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PSII photochemistry is the primary target of oxidative stress imposed by ozone in *Tilia americana*

E. Pellegrini*

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

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ABSTRACT

Trees are essential in the urban environment not only because of their aesthetic and social values, but also for their effects on air quality. Data of the present experiment show some of the integrated mechanisms that may confer sensitivity/tolerance in *Tilia americana* saplings exposed to ozone treatment (120 ppb, 5 h day^{-1} for 45 consecutive days) in order to improve the management of green spaces responding to oxidative stress. At the end of exposure, plants do not exhibit any foliar symptoms. Profiles related to membrane injury, photosynthetic gas exchange, chlorophyll *a* fluorescence, pigment content and enzymes/metabolites linked to the synthesis of phenylpropanoids show a vulnerability in terms of: (i) change in the ionic and solute reactions of the membrane cells (maximum value of +34%, 28 days after the beginning of fumigation, compared to controls); (ii) inhibition of the carbon assimilation (−41%), mostly as a consequence of non-stomatal limitation of assimilation rate; (iii) progressive inhibition of the Calvin cycle, as demonstrated by decreases in Rubisco carboxylation efficiency and regeneration capacity (−35 and −21%, respectively, at the end of exposure), quantum yield of electron transfer at PSII and in the fraction of energy passively dissipated as heat and fluorescence (−34% and −31%); (iv) senescence process (decline in demand for reducing power and energy at the end of treatment); (v) damage to the chlorophyll pigment system. However, the activation of xanthophylls cycle and phenylpropanoid metabolism, that can be considered a strategy in plants in order to regulate light absorbed energy and to scavenge reactive oxygen species does not preserve PSII photochemistry from impairment at the end of exposure.

Introduction

The current annual average of ozone (O_3) levels ranges from 20 to 45 ppb across the globe, which is roughly double in comparison to that of the Industrial Revolution (Gillespie et al., 2011). In Europe, the Mediterranean area experiences relatively high O_3 concentrations due to (i) favourable conditions for formation of this pollutant and (ii) combination of meso-scale re-circulatory processes and long-range transport (González-Fernández et al., 2013). Also in the summer of 2012, the levels of this pollutant continued to exceed the long-term objective (a maximum daily 8 h average concentration of O_3 over 120 μg m^{-3}) established to protect human health in every European member state, with the exception of the Estonia (EEA, 2013). During episodes, O_3 concentrations may peak at 200 ppb or more (Pellegrini et al., 2007) with significant impact on crops and other vegetation, decreasing, for example, the carbon sink strength of trees and forests (Matyssek et al., 2008). Recently, Richet et al. (2012) report that the mechanical properties of the wood are affected by high dose of this pollutant; however, mature trees prove to be resilient to oxidative stress (Cascio et al., 2010).

Rapid increases in human population and economic development have led to tremendous urbanization: more than 50% of the world human beings is now living in an urban area causing severe air pollution that can be considered a potential risk factor in vascular plants (Wang et al., 2012). Trees offer several ecosystemical benefits to urban environment, not only in relation to their aesthetic and social values but also for their effects on air quality (Nowak et al., 2000). In fact, they can (i) sequester and store atmospheric carbon dioxide (CO_2) and other gaseous air pollutants and particulate matter and (ii) provide a natural cooling mechanism (through evapotranspiration and shade) able to reduce air-conditioning energy needs and to avoid contaminant emissions (McPherson et al., 1998; Soares et al., 2011; Roy et al., 2012). But these functions can be modified by other adversely acting factors (like heat, low air humidity, periods of critical water stress, high pH of soils, limited soil volumes, soil compaction; Sjöman and Nielsen, 2010) that alter the behaviour of plants in terms of (i) distribution, (ii) growth and phenology, (iii) response to pests and diseases and (iv) defense and detoxification processes (Yang, 2009).

According to that, O_3 toxic potential to urban forests should be better evaluated, in order to choose species for urban planting

* Tel.: +39 0502210562; fax: +39 0502210559.
E-mail addresses: elisa.pellegrini@for.unipi.it, epellegrini@agr.unipi.it

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especially in cities of the Mediterranean basin. Although there is a large literature on the effects of O_3 on forest trees (Ferretti et al., 2007; Matyssek et al., 2010), minimal studies have been conducted in urban environment (Takagi and Gyokusen, 2004; De Nicola et al., 2011; Ugolini et al., 2012).

Practical observations indicate that the urban trees are short-living in comparison to their growing in natural stands (Whitlow et al., 1992). Life expectancies for new street tree plantings in the north-eastern United States are estimated to be 10 years, with up to 50% mortality occurring within a year of planting (Nowak et al., 2004). Many potential environmental stresses may induce injury or death that are the result of several processes occurring at the cellular, biochemical and physiological levels that induce changes in oxygen (O_2) metabolism. The consequent oxidative stress occurs when reactive oxygen species (ROS) are not rapidly scavenged and the rate of repair of damaged cell components fails to keep pace with the rate of damage (Mullineaux and Baker, 2010).

Tilia species (lime tree, basswood) are large deciduous trees recommended as ornamental plants when a deep shade is desired; the selection of this species as urban trees, based on their behaviour in response to O_3 stress, is important to optimize the production and the use in polluted environment, such as those in Mediterranean countries. Therefore, the aim of this study was to characterize the behaviour of *T. americana* saplings exposed to O_3 in a controlled environment in terms of ecophysiological (gas exchange and PSII performance) and biochemical (the concentration of a number of compounds and the activity of enzymes associated with photosynthetic apparatus and with phenylpropanoid metabolism) responses. This can constitute an instrument to support/disprove the hypothesis that *T. americana* (i) can be classified as tolerant to O_3 in terms of visible injury and (ii) can actively defend itself against oxidative stressors through several pathways (avoidance and repair strategy). On this species, our research group has carried out another study mainly focused on the response to irradiance of photosynthetic CO_2 assimilation under O_3 exposure (Pellegrini et al., 2013).

