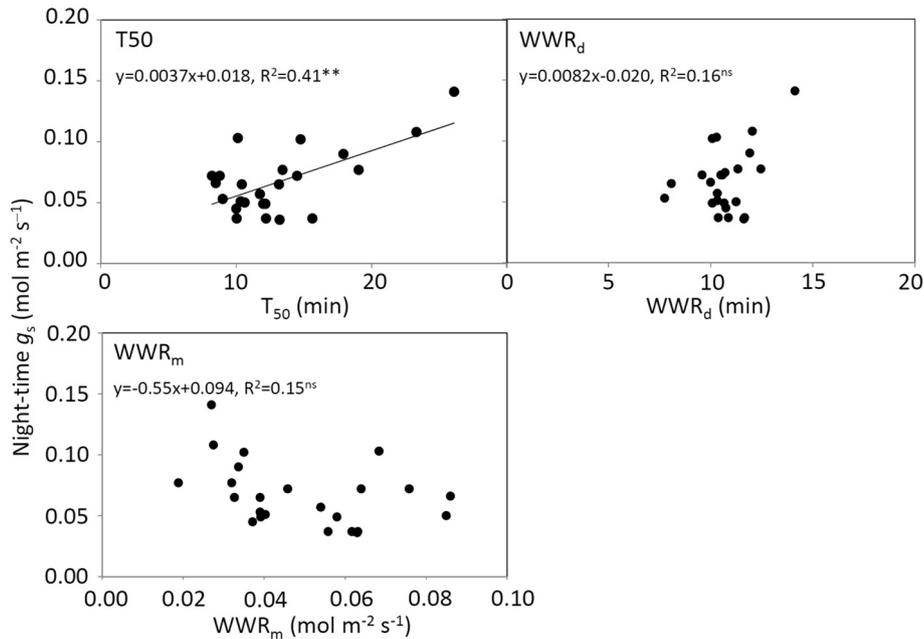


Fig. 8. Linear relationships between night-time stomatal conductance and stomatal parameters during the ‘wrong-way response’ (WWR) after leaf excision (WWR_d: duration of WWR, WWR_m: magnitude of WWR, T₅₀: time for 50% decrease of stomatal conductance after the WWR) in O₃-exposed poplar Oxford clone in 2016. Asterisks show the significance of regressions: ** p < 0.01, ns denotes not significant.

and Dalstein, 1999; Matyssek et al., 2010; McAdam et al., 2017). However, our results did not support this process. In fact, several studies also reported no increase of ABA concentration after O₃ exposure (Kobriger et al., 1984; Mao et al., 2017). Foliar ABA contents increased in N80 treated poplar leaves. There is some evidence linking soil nutrient status and the levels of ABA (Radin et al., 1982; Peuke et al., 1994; Wilkinson and Davies, 2002), which are involved in root development at high N condition (Kiba et al., 2011). Nitrogen-induced stimulation was similarly found in foliar IAA. Pavlíková et al. (2012) reported an increase of foliar IAA in *Festulolium* plants after ammonium nitrogen fertilization. It has been reported that O₃ may induce a reduction of IAA contents in leaves (e.g., Blomster et al., 2011). The present study indicated that O₃ decreased foliar IAA only in N80P80 treatments. However, there is little knowledge about interactive effects of O₃ and soil nutrient availability on foliar IAA contents. Further work would be required to elucidate the mechanism of this interaction.

4.2. Effects of ozone, nitrogen and phosphorus on night-time stomatal conductance and dynamic stomatal response

Ozone increased g_s at night in this O₃-sensitive poplar clone, as reported in Californian oaks (Grulke et al., 2007) and Siebold's beech (Hoshika et al., 2013b). The nocturnal g_s increased with increasing stomatal O₃ uptake (Fig. 6). As a result, in 2.0 × AA, poplars showed 0.10 to 0.15 mol m⁻² s⁻¹ of night-time g_s in September (Fig. 4). Previous meta-analytic reviews reported that night-time g_s generally has 5–40% of the magnitude of day-time g_s (Caird et al., 2007; Zeppel et al., 2014; Hoshika et al., 2018a). Our result showed that night-time g_s after O₃ exposure became 33–66% of the day-time g_s values in September. Such an incomplete stomatal closure due to elevated O₃ enhances nocturnal water loss, which may result in dysfunction of water regulation such as a reduction of hydraulic redistribution, with an increased risk of water deficit stress (Domec et al., 2012).

It has been reported that nutrient availability affects night-time g_s (Ludwig et al., 2006). In Oxford clone, N addition increased g_s at night, at least in June. Elevated nocturnal g_s during N enrichment was reported in *Arabidopsis thaliana* (Howard and Donovan, 2007) and *Populus tremula* × *P. tremuloides* (Kupper et al., 2012). Nitrogen addition stimulated new leaf formation in our poplars in early summer (Zhang et al., 2018a). Several studies reported that flushing leaves have higher night-time g_s than older leaves (Phillips et al., 2010). In fact, the observed positive effect of N application on nocturnal g_s in poplars was not found in the later growing season due to leaf aging. Phosphorus was found to decrease nocturnal g_s of our poplars. This is in agreement with the findings by de Dios et al. (2013) where nocturnal g_s of *Eucalyptus tereticornis* was lower at high than at low soil P content. de Dios et al. (2013) assumed that P dependency of night-time g_s may be due to changes in wood density by P treatments. In addition, P may allow a fine control of stomata apertures at night as it is involved in energy production as a component of ATP, which acts as an energy currency of guard cells for stomatal movement (Marten et al., 2007).

