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Physiological ecology of woody species in response to air pollution and climate changes

Effects of nitrogen and phosphorus imbalance on photosynthetic traits of poplar Oxford clone under ozone pollution

Lu Zhang[1] · Yasutomo Hoshika[2] · Elisa Carrari[2] · Lorenzo Cotrozzi[3] · Elisa Pellegrini[3] · Elena Paoletti[2]

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Abstract

Ozone (O_3) pollution and the availability of nitrogen (N) and phosphorus (P) in the soil both affect plant photosynthesis and chlorophyll (Chl) content, but the interaction of O_3 and nutrition is unclear. We postulated that the nutritional condition changes plant photosynthetic responses to O_3. An O_3-sensitive poplar clone (Oxford) was subject to two N levels (N0, 0 kg N ha^{-1}; N80, 80 kg N ha^{-1}), two P levels (P0, 0 kg P ha^{-1}; P80, 80 kg P ha^{-1}) and three levels of O_3 exposure (ambient concentration, AA; 1.5 × AA; 2.0 × AA) over a growing season in an O_3 free air controlled exposure (FACE) facility. The daily change of leaf gas exchange and dark respiration (R_d) were investigated at mid-summer (August). Chl *a* fluorescence was measured three times in July, August and September. At the end of the growing season, Chl content was measured. It was found that Chl content, the maximum quantum yield (F_v/F_m), Chl *a* fluorescence performance index (PI) and gas exchange were negatively affected by elevated O_3. Phosphorus may mitigate the O_3-induced reduction of the ratio of photosynthesis to stomatal conductance, while it exacerbated the O_3-induced loss of F_v/F_m. Nitrogen alleviated negative effects of O_3 on F_v/F_m and PI in July. Ozone-induced loss of net photosynthetic rate was mitigated by N in medium O_3 exposure (1.5 × AA). However, such a mitigation effect was not observed in the higher O_3 level (2.0 × AA). Nitrogen addition exacerbated O_3-induced increase of R_d suggesting an increased respiratory carbon loss in the presence of O_3 and N. This may result in a further reduction of the net carbon gain for poplars exposed to O_3.

Keywords Chlorophyll *a* fluorescence · Nitrogen · Ozone · Phosphorus · Photosynthesis · Stomatal conductance

Introduction

Tropospheric ozone (O_3) pollution is a serious concern to plant health in many areas of the world (Matyssek et al. 2013; Mills et al. 2018). Ozone pollution has been rising continuously since the preindustrial age (Vingarzan 2004), although control measures on precursor emission are now successful in some areas of the world, such as North America (Cooper et al. 2014) and Europe (Paoletti et al. 2014; Sicard et al. 2013).

Nitrogen oxides are primary sources for O_3 formation in the atmosphere (Crutzen 1970). In the United States, Ollinger et al. (2002) reported that high O_3 concentration was often observed in regions where nitrogen (N) deposition from the atmosphere was high. Nitrogen input generally stimulates plant growth (Wooliver et al. 2017). Another important macronutrient influencing the change of plant growth is phosphorus (P) (Marchner 2011). Phosphorus plays a role in photosynthesis, energy storage and transfer, cell division and the development of new tissue (Marchner, 2011). Phosphorus contents in soils are usually determined by land use and in particular fertilization practices, while global P deposition from the atmosphere represents a relatively small amount (3–3.5 Mton $year^{-1}$) mostly derived from wind-eroded particles and biomass burning aerosols (Peñuelas et al. 2012).

✉ Yasutomo Hoshika
yasutomo.hoshika@ipsp.cnr.it

[1] College of Horticulture and Landscape Architecture, Northeast Agricultural University, Changjiang Road 600, Harbin 150030, China

[2] National Research Council of Italy, Via Madonna del Piano 10, 50019 Florence, Italy

[3] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto, 80, 56124 Pisa, Italy

Poplars are widely used for wood production and as a model plant in plant physiology (Christersson 2010), and are generally susceptible to oxidative stress due to O_3 exposure (Hoshika et al. 2018). Poplar plantation is established in various soil conditions with low- (e.g., volcanic ash soils, Hoosbeek et al. 2004) and high-nutritional availability (e.g., agricultural field soils, Arevalo et al. 2011). Nutrient-rich conditions may reduce the production of secondary metabolites in relation to plant resistance to abiotic and/or biotic stress, i.e., the carbon-nutrient balance hypothesis (Bryant et al. 1983) thus changing the metabolic capacity for coping with O_3 stress (Schulze 1989), although this hypothesis explains mainly behavior of phenolic compounds (Koricheva 2002). Braun et al. (2017) reported that the basal area increment in beech and Norway spruce may be limited by O_3 and that a significant interaction exists with nutrition such as N and P. However, our knowledge of plant responses to O_3 under various nutritional conditions is still limited. Only a few studies have investigated the effects of different N loads on O_3 sensitivity in terms of photosynthetic traits, with mitigating (Fusaro et al. 2017; Häikiö et al. 2007; Watanabe et al. 2006), detrimental (Maurer et al. 1997; Yamaguchi et al. 2007) and neutral effects (Feng et al. 2011; Harmens et al. 2017). To our knowledge, the interaction between N, P and O_3 on photosynthetic traits has never been tested.