Materials and methods

Experimental design

One-year old rooted cuttings of *T. americana*, grown in plastic pots containing a mix of steam sterilized soil and peat (1:1), were placed for 1 month in a controlled environment facility at a temperature of $20 \pm 1\,^{\circ}C$, a RH of $85 \pm 5\%$ and a photon flux density (PFD) at plant height of 500 μmol photon $m^{-2}\,s^{-1}$ provided by incandescent lamps, during a 12 h photoperiod. Uniform-sized plants (35 cm tall; fourth leaf fully expanded) were placed in a controlled environment fumigation facility under the same climatic conditions as the growth chamber. The entire methodology has been performed according to Pellegrini et al. (2011b).

Plants were exposed to 120 (±13) ppb of O_3 (1 ppb = 1.96 μg m^{-3}, at 20 °C and 101.325 kPa) for 45 consecutive days (5 h day^{-1}, in form of a square wave between 09.00 and 14.00). They were watered twice a day with 200 ml of distilled water. Analyses were performed on recently fully expanded leaves at 8, 15, 28, 38 and 45 days from the beginning of exposure (FBE) corresponding to a cumulative O_3 uptakes (CUOs) of: 4.36, 9.31, 14.41, 16.57 and 33.80 mmol m^{-2}, respectively. The calculation for CUO was performed utilizing the equation, according to Lombardozzi et al. (2013): CUO (mmol m^{-2}) = CEO_3 g_s k_{O_3} 3600×10^{-6}, where $k_{O_3} = 1.67$ is the ratio of the leaf resistance for O_3 to the leaf resistance to water, g_s is the leaf-level stomatal resistance (in units of mol $H_2O\,m^{-2}\,s^{-1}$) and CEO_3 is the cumulative exposure to O_3 calculated as: CEO_3 (nmol mol^{-1} h) = [O_3] H D, where H is the number of daytime hours the plant was exposed to O_3, D is the total number of days, and [O_3] is the external O_3 concentration in ppb that plants were exposed to during daytime hours of the entire experimental period. 3600 is the number of seconds per hour and 10^{-6} is the conversion from nmol to mmol.

Physiological measurements

Foliar CO_2 and water vapour exchanges were measured with an open infra-red gas exchange system (CIRAS-1, PP-Systems) equipped with a Parkinson leaf chamber, able to clamp a single leaf. Details are reported in Pellegrini et al. (2011b). Measurements were performed at ambient CO_2 concentrations (340–360 ppm) at 80% RH. The chamber was illuminated by a quartz halogen lamp and the leaf temperature was maintained at $26 \pm 0.4\,^{\circ}C$. CO_2 assimilation rate (A) was measured at 1200 μmol photons $m^{-2}\,s^{-1}$ (determined as saturating by preliminary light response curve). The calculation of intercellular CO_2 concentration (C_i) was based on the equations described in von Caemmerer and Farquhar (1981). Non-stomatal limitations (L_m) were calculated as $L_m = C_i/C_a$, where C_a is the external CO_2 concentration (*sensu* Volkova et al., 2011). The response of leaf net CO_2 assimilation rate (A) to C_i (where $C_i < 200$ μmol mol^{-1}) was analyzed according to the mechanistic model of CO_2 assimilation proposed by Sharkey (1985). The slope d_A/d_{Ci} of the regression equation was taken as quantum efficiency (Φ_{CO_2}) of the leaf. The assimilation chamber conditions were maintained at RH $63 \pm 7\%$ and a temperature of $25 \pm 1.1\,^{\circ}C$. The maximum carboxylation rate of Rubisco (V_{cmax}), the light-saturated rate of electron transport (J_{max}) and the daytime respiration (R_d) was calculated in according to Dubois et al. (2007).

Modulated chlorophyll (chl) *a* fluorescence measurements and the status of the electron transport of PSII were carried out with a PAM-2000 fluorometer (Walz) on the same leaves used for gas exchange which were dark-adapted for 40 min using a dark leaf-clip. Minimal fluorescence, F_0, when all PSII reaction centres were open, was determined using the measuring modulated light, which was sufficiently low (<1 μmol $m^{-2}\,s^{-1}$) without inducing any significant variable fluorescence. The maximal fluorescence level, F_m, when all PSII reaction centres were closed, was determined by applying a saturating light pulse (0.8 s) at 8000 μmol $m^{-2}\,s^{-1}$ in dark adapted leaves. Fluorescence induction was started with actinic light (about 400 μmol $m^{-2}\,s^{-1}$) and superimposed with 800 ms saturating pulses (10,000 mol $m^{-2}\,s^{-1}$ PFD) at 20 s intervals to determine maximal fluorescence in the light-adapted state (F'_m). Minimal fluorescence in the light-adapted state (F'_0) was determined immediately after turning off the actinic source in the presence of a far-red (>710 nm) background for 10 s to ensure maximal oxidation of PSII electron acceptors. The intensity of actinic light was maintained at about 400 μmol $m^{-2}\,s^{-1}$ and saturating flashes of white light 15,000 μmol $m^{-2}\,s^{-1}$ and 800 ms duration were given every 20 s. The saturation pulse method was used for analysis of quenching (q_P) and no-photochemical quenching (q_{NP}) components as described by Schreiber et al. (1986). The actual quantum yield of PSII (Φ_{PSII}) was computed as $(F'_m - F_s)/F'_m$, where F_s achieved ($F_t - F'_0$), is the steady-state fluorescence yield in the light-adapted state, as in Rohacek (2002). The quantum yield of regulate (Φ_{NPQ}) and non-regulated (Φ_{NO}) energy dissipation in PSII were estimated as reported in Kramer et al. (2004).

