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Ecophysiology of *Tilia Americana* under ozone fumigation

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ABSTRACT

The negative effects of the pollutant gas ozone are widely studied in many plant species, but the intimate mechanisms of toxicity have not been completely defined. Generally this contaminant or its free radical by–products impair membrane functions, leading to declines in physiological processes, accelerated foliar senescence and premature leaf abscission. Trees of the genus *Tilia* do not show any foliar injury induced by ozone under natural conditions. In this study, we investigated the effects of this pollutant on ecophysiological and biochemical parameters of *T. Americana* saplings exposed to a fumigation (120 ppb for 45 consecutive days, 5 h d^{-1}). At the end of treatment, even if plants did not exhibit any visible foliar injury, several parameters were significantly affected: stomatal conductance for water vapor (–15% compared to control), net photosynthesis (–39%), intercellular CO_2 concentration (+30%), as well as chlorophyll fluorescence indexes. After 45 days of fumigation neo–, viola– and anteraxanthin content significantly decreased (–25%, –34% and –63%, respectively, in comparison with controls), but no zeaxanthin induction was detected, suggesting that exposure did not activate the xanthopyll cycle. Under these circumstances, this species should be regarded as "middle tolerant/sensitive".

Keywords: *Air pollution, chlorophyll a fluorescence, CO_2 assimilation rate vs. irradiance response curves, oxidative stress, photosynthetic gas exchange*

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1. Introduction

Ozone (O_3) air pollution causes many negative physiological and biochemical effects in plants and photosynthetic processes (Flowers et al., 2007; Lombardozzi et al., 2012). Generally at the physiological level, this pollutant can induce reductions in: (i) stomatal conductance and rate of CO_2 photoassimilation (Andersen, 2003); however, it is not still clear whether the O_3–induced decrease is a direct effect of the oxidant agent on the stomatal or an indirect one via photosynthetic activity; (ii) photosynthetic efficiency as an effect on the light reactions of photosynthesis, which would be reflected by increases in chlorophyll fluorescence and heat dissipation (Pellegrini et al., 2011b); (iii) carboxylation efficiency (Farage and Long, 1999) by an alteration of the biochemical activity of the Calvin cycle (Calatayud et al., 2003). At the biochemical level, O_3 causes a depression of photosynthetic pigments (Saitanis et al., 2001). In particular, a reduction in the chlorophyll content of leaves, following exposure of plants to O_3, has been reported in many species and, for this reason, Knudson et al. (1977) proposed that this parameter could be a useful indicator for the evaluation of injury induced by oxidative stressors. The dynamic effect of this pollutant on the photosynthesis of trees in urban areas has received little attention so far, even if during the warm season O_3 concentrations may be – for many hours – well above 100 ppb. Model predictions using IPCC scenarios suggest that background levels of this pollutant will increase worldwide in the future (Vingarzan, 2004). Furthermore, O_3 is itself a greenhouse gas contributing to radiative forcing (ICPP, 2007). At the moment, the data in literature in terms of resistance to oxidative stress of species of the genus *Tilia* are scarce: Novak et al. (2003) – assessing the foliar sensitivity of 12 native tree, shrub, and herbaceous species to O_3 in the Switzerland – reported that *T. platyphyllos* and *T. cordata* were among least sensitive; Bussotti et al. (2003) included *T. cordata* into the symptomatic plants, indicating that visible injury was also confirmed under experimental conditions. We investigated the effects of exposures to O_3 based on gas exchange measurements and chlorophyll *a* fluorescence analysis in *T. americana*, a species widespread in urban environment. The aim of the study was to determine if the physiological tools can constitute an instrument in the early subliminal detection of environmental stress and an improvement for the management in urban environment. Preliminary results have been previously communicated (Pellegrini et al., 2010).