The aim of the present work was to explore the interactive impacts of O_3, N and P on the photosynthetic traits (gas exchange, chlorophyll (Chl) *a* fluorescence and content) of an O_3-sensitive poplar clone (*Populus maximoviczii* Henry × *Populus berolinensis* Dippel) upon exposure to ambient, 1.5 fold ambient and twice ambient O_3 conditions in open air. In our previous paper with the same poplar clone (Zhang et al. 2018), we showed that O_3-induced biomass decline was greater at high N supply, while P alleviated such biomass losses, but not under high N supply. We postulated that higher nutritional availability may exacerbate the negative impacts of O_3 on photosynthetic traits.

Materials and methods

Plant material, ozone exposure and nutritional treatments

Rooted cuttings of the Oxford poplar clone (*Populus maximoviczii* Henry × *Populus berolinensis* Dippel) were propagated in December 2015, and transplanted into 10 L plastic pots filled with a mixture of commercial river sand, sphagnum peat moss and soil, in a proportion 1: 1: 1 (v: v: v) in April 2016. Soil was collected in a semi-natural area nearby the experimental site (43° 46′ 56″N, 11° 10′ 24″E), characterized by a sandy-loam texture slightly acidic. The plants were irrigated to field capacity every 2–3 days to avoid water stress.

Plants were fumigated from 1st May to 1st October, 2016 (24 h per day) under three O_3 levels: ambient air concentration (AA), 1.5 × AA and 2.0 × AA, in a free air controlled exposure (FACE) system located in Florence, Italy (43° 48′ 59″N, 11° 12′ 01″E, 55 m a.s.l.). More technical information about this facility are available in Paoletti et al. (2017). There were three replicated blocks in each O_3 concentration, and 12 plants in each block. Three of the plants in each block were randomly subject to each of four nutritional treatments as explained below. Ozone was produced by a generator (TGOC13X, Triogen ltd., Glasgow, UK), mixed with ambient air and distributed into the O_3 FACE using a system of 25 Teflon tubes in each block hanging from a fixed grid above the plants (Paoletti et al. 2017). Ozone concentration at plant height was continuously measured by O_3 analyzers (Model 202, 2B Technologies Inc., Boulder, Colorado, USA) and adjusted to the target level by a PID system. The AOT40 (Accumulated Ozone exposure over a Threshold of 40 ppb) during the experimental period was 14.4 ppm.h in AA, 43.8 ppm.h in 1.5 × AA, and 71.1 ppm.h in 2.0 × AA (Zhang et al. 2018).

Two N concentrations (N0: 0 kg N ha^{-1}, i.e. 0 mg N $seedling^{-1}$; N80: 80 kg N ha^{-1}, i.e. 392.5 mg N $seedling^{-1}$) were added to the pot soil using NH_4NO_3 (0 and 5 mM solution) according to Thomas et al. (1994). The level of N80 may be considered as a realistic N deposition because the background deposition in some areas, such as the Sichuan basin in China and the California central valley in USA, may be higher than 80 kg N ha^{-1} yr^{-1} (Fenn et al. 2003; Peng et al. 2017). Two P concentrations (P0: 0 kg P ha^{-1}, i.e. 0 mg P $seedling^{-1}$; P80: 80 kg P ha^{-1}, i.e. 392.5 mg P $seedling^{-1}$) were supplied to the pot soil as KH_2PO_4 (0 and 1.0 mM solution) according to Lewis and Strain (1996). Based on P affinity constant and adsorption maxima, these levels of P were selected to simulate a realistic increase in soil available P. In total, there were four combinations of nutrient treatment, i.e. N0P0, N0P80, N80P0, N80P80. In detail, NH_4NO_3 or KH_2PO_4 solution (200 ml) with different concentrations as described above were added into the soil twice a week during the whole treatment period, with an aim of reaching the target concentrations at the end of the experiment, for a total of 11 applications. In order to maintain the same level of K among all treatments, KCl was added into the soil that did not receive KH_2PO_4 (Tissue and Lewis 2010).