Biochemical analyses

TBARS (thiobarbituric acid reactive substances) were determined following Hodges et al. (1999) and the procedure is described in Pellegrini et al. (2011a). To correct the measure for possible interference by MDA-sugar complexes, which also absorb around

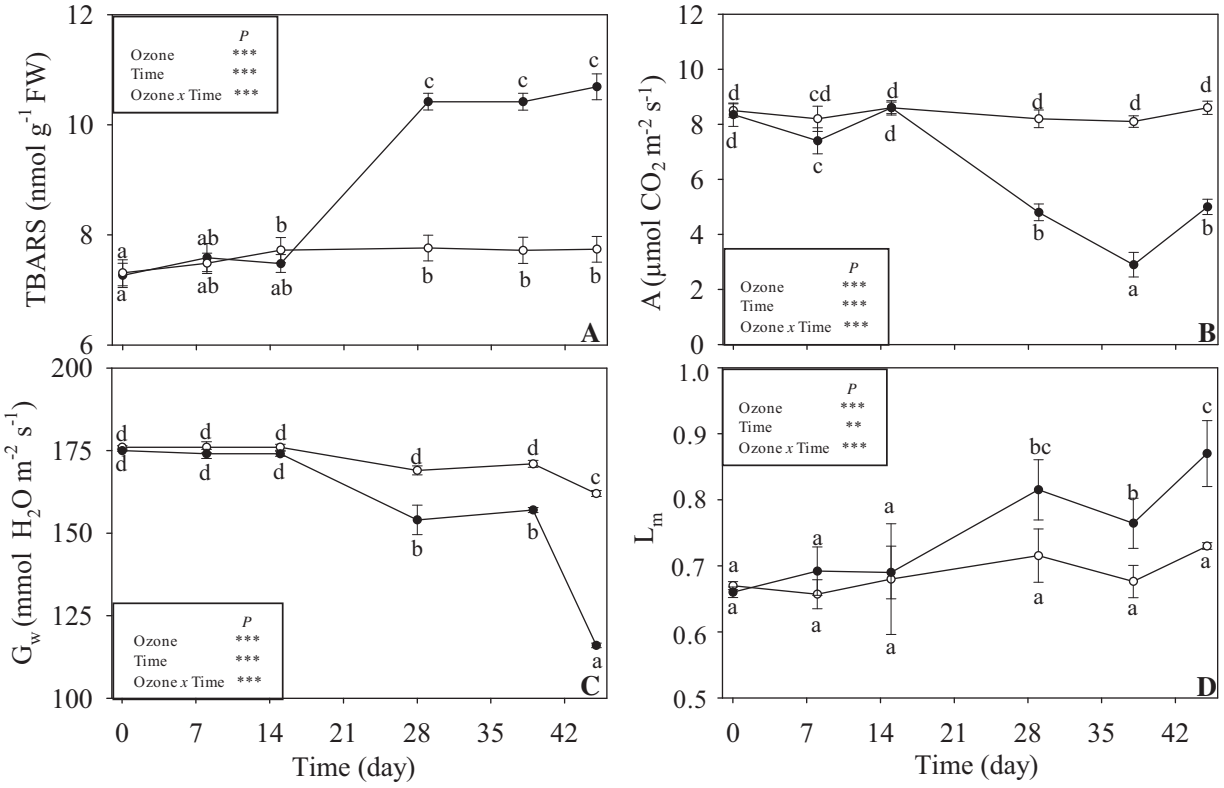

Fig. 1. Profiles of TBARS (thiobarbituric acid reactive substances) (A), CO_2 assimilation rate (A) (B), stomatal conductance to water vapour (G_w) (C) and non-stomatal limitations (L_m) in *Tilia americana* leaves maintained in filtered air (open circle) and exposed to ozone (120 ppb for 45 consecutive days, 5 h d^{-1}, closed circle). Data are shown as mean ± standard error ($n = 3$). The measurements are carried out 8, 15, 28, 39 and 45 days from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of two-way (TBARS) and repeated measures ANOVA (A, G_w and L_m parameters) of are reported, asterisks showing the significance of factors/interaction for: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$.

532 nm, an aliquot of the sample extract was incubated without thiobarbituric acid (TBA) and the absorbance of the solution at 532 nm was subtracted from that containing TBA reagent. Moreover, the absorbance of the sample was also read at 440 nm in addition to 532 and 600 nm. Calculations were performed utilizing the equation TBARS (nmol ml^{-1}) = $(A - B/157{,}000) \times 10^6$, where $A = [(A_{532+TBA}) - (A_{600+TBA}) - [(A_{532-TBA}) - (A_{600-TBA})]$ and $B = [(A_{440+TBA}) - (A_{600+TBA}) \times 0.0571]$.

Pigment analysis was performed by HPLC according to Pellegrini et al. (2011b). 30 mg of leaves previously utilized for gas exchange analysis and fluorescence measurements were homogenized in 3 ml of 100% HPLC-grade methanol overnight. The supernatant was filtered through 0.2 μm Minisart SRT 15 filters and immediately analyzed. The extraction was carried out as quickly possible, in dimmed green light. HPLC separation was performed at room temperature with a Dionex column (Acclaim 120, C18, 5 μm particle size, 4.6 mm internal diameter × 150 mm length). The pigments were eluted using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 12 min to elute all xanthophylls, including the resolution of lutein from zeaxanthin, followed by a 3 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped for 15 min to elute chlorophyll *b* and chlorophyll *a* and *β*-carotene, followed by 2 min linear gradient to 100% solvent A. The flow-rate was 1 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection. The pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts of pure standard were injected into the HPLC system and an equation, correlating peak area to pigment concentration, was formulated. De-epoxidation index (DEPS) was calculated as DEPS = 100 × (*V* + 0.5 A)/(*V* + *A* + *Z*), where *V*, *A* and *Z* are respectively violaxanthin, antheraxanthin and zeaxanthin (*sensu* Adams and Demmig-Adams, 1995).

Procedures for PAL activity and phenolic content are described in Francini et al. (2008). The PAL activity was assayed in leaves (1 g) ground in pre-chilled mortar with liquid nitrogen. The powder was immediately added to 3 ml of extraction buffer containing 100 mM potassium borate (pH 8.8), with 14 mM 2-mercaptoethanol. The homogenate was then centrifuged at 13,000 × *g* for 30 min, and the supernatant was used as enzymatic extract. The PAL assay was determined using a reaction mixture containing 100 ml of enzymatic extract, 1 ml of 100 mM potassium borate buffer (pH 8.8) and 200 ml of 100 mM L-phenylalanine. The reaction was incubated at 40 °C for 120 min, the reaction was stopped by adding 50 ml of 5 N HCl. The cinnamic acid produced was measured at 290 nm. The molar extinction coefficient for transcinnamic acid was determined to be 17,400 M^{-1} cm^{-1} (Cheng and Breen, 1991). Proteins were determined according to Bensadoun and Weinstein (1976), using bovine serum albumin as standard.