2. Materials and Methods

One–year old rooted cuttings of *T. americana* were placed for 1 month in a controlled environment facility at a temperature of 20±1 °C, a RH of 85±5% and a photon flux density at plant height of 500 µmol photon m^{-2} s^{-1} provided by incandescent lamps, during a 12 h photo–period. Uniform individuals, selected when the second leaf was fully expanded, were grown in plastic pots containing a mix of steam sterilized soil and peat (1:1). Uniform–sized plants 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 daily treated for 45 days with a single pulse of O_3 between 09:00 and 14:00 in the form of a square wave [a periodic waveform consisting of instantaneous transitions between two levels: zero ozone (control), and the fixed target of ozone, respectively], with target concentration of 120 ppb (for O_3, 1 ppb=1.96 µg m^{-3}, at 20 °C and 101.325 kPa) equivalent to an AOT40 (Accumulated exposure Over Threshold of 40 ppb) of 400 ppb h d^{-1}; control plants were maintained under the same

experimental conditions, but in chambers receiving the charcoal–filtered air. Concerning the AOT40 value adopted for our study (18 000 ppb h), long–term ozone European metrics (i.e. EEA, 2012) put in evidence that such an AOT40 value is not unrealistic under present ozone scenarios, especially in the Mediterranean area. As a consequence, it has been reported by Ferretti et al. (2007) that current O_3 concentration–based critical levels are neither realistic, nor attainable for crops and forest vegetation in Italy and that a unique set of critical levels appears not suited for the whole Europe.

Analyses were performed on plants at 15 and 45 days from the start of fumigation. Measurements of leaf gas exchanges were carried out by an infra–red gas–analyzer (CIRAS–1, PP–Systems) equipped with a Parkinson leaf chamber that controlled leaf temperature (25 °C), RH (80%), light (1 500 µmol photons m^{-2} s^{-1}) and CO_2 concentration (350 ppm). Response to irradiance (20–1 200 µmol m^{-2} s^{-1}) of photosynthetic CO_2 assimilation was calculated using the Smith equation (Tenhuen et al., 1976). Modulated chlorophyll a fluorescence measurements and the status of the electron transport of PSII were carried out with a PAM–2000 fluorometer (Walz) on dark–adapted leaves for 40 min using a dark leaf clip. In vivo analyses were carried out between 10:00 and 12:00 am (solar time). Further details are reported in Pellegrini et al. (2011a). Chloroplast pigments were determined in methanol extracts using the HPLC methods described by De las Rivas et al. (1989).

A minimum of six plants per treatment were used in each of the three repeated experiments. For statistical significance between the control and treated plants, Student's *t*–test was used.

3. Results

At the end of the treatment, plants did not develop any injury symptoms on leaves, but several physiological parameters were significantly affected. The CO_2 assimilation rate vs. irradiance response curves, obtained after 15 and 45 days of treatment, are shown in Figure 1. Controls in both times of analysis showed typical light response curves; treated plants strongly decreased their photosynthetic rate along the whole light response curve and this reduction was already evident after 15 days of exposure: the maximum net carbon assimilation (A_{max}) obtained when the curve reaches its plateau, significantly decreased in comparison to controls (–16%), although the light saturation point (LSP) reaching for irradiance values (above 999±4.5 µmol m^{-2} s^{-1}) did not show differences in comparison to control (Table 1). In treated leaves, Φ_a was significantly lower than controls in both measurements (–10 and –12%, respectively, after 15 and 45 days of exposure). No recovery was observed by increasing fumigation time up to 45 days in terms of A_{max} and LSP values (–16 and 39%, respectively) (Table 1).