The soil N and P concentrations and the N:P ratio of leaves are shown in our previous paper (Zhang et al. 2018). Soil N concentration (mean ± SE) in N0 was 1.7 ± 0.1 g kg^{-1}, and that in N80 treatments was 2.7 ± 0.1 g kg^{-1}. These are in agreement with normal soil N ranges (0.2 to 5 g kg^{-1}, Bowen 1979). Soil P concentrations (mean ± SE) were 0.5 ± 0.1 g kg^{-1} in P0 and 1.0 ± 0.1 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). Leaf N:P ratios (mean ± SE) were 12.1 ± 1.1 at N0P0, 8.3 ± 1.1 at N0P80, 18.8 ± 1.5 at N80P0, and 10.6 ± 1.4 at N80P80 (Zhang et al. 2018). Koerselman and Meuleman (1996) suggested an optimal aboveground biomass N:P ratio of 14–16.

Gas exchange and Chl a fluorescence measurements

Daily course of leaf gas exchange was measured for fully-expanded sun leaves (5-8th order from the tip of terminal shoots, flushed in July 2016), using a portable infra-red gas analyzer (CIRAS2, PP-systems, UK). No typical symptoms of senescence (e.g., yellowing) were observed, although some O_3-like visible foliar injury (dark stippling in the interveinal area) was found in 1.5 × AA and 2.0 × AA. Measurements were carried out in 1 to 3 plants per plot every 3 h (morning: 9:00, noon: 12:00, afternoon: 15:00) on 25 August 2016. Leaves in the cuvette were oriented so as to be fully and directly exposed to sunlight. Net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured at a CO_2 concentration (mean ± SE) of 385.9 ± 0.5 µmol mol^{-1}. The temperature in the cuvette was adjusted manually to the ambient conditions, which were measured by a thermometer under shaded condition at plant canopy height. Relative humidity in the cuvette was adjusted to be similar to the ambient humidity. Dark respiration rate was determined in the laboratory by putting the plants in shade for 30 min. The CO_2 efflux from the leaf was recorded after the value was stable. Measurements were carried out at the control values of CO_2 concentration (386.1 ± 0.3 µmol mol^{-1}), leaf temperature (25.2 ± 0.1 °C) and leaf-to-air vapour pressure deficit (VPD 1.5 ± 0.0 kPa) inside of the leaf chamber.

Chl *a* fluorescence was measured three times using a HandyPEA portable fluorimeter (Hansatech Instruments, Pentney, Norfolk, UK) in July, August and September, 2016. All measurements were carried out in the morning (8:00–10:00). Three plants per treatment were selected and one of the leaves in the intermediate level of the plant canopy was measured after 30 min of dark adaption with leaf clips. Fluorescence transient (FT, that expresses fluorescence induction curve from F_0 to F_m in dark-adapted leaves) was analyzed (Strasser et al. 2000). It is well known that FT has a polyphasic behavior (Bussotti et al. 2011). The different phases include step O (20–50 µs), step K (300 µs), step J (2 ms), step I (30 ms) and step P (peak, the highest fluorescence intensity). Here the maximum quantum yield (F_v/F_m) and Chl *a* fluorescence performance index (PI) were assessed. PI is an integrative index including three independent parameters: the density of active reaction centers on an absorption basis, the quantum yield of primary photochemistry, and the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-. PI decreases when the photosynthetic system was damaged by the environmental stress conditions (Appenroth et al. 2001; van Heerden et al. 2003, 2004). The equations for calculating those parameters of Chl *a* fluorescence are as follows (Strasser et al. 2000):

$$F_v/F_m = \left[1 - \left(F_0/F_m\right)\right]$$

$$RC/ABS = \left[1 - \left(F_0/F_m\right)\right]/\left(M_0/V_J\right)$$

$$PI = (RC/ABS)\left[\left(F_v/F_m\right)/\left(1 - F_v/F_m\right)\right]\left[\left(1 - V_J\right)/V_J\right],$$

where RC is the number of active PSII reaction centers, ABS is the quantity of light absorbed by the antenna, V_J is the relative variable fluorescence at 2 ms of the fluorescence rise [denoted as $V_J = (F_{2ms} - F_0)/(F_m - F_0)$], and M_0 is the slope at the origin of the relative variable fluorescence [denoted as $M_0 = 4(F_{300\,\mu s} - F_0)/(F_m - F_0)$].