Total phenols were extracted from fresh leaves in boiling 80% ethanol. Phenolic extract was mixed with equal volume of 1 M Folin Ciocalteau and 1.5 ml of 7.5% of Na_2CO_3. Absorbance of the blue coloured solution was measured at 760 nm. Total phenols were estimated by comparison with the standard curve obtained with gallic acid.

Anthocyanins were extracted into methanolic HCl and the concentration of cyanidin-3-glucoside equivalents (ε 29,600) determined spectrophotometrically at 535 nm (Francini et al., 2008).

Lignin content was estimated as described by Kevers et al. (1985) and assayed in leaves (1 g) ground in pre-chilled mortar with liquid nitrogen. The powder was immediately added to 3 ml of water. The

homogenate was then centrifuged at 10,000 × *g* for 10 min and the supernatant was mixed with 2.5 ml of acetyl bromide (v/v in glacial acetic acid), 1 ml of perchloric acid and incubated for 30 min at 70 °C. Samples were rapidly cooled on ice, mixed with 2.5 ml of 2 M NaOH and 12 ml of glacial acetic acid. The absorbance of the solution was determined at 280 nm. The molar extinction coefficient of alkaline spruce lignin was determined to be 24 $mmol^{-1}$ cm^{-1}.

Data analysis

A minimum of six plants per treatment were used in each of the three repeated experiments ($n = 18$). Following performance of the Shapiro–Wilk *W* test, data were analyzed using repeated measures (in the case of the measurements carried out for more of 2 time-points) or two-way analysis of variance (ANOVA) and comparison among means was determined by Fisher LSD post-test ($P \leq 0.05$) using as replicates the averages of the three experiments ($n = 3$). Means related to PAL activity, phenols, anthocyanins and lignin content were compared by paired-sample *t*-test ($P \leq 0.05$) using as replicates the averages of the three experiments ($n = 3$). Curvilinear correlations were applied to: *A* vs. R_d and J_{max} vs. V_{cmax}. Analyses were performed by NCSS 2000 Statistical Analysis System Software.

Results

Markers of ozone injury

No foliar symptoms were observed in both fumigated and unfumigated (control) plants. Membrane integrity was significantly affected by O_3 only after prolonged exposure (Fig. 1A), such as confirmed by an evident increase of TBARS levels (+34% in comparison with air filtered material) observed after 28 days of exposure and maintained until the end of fumigation (about 1-fold).

Profiles of gas exchange and chlorophyll a fluorescence

Gas exchange profiles indicate that there was a changing pattern of *A*, G_w and L_m along the exposure (Fig. 1B-D): the response to O_3 was significantly modified starting from 28 days FBE. Photosynthetic rates were 41% lower in O_3-treated plants at a CUO of 14.41 mmol m^{-2} and 60% lower at a CUO of 16.57 mmol m^{-2} (Fig. 2). Instantaneous conductance of water vapour also decreased in O_3-treated compared to control plants over the course of 45-day experiment: at a CUO of 14.41 mmol m^{-2}, G_w in treated plants decreased by 6% and was 8% lower at CUO of 16.57 mmol m^{-2}. At the end of the treatment (CUO of 33.80 mmol m^{-2}), O_3-induced decline in *A* (−42%) was twinned with a severe depression in G_w (−28%). Anyway, biochemical limitations to photosynthesis prevailed over stomatal ones. Referring to non-stomatal photosynthetic parameters derived from

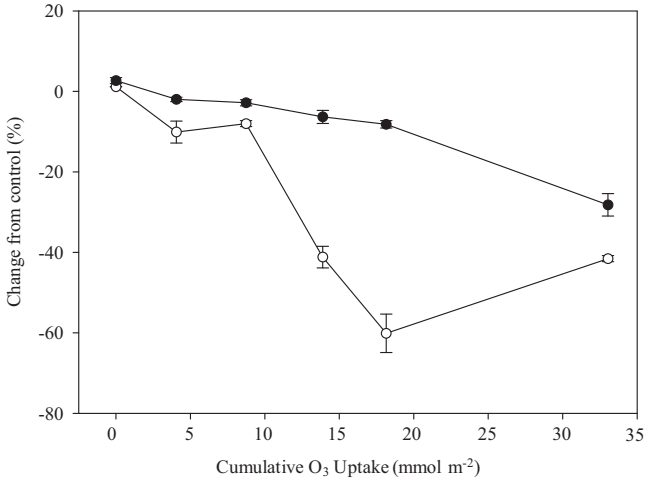

Fig. 2. Percent changes in photosynthesis (open circle) and stomatal conductance (closed circle) over a range of cumulative ozone uptakes (CUOs) in *Tilia americana* leaves exposed to ozone (120 ppb for 45 consecutive days, 5 h day^{-1}). Data are shown as mean ± standard error ($n = 3$).

CO_2 response curve of CO_2 assimilation rate (Table 1), V_{cmax} and J_{max} were not affected in the first 15 days of fumigation. After a prolonged exposure, these values became lower than those observed in controls (−35 and −21%, respectively). Both Φ_{CO_2} and R_d decreased regardless the time of exposure (−13 and −21%, for the first, and −175 and −36%, for the last parameter, 15 and 45 days FBE). The correlation of V_{cmax} against J_{max} revealed a strong linear relationship between these variables in treated materials ($y = 1.41x + 22.8$, $R^2 = 0.99$); in untreated materials no significant correlation was observed between them ($y = 0.18x + 73.3$, $R^2 = 0.08$) (data not shown). Strong linear relationships between A and R_d in both treated and untreated materials were reported respectively after 15 (control: $y = 0.68x + 9.9$, $R^2 = 0.68$; treated with O_3: $y = 7.67x + 42.6$, $R^2 = 0.95$) and 45 days FBE (control: $y = 1.84x + 12.6$, $R^2 = 0.87$; treated with O_3: $y = -0.37x + 3.9$, $R^2 = 0.56$) (data not shown).