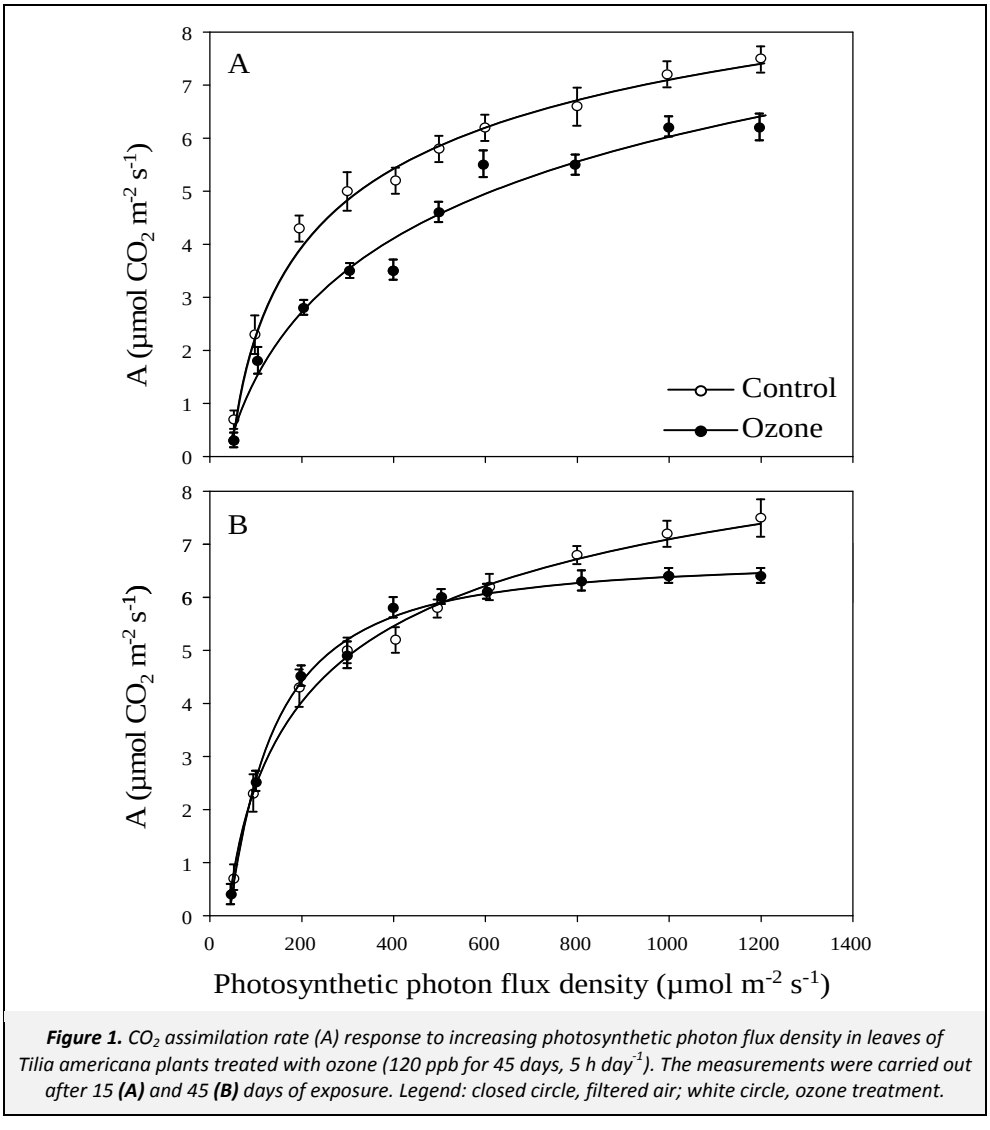

Figure 1. *CO_2 assimilation rate (A) response to increasing photosynthetic photon flux density in leaves of Tilia americana plants treated with ozone (120 ppb for 45 days, 5 h day^{-1}). The measurements were carried out after 15* ***(A)*** *and 45* ***(B)*** *days of exposure. Legend: closed circle, filtered air; white circle, ozone treatment.*

Table 1. *Light response curve parameters (mean ± standard deviation) in leaves of Tilia americana plants exposed to an ozone treatment (120 ppb for 45 consecutive days, 5 h day^{-1}). Measurements were carried out on plants maintained in filtered air (control) and after 15 and 45 days of fumigation (ozone). Legend: A_{max}: maximum net carbon assimilation (μmol CO_2 m^{-2} s^{-1}); Φ_a: apparent quantum yield; LSP: light saturation point (μmol photons m^{-2} s^{-1}). The significance of differences between control and ozone-exposed plants is determined with the t-Student's test: significant, P≤0.001; not significant, P>0.05*

	Days	Control	Ozone	*P*
A_{max}	15	7.4±0.19	6.2±0.10	≤0.001
	45	7.5±0.10	6.3±0.06	≤0.001
Φ_a	15	0.042±0.0004	0.038±0.0005	≤0.001
	45	0.042±0.0003	0.037±0.0007	≤0.001
LSP	15	997±2.8	999±4.5	>0.05
	45	996±2.3	604±5.0	≤0.001

At the end of fumigation (Table 2), the light saturated CO_2 assimilation rate (*A*) and stomatal conductance (G_w) significantly decreased (respectively, –39% and –15% compared to controls); the values of internal CO_2 concentration (C_i) were higher in treated plants respect to controls (+30%). The maximum quantum yield (F_v/F_m) was slightly reduced compared to controls (–7%), which was mainly due to the increase in minimum level of fluorescence (F_0, +36%) (Table 3). Also modulated chlorophyll *a* fluorescence at the end of actinic irradiation changed: the apparent relative electron transport rate (ETR) was strongly and significantly reduced in treated plants (–45% in comparison to controls). A similar pattern was recorded for the actual quantum yield (Φ_{PSII}) that significantly decreased at the end of the treatment (–34% compared to controls). The reduction state of the primary stable quinone acceptor of PSII (Q_A) can be estimated as 1 – *qP*: in plants exposed to O_3, the values increased (+60% compared to control).