Measurement of Chl content

Chl content was assessed according to Cotrozzi et al. (2017), with minor modifications. Samples (30 mg fresh weight, FW) were collected at early morning at harvest (October 1st) as one intermediate-canopy, fully-expanded leaf per each of the three plants in each treatment, immediately treated by liquid nitrogen and stored at − 80 °C until analysis. They were ground with mortar and pestle, homogenized in 0.3 mL of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV–VIS detector, Dionex, Sunnyvale, CA, USA) separation was performed at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter 150 mm length). Chls were eluted at a flow rate of 1 mL min^{-1} using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min followed by a 1.5 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped to elute Chl *a* and *b*. Chls were detected by their absorbance at 445 nm. Pure authentic standards were used to quantify the pigment contents of each sample. Data were extrapolated by Chromeleon software version 6.60 (Dionex Corporation, Sunnyvale, CA, USA).

Statistical analysis

The study was conducted in a well-replicated split-plot design, with O_3 (AA, 1.5 × AA, and 2.0 × AA) as whole-plot factor, and N (N0 and N80) and P (P0 and P80) as split-plot randomized factors. Data were tested for normal distribution by Kolmogorov–Smirnov test. Three-way ANOVA was

applied to assess the effect of O_3 in plants under different N and P conditions ($O_3 \times N \times P$). Post-hoc differences were tested by Tukey test ($p < 0.05$). The relative effect of O_3 on each parameter was calculated as $[(Value_{AA} - Value_{2.0\times AA})/Value_{AA}]$. $Value_{AA}$ means the value of a certain parameter under AA and $Value_{2.0\times AA}$ means the corresponding value under 2.0 × AA. Simple regression analysis was applied to assess relationships between P_N and g_s at three levels of O_3 in each nutritional treatment. If all regressions at the three O_3 levels were statistically significant, we applied an analysis of covariance (ANCOVA) to assess effects of O_3 on the relationship between P_N and g_s.

Results

Gas exchange

From morning to afternoon, P_N and g_s decreased gradually (Fig. 1). In the morning, elevated O_3 significantly decreased both P_N and g_s relative to AA (P_N − 5% in 1.5 × AA and − 26% in 2.0 × AA; g_s − 1% in 1.5 × AA and − 23% in 2.0 × AA). Nitrogen addition significantly increased P_N relative to N0 (+ 20%). There was a significant interaction between O_3 and N on P_N. The loss of P_N caused by medium O_3 exposure (1.5 × AA) was alleviated by N addition, although such an alleviating effect of N was not found in 2.0 × AA. At noon, P_N and g_s were significantly decreased by O_3 (P_N − 18% in 1.5 × AA and − 38% in 2.0 × AA; g_s − 35% in 1.5 × AA and − 45% in 2.0 × AA) and increased by N80 (P_N + 32%; g_s + 27%). However, the $O_3 \times N$ interaction was not significant. P_N and g_s in the afternoon were not affected by any treatments. The Ci/Ca ratio was decreased by N addition (–5%) in the morning (Fig. 1). Regardless of measurement time of day, a significant interaction was found between O_3 and P, indicating that increases of the Ci/Ca ratio due to O_3 exposure were limited by P treatments (i.e., P0 treatments: + 6% in the morning, + 6% at noon and + 10% in the afternoon in 2.0 × AA relative to AA; on the other hand, P80 treatments: − 2% in the morning, − 3% at noon and − 7% in the afternoon in 2.0 × AA relative to AA). Dark respiration rate (R_d) was significantly increased by O_3 (Fig. 2, + 58% in 1.5 × AA and + 108% in 2.0 × AA). Nitrogen increased R_d by 59% while P did not change it significantly. There was a significant interaction of O_3 and N in that the increase of R_d due to O_3 exposure was enhanced due to the N treatment.