In Fig. 3, some crucial chl *a* fluorescence-derived parameters were reported. The ratio F_v/F_m, which indicates the photochemical efficiency of PSII in dark-adapted leaves, in control, ranged from 0.80 to 0.82 with a mean value of 0.81 (Fig. 3A). After 28 days FBE, the ratio decreased and reached a mean value of 0.71, indicating that O_3 impaired the efficiency of PSII. This reduction was attributable to an increase in F_0 associated to F_m similar to controls (data not shown) and was maintained until the end of treatment (about 1-fold compared to controls). The actual quantum yield of PSII was also decreased in O_3-treated compared to control plants over the course of 45 days of experiment starting to 28 days FBE (−41%) and changing at the different rates after

Table 1
Foliar gas exchange parameters estimated from CO_2 response curves of CO_2 assimilation rate in *Tilia americana* plants exposed to ozone (120 ppb for 45 consecutive days, 5 h day^{-1}). Controls were kept in charcoal-filtered air. Data are shown as mean ± standard error ($n = 3$). Measurements are made 15 and 45 days from the beginning of exposure. For each parameter, different letters indicate significant differences ($P \leq 0.05$). Asterisks show the significance of factors/interaction in the two-way ANOVA for: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. Abbreviations: Φ_{CO2}, quantum efficiency; V_{cmax}, maximum rate of carboxylation (μmol CO_2 m^{-2} s^{-1}); J_{max}, light-saturated rate of electron transport (μmol electrons m^{-2} s^{-1}); R_d, daytime respiration (μmol CO_2 m^{-2} s^{-1}).

Days of treatment		Φ_{CO_2}	V_{cmax}	J_{max}	R_d
15	Control	0.032 ± 0.0016 c	43.7 ± 1.34 bc	79.8 ± 0.23 b	−1.6 ± 0.09 d
	Ozone	0.028 ± 0.0013 b	40.5 ± 0.09 b	80.0 ± 0.10 b	−4.4 ± 0.05 a
45	Control	0.033 ± 0.0009 d	46.6 ± 1.34 c	83.0 ± 0.45 b	−2.2 ± 0.08 c
	Ozone	0.026 ± 0.0011 a	30.4 ± 1.23 a	65.8 ± 0.87 a	−3.0 ± 0.32 b
Ozone		***	**	*	***
Time		**	***	***	***
Ozone × time		***	***	***	**

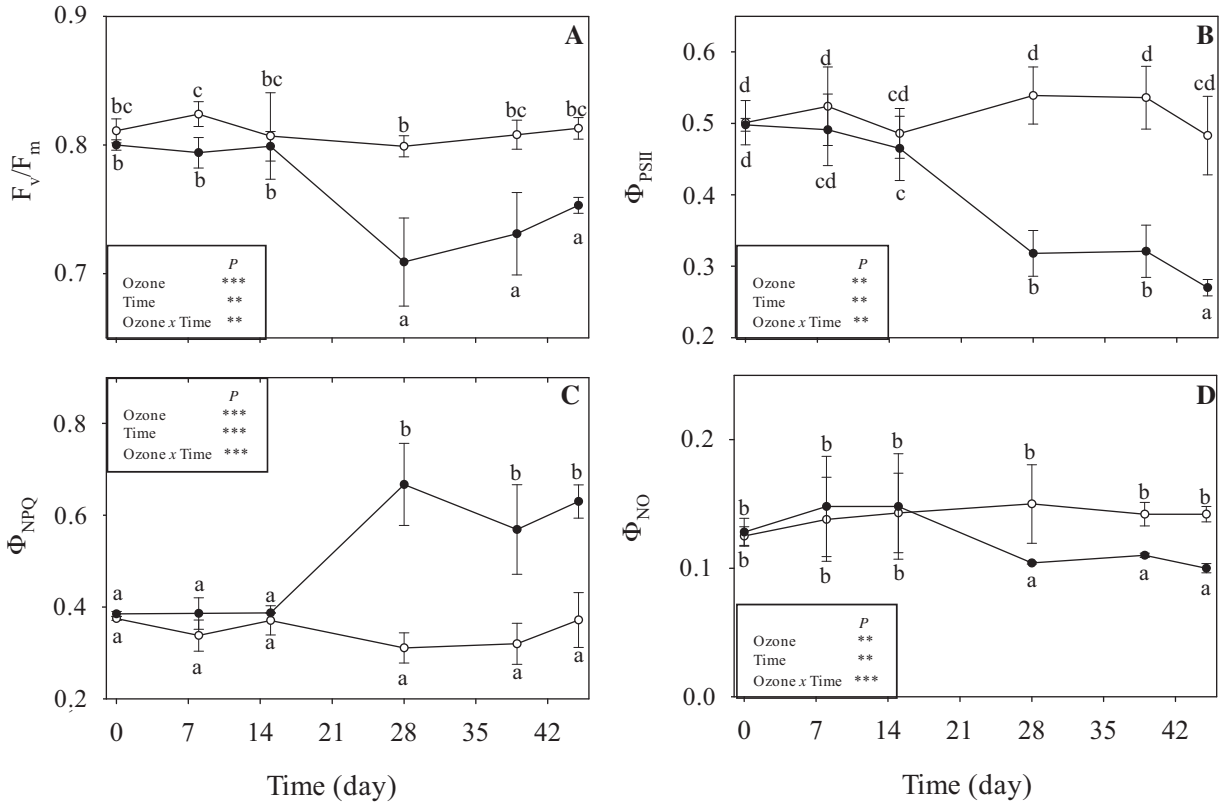

Fig. 3. Profiles of chlorophyll *a* fluorescence parameters (arbitrary units) in *Tilia americana* leaves maintained in filtered air (open circle) and exposed to ozone (120 ppb for 45 consecutive days, 5 h day^{-1}, closed circle). Data are shown as mean ± standard error ($n = 3$). The measurements are carried out 8, 15, 28, 39 and 45 days from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of repeated measures ANOVA are reported, asterisks showing the significance of factors/interaction for: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$. (A) Variable and maximal fluorescence ratio (F_v/F_m); (B) actual quantum yield of PSII (Φ_{PSII}); (C) quantum yield of regulated energy dissipation in PSII (Φ_{NPQ}); (D) quantum yield of non-regulated energy dissipation in PSII (Φ_{NO}).

a prolonged exposure (−40 and −44%, after 39 and 45 days FBE, Fig. 3B). In parallel with the reduction of F_v/F_m and Φ_{PSII}, a significant increase in Φ_{NPQ} was observed in fumigated plants at the same time points. After 28 days FBE, the fraction of energy dissipated in form to heat via the regulated no photochemical quenching mechanisms was increased about 2-fold in comparison to controls and was 69% higher at the end of the treatment (Fig. 3C). In the first 15 days of fumigation, Φ_{NO} was not affected by O_3 (Fig. 3D), as also observed for Φ_{PSII} and Φ_{NPQ}; after long-time exposure, these values became lower than that reported in controls plants (−31, −23 and −30%, respectively after 28, 39 and 45 days FBE).