One of the most common hypotheses for the evolutionary advantage of night-time stomatal aperture is nutrient acquisition in low-nutrient environments or desert (e.g., Daley and Phillips, 2006). However, the response of night-time g_s to nutrient availability is rather species-specific (Caird et al., 2007). Kupper et al. (2012) reported that poplars, which are adapted to grow in fertile soil, enhanced night-time g_s in periods of high N demand for intensive leaf growth, although night-time g_s did not increase under low N conditions. Interestingly, in our poplars, O₃-induced increase of night-time g_s was exacerbated in low N condition (N0), while such night-time stomatal opening was limited by N-rich condition (N80) in August (Fig. 4). Ozone stimulated new leaf formation to replace old damaged leaves (Fig. S1), as a result of root N uptake and retranslocation of N from old leaves (Pell et al., 1995). Since elevated O₃ generally reduces the retranslocation rate of N (Kam et al., 2015), night-time stomatal opening may therefore assist with root N

uptake for further leaf production especially in low soil N availability. However, O₃-induced elevated night-time g_s was found regardless of the N treatment in September.

The transient WWR of stomata has been used to assess O₃-induced stomatal sluggishness in previous studies (Paoletti, 2005; Hoshika et al., 2013a, 2014). Those studies demonstrated slower closing responses of stomata to the severe water stress imposed by leaf severing. In addition to the previous findings, the present study found that such a sluggish response of stomata occurred for poplar only in the late growing season (Fig. 7). The significant relationship between T₅₀ and night-time g_s implies that the increase of night-time g_s due to elevated O₃ may be caused by a slower execution of the guard cell osmoregulation (Powles et al., 2006). A question raised, however, is whether stomata could eventually close during night-time or not. The time course of night-time g_s showed a downward convex curve (Fig. 5) as reported before in *Eucalyptus* (de Dios et al., 2013). Night-time g_s reached the minimum value several hours after sunset, i.e. at 2:00 h to 3:00 h (solar time) regardless of the O₃ treatments, and again slightly increased later in the night. Ozone therefore did not affect such a 'circadian rhythm'. The difference in g_s between AA and 1.4 × AA was fairly constant over the night (60 to 80% increase in 1.4 × AA relative to AA). This suggests that O₃ permanently impaired the stomatal closing response at night.

Several hypotheses have been proposed to explain a slower or less efficient stomatal control after O₃ exposure (Paoletti and Grulke, 2005). One of the main hypotheses is a modulation of ABA and ethylene biosynthesis due to O₃ exposure (Wilkinson and Davies, 2010) or their interaction with IAA (Eamus and Wilson, 1984). However, such an interaction between ethylene emission and ABA or their interaction with IAA was not relevant for the sluggish stomatal response at night in our experiment, although ABA and IAA had some effects on stomatal water relation (i.e., WWR_m). The observed stomatal sluggishness at night was related only to ethylene emission (Table 2). It has been reported that ethylene emissions cause stomatal dysfunction in several plant species, which results in the inhibition of stomatal closure (Azuma et al., 2003; Chen et al., 2013). However, the mechanisms are still not clear.

In conclusion, we found evidence that O₃ induced sluggish closure of stomata at night and such night-time stomatal opening were limited by N treatments in August, but not limited in September. Our previous biomass assessment study suggested an increased demand for N for poplar growth under elevated O₃, because O₃ decreases N use efficiency (Zhang et al., 2018a). The increased nocturnal g_s may therefore be associated with N acquisition to cope with O₃ stress. The increase of night-time g_s was related to ethylene emissions after O₃ exposure. The incomplete stomatal closure at night coincided with impairment of the execution rather than the induction of the closing response of stomata. The enhanced nocturnal stomatal opening in elevated O₃ may increase total water requirements. The assessment of plant-level nocturnal water use after O₃ exposure will be the next step of this study.

In general, O₃ concentration can remain high at night in mountainous areas and plants are sensitive to O₃ during night rather than daytime since plant defenses are lower at night (Musselman and Minnick, 2000). However, current O₃ risk assessments do not consider the night-time stomatal aperture (CLRTAP, 2017). Hoshika et al. (2018a, 2018b) pointed out the importance of nocturnal g_s for modeling O₃ risk assessments. Our results indicate that night-time g_s in poplars increased exponentially after 40 mmol m⁻² POD₀ in poplar. Our result is essential to achieve the parameterization of species-specific nocturnal stomatal O₃ flux in the modeling for the risk assessment of forests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.07.288>.

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