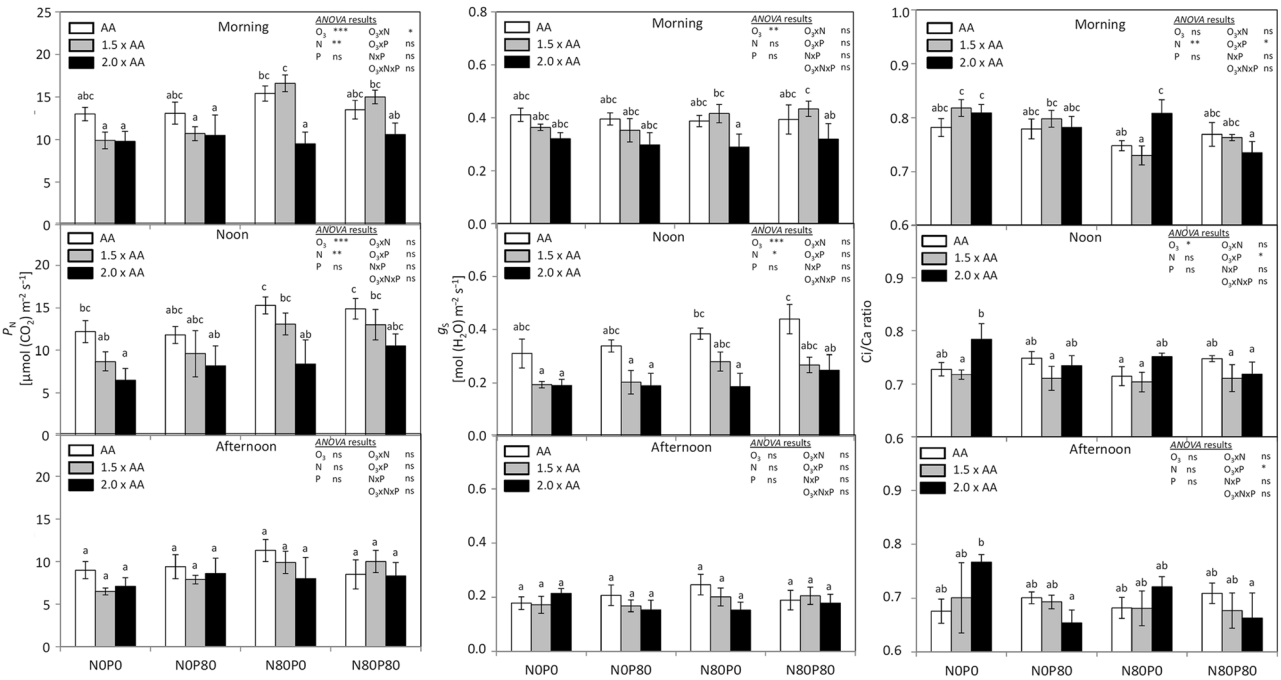

Fig. 1 Daily changes of net photosynthetic rate (P_N, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}) and the ratio of leaf-internal to ambient CO_2 concentration (Ci/Ca ratio) of poplar Oxford clone under different O_3 concentrations (AA, ambient O_3 concentration; 1.5 × AA; 2.0 × 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)]. The bars represent mean ± S.E. ($n = 3$). Asterisks show the significance of a 3w 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)

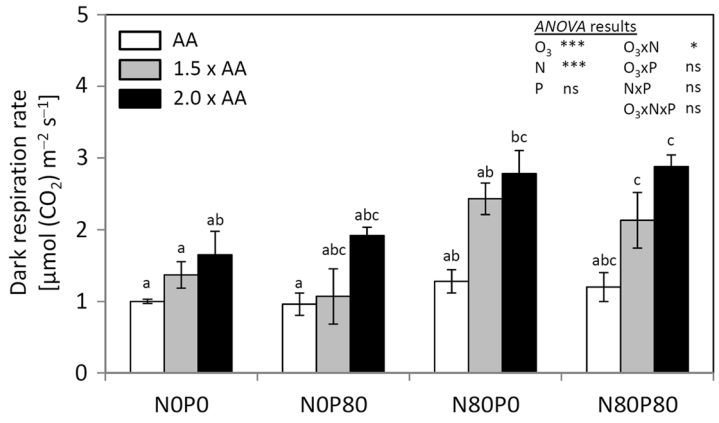

Fig. 2 Dark respiration rate (μmol m^{-2} s^{-1}) in *Populus maximoviczii* Henry × *P. berolinensis* Dippel (clone Oxford) leaves under free air O_3 exposure [applied for five consecutive months: ambient air (AA), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA)] and subject to four combinations 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)]. Data are shown as mean ± S.E. ($n = 3$). Different letters show significant differences among treatments ($p < 0.05$, Tukey test). Asterisks show the significance of a 3w ANOVA: ***$p < 0.001$, *$p < 0.05$

Chl fluorescence

The effect of the combination of O_3, N and P treatments on the chl fluorescence parameters of poplar leaves are shown in Fig. 3. At any measurement time, F_v/F_m was decreased by elevated O_3 (1.5 × AA − 1% in July, − 1% in August, and − 2% in September; 2.0 × AA − 2% in July, -1% in August, and − 9% in September). The values of F_v/F_m were slightly but statistically significantly increased by N supply in July (+ 1%). Phosphorus addition decreased F_v/F_m in July (–1%) and September (–1%) but increased it in August (+ 1%). We found an interactive effect of O_3 × N on F_v/F_m in July, suggesting a mitigation effect of N on the O_3 damage to this Chl fluorescence parameter. However, such an effect was not clear in the other months. Ozone-induced loss of F_v/F_m was higher in P enrichment as confirmed by a significant interaction between O_3 and P on F_v/F_m in July and August. This suggests that P exacerbated negative effects of O_3 on F_v/F_m. We also found an interactive effect of N × P on F_v/F_m in September, confirming that N addition increased the F_v/F_m values at P0 (+ 2%) and decreased them at P80 (–3%). The interaction of the three factors (O_3 × N × P) was significant for F_v/F_m in September. This indicates that the O_3-caused losses of F_v/F_m were aggravated when adding N and P together.