Leaf pigments

Fig. 4 shows the trend of carotenoids that vary significantly only after 15 days FBE. Lutein and β-carotene decreased as the O_3 stress progressed (Fig. 4A and B) (lutein: −29, −45 and −54% at 28, 39 and 45 days FBE, respectively; β-carotene: −25, −39 and −39%). On the contrary, the sum of anteraxanthin and zeaxanthin concentrations enhanced (Fig. 4C) (+14, +20 and +24% at 28, 39 and 45 days FBE, respectively).

After 28 days FBE, the chl *a*/chl *b* ratio were higher than that observed in control plants (about 2-fold) indicating a reduction of the light-harvesting complex of photosystem II (LHCII); this increase was maintained until the end of treatment (+87 and +49%, respectively 38 and 45 days FBE, Fig. 5A). The concomitant decrease of the total chl/carotenoids ratio at the same time points (−24, −25 and −25% in comparison to filtered air material) confirmed that during a prolonged exposure (Fig. 5B) there was a need for plants to invest in an enhancement of photoprotective de-excitation pathways mediated by carotenoids. The effect of oxidative stress regarding the DEPS index was also observed starting from 28 days FBE, with an increase of this parameter (+12%, in comparison to controls), showing a marked activation of the xanthophyll cycle until the end of treatment (about 1-fold Fig. 5C).

Metabolites and enzymes involved in phenylpropanoid pathway

After a prolonged O_3 exposure, the activity of PAL significantly increased in comparison to controls (+64%); in parallel a

Table 2
Phenylalanine ammonia lyase (PAL) activity, total phenolic, anthocyanins and lignin content in *Tilia americana* plants exposed to ozone (120 ppb for 45 consecutive days, 5 h day^{-1}). Controls were kept in charcoal-filtered air. Data are shown as mean ± standard error ($n = 3$). Measurements were made 45 days from the beginning of exposure. For each parameter, significant differences are for: $^{***}P \leq 0.001$, $^{*}P \leq 0.05$.

	PAL activity (uU mg protein^{-1})		Total phenolic content (mg g^{-1} FW)		Anthocyanins content (mg g^{-1} FW)		Lignin content (mg g^{-1} FW)	
Control	10.2 ± 1.08	***	192 ± 16.0	*	0.49 ± 0.028	*	1.48 ± 0.129	***
Ozone	16.7 ± 1.68		277 ± 29.3		0.38 ± 0.049		2.20 ± 0.130	

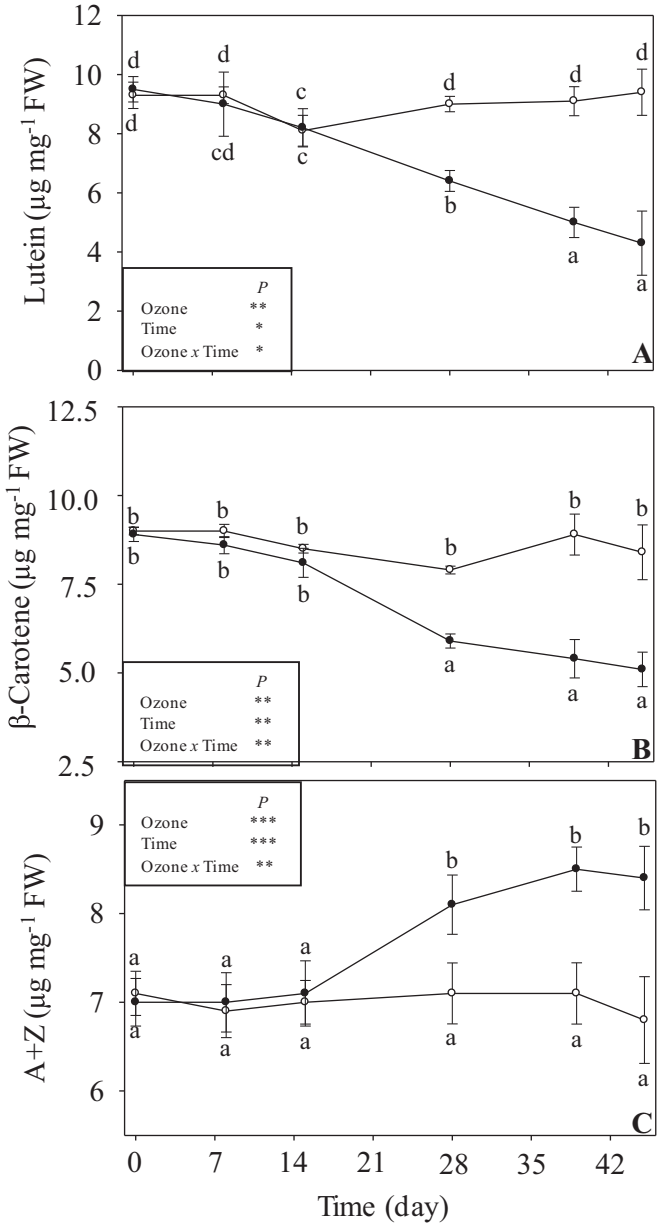

Fig. 4. Profiles of lutein (A), β-carotene (B) and anteraxanthin + zeaxanthin (A + Z) (C) concentrations in *Tilia americana* leaves maintained in filtered air (open circle) and exposed to ozone (120 ppb for 45 consecutive days, 5 h d^{-1}, closed circle). Data are shown as mean ± standard error (n = 3). The measurements are carried out 8, 15, 28, 39 and 45 days from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of two-way ANOVA are reported, asterisks showing the significance of factors/interaction for: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