Chl *a* fluorescence performance index (PI) was reduced by O_3 in July (–26% in 1.5 × AA, − 23% in 2.0 × AA) and September (–26% in 1.5 × AA, − 62% in 2.0 × AA), although increases of PI were found in elevated O_3 in August (+ 15% in 1.5 × AA, + 27% in 2.0 × AA) (Fig. 3). Nitrogen supply significantly increased PI in August (+ 23%) and September (+ 30%). Phosphorus addition decreased PI in September (–20%). Similarly to F_v/F_m, a mitigation effect of N on O_3-induced losses of PI was found in July, as confirmed by the statistical significance of O_3 × N. The analysis shows that there was a significant interaction on PI between N and P in September. Nitrogen increased PI values at low P (+ 69%) and decreased them at high P treatments (–6%).

Total Chl content

The three-way ANOVA test of the total Chl content revealed that all the effects and their interactions were significant (Fig. 4). Compared to AA, O_3 *per se* slightly decreased the total Chl content (–10% and − 14% in 1.5 × AA and 2.0 × AA). Nitrogen addition significantly increased the total Chl content when all N80 treatments were compared to all N0 ones. The relative rise was higher in N80P0 than in N0P0 under 2.0 × AA conditions (+ 63%). However, total Chls were not affected by N enrichment under 1.5 × AA (except under N80P80). Compared to P0, P addition decreased the total Chl content, independently of O_3 levels and N addition (except in N80P80 and N0P80 under AA and 2.0 × AA): the relative loss was highest in N80P80 than in N80P0 under 1.5 × AA conditions (–48%).

Discussion

The present study showed that N addition significantly increased P_N of poplars in the morning (Fig. 1), which is consistent with other studies (e.g., Yamaguchi et al. 2007). In addition, the N supply (80 kg ha^{-1} $year^{-1}$) reduced the relative loss of P_N induced by 1.5 × AA O_3 level, although