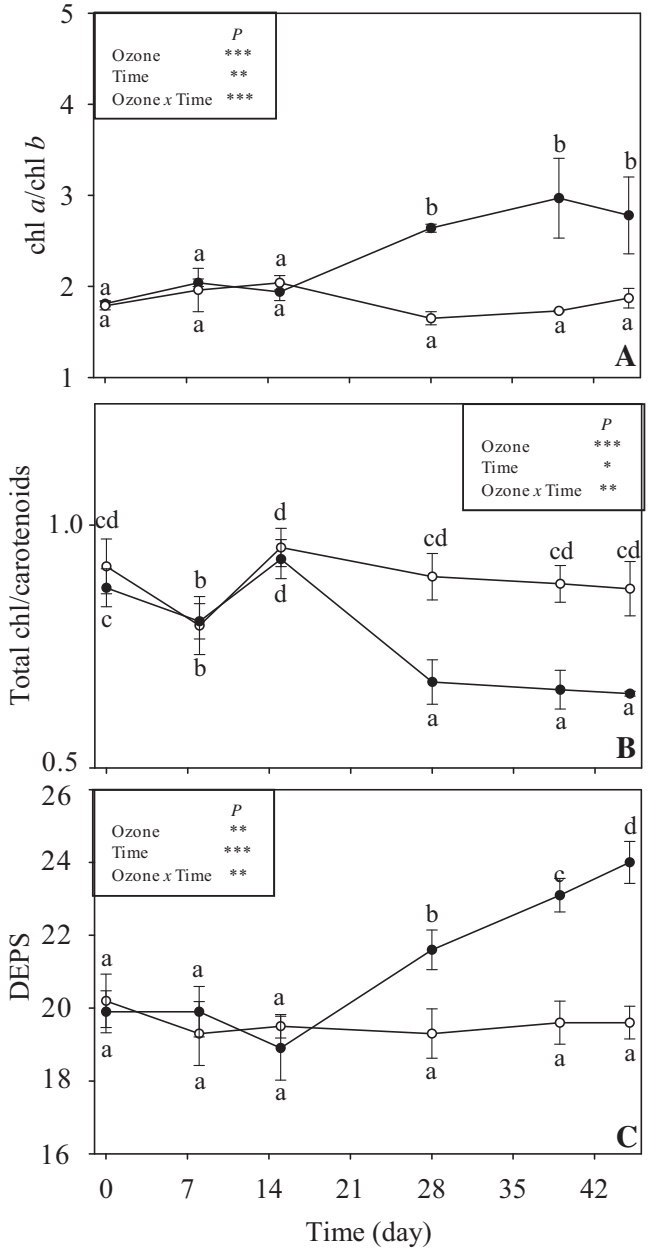

Fig. 5. Profiles of ratio of chlorophyll *a* to chlorophyll *b* (chl *a*/chl *b*) (A), ratio of total chlorophylls to total carotenoids (total chl/carotenoids) (B) and de-epoxidation index (DEPS) (C) in *Tilia americana* leaves maintained in filtered air (open circle) and exposed to ozone (120 ppb for 45 consecutive days, 5 h d^{-1}, closed circle). Data are shown as mean ± standard error (n = 3). The measurements are carried out 8, 15, 28, 39 and 45 days from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of two-way ANOVA are reported, asterisks showing the significance of factors/interaction for: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

pronounced increase of the content of phenols and lignins was observed (+44 and +47%) (Table 2). On the contrary, the values of anthocyanins showed a marked decrease after 45 FBE (−22% in comparison to control plants) (Table 2).

Discussion

Data of the present experiment show some of the integrated mechanisms that may confer sensitivity/tolerance in *T. americana* saplings exposed to O_3 in order to improve the management in urban environment of green spaces responding to climate change. Following Levitt (1980), these mechanisms involve morphological, physiological and biochemical-related features aimed at (i) avoiding, (ii) preventing and (iii) countering oxidative damage.

The degree to which plants develop visible injury is commonly utilized in many intra- and interspecies comparisons as an indicator of their O_3 sensitivity (He et al., 2007). In our case, the lacking of visible injury throughout the duration of the experiment should confirm a high O_3 tolerance of *T. americana*. This conclusion is supported by similar findings described in Smith (1981) and Novak et al. (2003) on *Tilia* genus. To the best of our knowledge, there are not biochemical and physiological studies that can support/disprove this hypothesis on this species that can be considered O_3-tolerant in term of visible injury.

Until 15 days FBE, biochemical and physiological measurements are also consistent with previous conclusions. After this time, although in the absence of visible injury, TBARS analysis shows lipid peroxidation of the membrane: the increase of TBARS levels in O_3-treated plants indicates the changes in the ionic and solute reactions of the membrane cells (e.g. Calatayud and Barreno, 2001; Pellegrini et al., 2011b).

Significant reductions in A and G_w and related changes in Rubisco carboxylation efficiency (V_{cmax}) and regeneration capacity (J_{max}) have been observed in fumigated plants. Rubisco-related biochemical limitations are involved in the decline of CO_2 assimilation in treated plants, as already reported in *Gingko biloba* (He et al., 2007) and in *Quercus* species (Calatayud et al., 2011). Indeed, our study indicates that photosynthesis and stomatal conductance change at different rates over the same CUO suggesting that an additional mechanism is causing the observed decreases in CO_2 assimilation rate, as has already been reported in the literature for chronic O_3 exposure (Lombardozzi et al., 2012). Many Authors (Calatayud et al., 2007; Pellegrini et al., 2011b; Pina and Moraes, 2010) similarly found that O_3-induced differences in photosynthesis are the result of non-stomatal factors, potentially driven by either photosystem oxidation, (i) limiting the energy for RuBP regeneration from the lower pools of Calvin cycle intermediates, (ii) decreasing the efficiency of Rubisco due to direct enzyme oxidation or (iii) reducing CO_2 transport to the enzymes. The O_3-induced decrease in V_{cmax} and J_{max} observed in this experiment suggests that O_3 could reduce the biochemical capacity of *T. americana* to fix CO_2 in order to optimize the utilization of available resources within the photosynthetic apparatus (Zheng et al., 2002; Goumenaki et al., 2010). This assumption only applies for the first 15 days of treatment. The relationship between R_d and A can be explained by an economic carbon budget optimization at the plant level. When carbon fixation is low, e.g. O_3 induced stress as is this case here, the plant should use all available carbon as economically as possible, in order to maintain a positive net CO_2 budget. Also in this case, the observed relationship indicates that this optimization is maintained only until 15 days FBE. In *Prunus* plants affected by drought stress, the optimization of the carbon budget is a general characteristic independent by the sensitivity of the species to drought stress (Rohui et al., 2007).