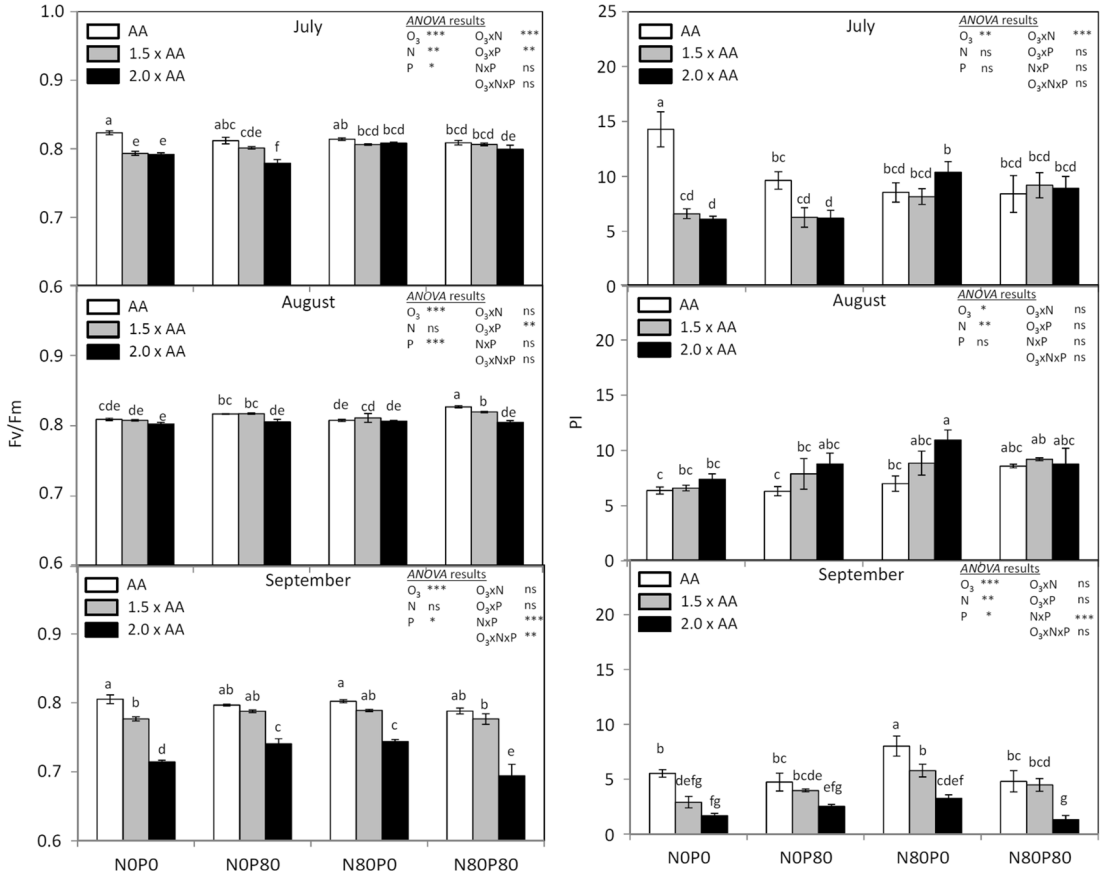

Fig. 3 Monthly values of the maximum quantum yield of primary photochemistry (F_v/F_m) and the performance index (PI) under different O_3 concentrations (AA, ambient O_3 concentration; 1.5×AA; 2.0×AA) and subjected to four combinations of nutrient treatment in the soil [two levels of N (0 and 80 kg N ha^{-1}; N0 and N80) and two levels of P (0 and 80 kg P ha^{-1}; P0 and P80)]. The bars represent mean ± S.E. ($n = 3$). Different letters show significant differences among treatments ($p < 0.05$, Tukey test). Asterisks show the significance of a 3w ANOVA: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

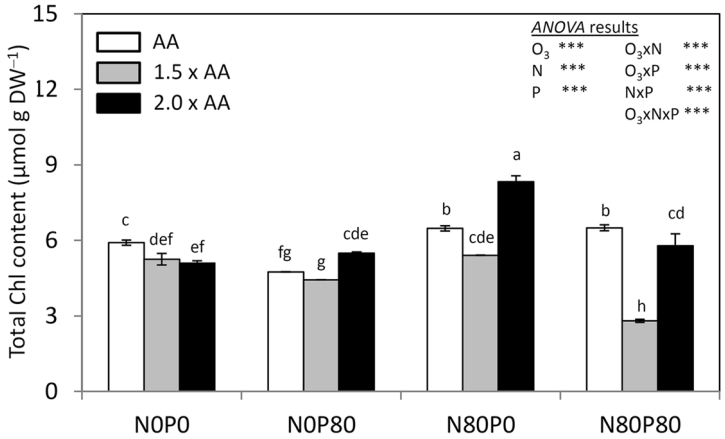

Fig. 4 Total Chl content (µmol g DW^{-1}) in *Populus maximowiczii* Henry × *P. berolinensis* Dippel (clone Oxford) leaves under free air O_3 exposure [applied for five consecutive months: ambient air (AA), 1.5× and 2.0× ambient O_3 (1.5×AA and 2.0×AA)] and subject to four combinations of nutrient treatment 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)]. Data are shown as mean ± S.E. ($n = 3$). Different letters show significant differences among treatments ($p < 0.05$, Tukey test). Asterisks show the significance of a 3w ANOVA: ***$p < 0.001$

such a mitigation was not observed in the higher level of O_3 concentration (2.0 × AA). Nitrogen addition may stimulate leaf turnover forming new leaves with elevated photosynthesis, which might counteract the decrease of P_N induced by O_3 in damaged older leaves (Maurer and Matyssek 1997; Pell et al. 1995). However, such a response did not fully compensate the loss of carbon gain due to O_3 exposure. We also found that R_d was increased by O_3, as previously reported in Japanese and European beeches (Hoshika et al. 2013; Kitao et al. 2009). It has been postulated that the increase in R_d under elevated O_3 may be attributed to demand for O_3 detoxification and repair of damaged tissues (Matyssek and Sandermann 2003). Interestingly, O_3-induced increase of R_d was more evident with N addition. This suggests that O_3 stress is more severe under high N availability. Nitrogen addition may thus lower the O_3 tolerance of Oxford poplar clone. The respiratory carbon loss can be considered a major cause for the reduction of carbon gain due to elevated O_3 (Watanabe et al. 2014a). In our previous paper (Zhang et al. 2018), we found that O_3-induced decrease of poplar biomass was greater at high N supply. The increased respiratory carbon loss by O_3 × N treatments may result in a further decrease in net carbon gain under O_3.

Stomatal conductance is generally correlated with photosynthetic rate to keep a nearly constant ratio of Ci/Ca (Larcher 2003). However, in particular in P0 treatments, elevated O_3 caused a significant increase of the Ci/Ca ratio, which explains a relative decrease in stomatal limitation to photosynthesis and an increase in non-stomatal limitations such as biochemical limitations (Farage and Long 1995; Watanabe et al. 2014b). Such an increase of the Ci/Ca ratio after O_3 exposure was probably a result of a decoupling of g_s from P_N, i.e., g_s remained relatively high (0.3–0.4 mol m^{-2} s^{-1}) even though a significant decrease of P_N was found in 2.0 × AA (Fig. S1). The decoupling of g_s from P_N may be attributed to an impairment of stomatal control after O_3 exposure (i.e., stomatal sluggishness, Paoletti 2005), which results in decreases in the intrinsic water use efficiency (iWUE, denoted as the ratio of P_N to g_s). However, such a response was not found in P80 treatments. With P addition, elevated O_3 exhibited declining P_N and g_s, while maintaining the Ci/Ca ratio as in the control. Phosphorus plays an important role in energy production as components of ATP, which acts as an energy currency of guard cells for stomatal movement (Marten et al. 2007; Suetsugu et al. 2014), involving in controlling key enzyme reactions and in the regulation of metabolic pathways (Larcher 2003). Phosphorus may therefore improve the O_3-induced reduction of iWUE.

Chl *a* fluorescence is a useful tool to unveil the change of the excitation energy in PSII and estimate the status of PSII (Kalaji et al. 2014). Ozone caused a reduction in F_v/F_m and PI throughout most of the experimental period (Fig. 3, S2), although a temporal increase of PI of poplar leaves under elevated O_3 was found in August. This decrease of Fv/Fm may relate to photoinhibition (Bussotti et al. 2011; Zhang et al. 2010). The reduction of PI indicates the development of O_3 damage with the increase in energy dissipation through thermal losses (Bussotti et al. 2011). The temporarily higher PI under O_3 stress in August indicated an enhanced efficiency of electron trapping and transport which might be related to the repair processes (Bussotti et al. 2011). Nitrogen addition ameliorated the O_3-induced decrease of F_v/F_m and PI in July as previously observed in aspen (Häikiö et al. 2007) and manna ash (Fusaro et al. 2017). Nitrogen-fertilized plants are considered more tolerant to photoinhibition due to their higher photosynthetic energy consumption (e.g. Ferrar and Osmond 1986), although Kato et al. (2002) suggested a similar susceptibility to photoinhibition between high and low N-fertilized *Chenopodium album* plants because the low N-fertilized plants may dissipate large excess energy by non-photochemical quenching to compensate for the lower photosynthetic energy utilization. At the end of the growing season, however, an O_3-induced decrease of F_v/F_m was found regardless of N availability, suggesting a significant decrease of photosynthetic efficiency. On the other hand, the addition of P exacerbated the loss of F_v/F_m in July and August, which indicates a lower capacity of protection against photoinhibition. Asada (1999) pointed out a contribution of energy dissipation by the water–water cycle to the protection against photoinhibition. Phosphorus enrichment may decrease the content of ascorbate peroxidase (Desai et al. 2014), which is essential for the water–water cycle (Awad et al. 2015).

The effects of O_3 on total Chl content were changed by N and/or P treatments. The nutrient-rich conditions resulted in even higher Chl concentration in 2.0 × AA relative to AA. Similarly, Han et al. (2009) reported that fertilized *Platanus occidentalis* trees formed leaves with 1.5 times higher Chl content after O_3 exposure, suggesting an overcompensation against O_3 stress (Kitao et al. 2015). Such an overcompensation may be interpreted as a hormetic response (Sugai et al. 2018). The result, however, indicated that this increase of Chl content did not eventually protect the electron transport activity in PSII from O_3 damage.

In conclusion, as we postulated, the availability of N and P influenced the photosynthetic responses to O_3. Our results indicate that P ameliorated the O_3-induced reduction of iWUE while it aggravated the O_3-induced damage to Chl *a* fluorescence. N could alleviate negative effects of O_3 on Chl *a* fluorescence parameters in early summer. Although N addition partly mitigated O_3-induced loss of P_N (only in 1.5 × AA), such a mitigation effect was not observed in the higher O_3 level (2.0 × AA). In addition to the decrease of P_N, N exacerbated an increase of R_d due to O_3 exposure, which results in further reductions in net carbon gain. Overall, O_3 decreased the photosynthetic capacity of poplars regardless

of the different nutritional conditions. Further studies on the responses of the antioxidant metabolism to O_3, N and P may help reconcile the contrasting effects on plant O_3 sensitivity observed so far.

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