The decline in demand for reducing power and energy (NADPH and ATP) may also trigger a series of senescence events such as those observed in *T. americana* after a prolonged O_3 exposure. In particular, the decline in the quantum yield of electron transfer at PSII (Φ_{PSII}) and in the fraction of energy passively dissipated as heat and fluorescence (Φ_{NO}) may indicate a progressive inhibition of the Calvin cycle and the concomitant reduction of Φ_{PSII}/Φ_a and F_v/F_m can indicate a photoinhibitory damage to PSII reaction centres (Maxwell and Johnson, 2000), confirming that the primary target of oxidative stress is the PSII photochemistry.

Therefore, it is likely that photosynthesis in plants treated with O_3 shows a photoprotective mechanism (as indicated by higher Φ_{NPQ}) that can prevent (or reduce) the potentially damaging accumulation of excitation energy with a lower partitioning of photochemical activity in non-carbon-assimilative processes. In particular, *T. americana* may try to avoid thylakoid over-excitation distributing the excess of energy into by thermal dissipation in a ΔpH-indipendent process (increasing the DEPS index, Pellegrini et al., 2011b) or into an alternative ways reducing the pressure of excitons or activating antioxidant molecules (decreasing the anthocyanins content, Steyn et al., 2002).

After a prolonged O_3 exposure, the lower photosynthetic performance is associated with an alteration of chlorophyll. In particular, the "chl *a*/chl *b*" ratio, often used as an indirect indicator of the size of LHCII (Ferraro et al., 2003), shows a significant increase at the end of treatment indicating a moderate LHCII reduction. Similar results have been obtained by de Oliveira et al. (2009) in coffee seedlings submitted to chilling. The ratio "total chl/carotenoids" is a certain measure for the greenness of leaves with respect to the relative amounts of the yellow carotenoids and represents – together with the "chl *a*/chl *b*" ratio – a good marker for adaptation of chloroplasts (González-Rodríguez et al., 2001). The decrease of the value of this ratio observed at the end of treatment (as a consequence of increased carotenoids content, in particular to xanthophylls cycle pigments) can be considered an early stress indicator. This result confirms that leaves treated with O_3 enhance the need for carotenoid-mediated photoprotection and that the oxidative stress induces photoxidation and consequently a partial breakdown of chlorophylls.

Lutein is the most abundant xanthophyll in the photosynthetic apparatus of higher plants and has the specific property of quenching harmful $^3Chl^*$, preventing ROS formation. In our case, the drop of lutein level, as a consequence of O_3 exposure, is compensated by the increase of anteraxanthin + zeaxanthin concentration that causes a lower ROS yield. Dall'Osto et al. (2006) conclude that zeaxanthin is effective in photoprotection of plants lacking of lutein, including ROS scavenging and direct quenching of chl fluorescence. Fini et al. (2012) report that in *Fraxinus ornus* subjected to drought stress zeaxanthin may have served a function as chloroplastic antioxidant other than contributing to thermal dissipation of excess radiant energy. However, the increase in zeaxanthin concentration and DEPS index do not preserve PSII photochemistry of *T. americana* from impairment at the end of exposure.

At the end of the fumigation, the deep reduction in carbon assimilation of plants treated with O_3 occurs with a typical increase of PAL activity that may play a key role in the synthesis of phenylalanine and trans-cinnamic acid, precursor of most, or all phenylpropanoids. Similar results have been previously observed in birch (Pellinen et al., 2002) and in poplar (Koch et al., 1998). This rise is twinned with an overproduction of total phenols and lignins and a reduction of anthocyanins content. Generally, the increase in phenylpropanoid concentration can be considered a repair process in post-stress phase that can equip fumigated plants with an additional antioxidant system capable to avoid and scavenge ROS (Pasqualini et al., 2003; Vollenweider et al., 2003). As observed by Fini et al. (2012), the biosynthesis of phenylpropanoids increases more in stress-sensitive than in stress-tolerant species, even if there is still uncertainty about how phenylpropanoids act as ROS scavengers *in planta* and few conclusive evidence of their *in vivo* antioxidant functions. In our case, the increase of lignin content and the consequent cell structural modifications suggests that these metabolites might be formed in consequence to leaf senescence induced by O_3. These findings are in agreement with the results reported by Severino et al. (2007).

On the basis of the data given here it is verified that in *T. americana* exposed to O_3: (i) the biochemical and physiological changes occur irrespective of the appearance of symptoms; (ii) PSII photochemistry is the primary target of oxidative stress; (iii) photosynthesis can be down-regulated as response to oxidative stress, at least for some period; (iv) increase in zeaxanthin concentration and activation of the phenylpropanoids metabolism do not preserve PSII photochemistry from impairment at the end of exposure.

In conclusion, although the response mechanisms that confer sensitivity/tolerance of *T. americana* to O_3 are complex, because involve highly integrate morphological-, physiological- and biochemical-related features, results of this study support the initial hypotheses. In fact, this species (i) can be considered tolerant to O_3 treatment in terms to visible injury, (ii) is able to control the entry of the pollutant in the leaf (avoidance strategy) and (iii) to remove the reactive oxygen species produced by oxidative stress (repair strategy).

To our knowledge, this is the first time that the behaviour of *T. americana* exposed to O_3 is characterized in terms of biochemical and physiological responses and these findings can be useful to give guidelines in the context of the arboriculture in urban environment and ameliorate the management in urban green spaces in the "climate change era". Nevertheless, the comparison of O_3-relevant traits between saplings and large trees remains an important issue.

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