Table 2. *Gas exchange parameters (mean ± standard deviation) in leaves of Tilia americana plants exposed to a ozone treatment (120 ppb for 45 consecutive days, 5 h day^{-1}). Measurements were carried out on plants maintained in filtered air (control) and after 45 days of fumigation (ozone). Legend: A: CO_2 assimilation rate; G_w: stomatal conductance to water vapor; C_i: apparent internal CO_2 concentration. The significance of differences between control and ozone-exposed plants is determined with the t-Student's test: significant at P≤0.001 or P≤0.01*

Parameters	Control	Ozone	*P*
A (μmol CO_2 m^{-2} s^{-1})	6.2±0.22	3.8±0.47	≤0.01
G_w (mmol H_2O m^{-2} s^{-1})	162±0.7	138±3.1	≤0.001
C_i (ppm)	235±5.1	306±7.9	≤0.001

Table 3. *Chlorophyll a fluorescence parameters (arbitrary units; mean ± standard deviation) in leaves of Tilia americana plants exposed to a ozone treatment (120 ppb for 45 consecutive days, 5 h d^{-1}). Measurements were carried out on plants maintained in filtered air (control) and after 45 days of fumigation (ozone). Significance of differences between control and ozone-exposed plants is determined with the t-Student's test; significant, P≤0.001, P≤0.01, P≤0.05; not significant, P>0.05. Abbreviations: F_0, minimal fluores-cence; F_m, maximal fluorescence; F_v/F_m, variable and maximal fluorescence ratio; 1-qP, reduction state of QA; qN, total nonphotochemical quenching; ΦPSII, actual quantum yield of PSII; ETR, apparent electron transport rate through PSII*

Parameters	Control	Ozone	*P*
F_0	0.107±0.0176	0.146±0.0076	≤0.01
F_m	0.572±0.0766	0.592±0.052	>0.05
F_v/F_m	0.813±0.0129	0.753±0.0125	≤0.001
ETR	120±30.1	66±13.2	≤0.05
1-qP	0.221±0.0617	0.354±0.0461	≤0.05
qN	0.683±0.0281	0.783±0.0500	>0.05
ΦPSII	0.456±0.0849	0.303±0.0403	≤0.05

Table 4 shows the parameters related to leaf pigment content. During the treatment, significant differences with controls occurred in leaf chlorophylls; in particular, a significant decrease was observed in chlorophyll *a* after 45 days (–32%, in comparison with controls) and in chlorophyll *b* for both times of analysis (–7%, –8%, respectively, in comparison with controls). Table 5 shows the results of parameters related to xanthophylls cycle pigments and carotenoids content: significant differences with controls occurred in carotenoids and xanthophyll concentration: in particular, a significant decrease was observed in lutein and β–carotene content after 15 days (–24% and –41%, respectively, in comparison with controls) and these parameters remained constantly low also at the end of treatment (–56% and –32%, respectively, in comparison with controls). Only after 45 days of fumigation, neo–, viola– and anteraxanthin contents significantly decreased (–25%, –34% and –63%, respectively, in comparison with controls), but no zeaxanthin induction was detected. Only at the end of the treatment the de–epoxidation index (DEPS) was depressed (–34%) in O_3–exposed individuals.

Table 4. *Chlorophyll a and chlorophyll b content (μg mg^{-1} FW) (mean ± standard deviation) in leaves of Tilia americana plants exposed to 120 ppb O_3 for 45 consecutive days (5 h day^{-1}). For each parameter, the significance of differences following the t-Student's test is: significant, P≤0.01; not significant P>0.05*

	Days	Control	Ozone	*P*
Chlorophyll a	15	9.67±0.203	9.20±0.361	>0.05
	45	9.47±0.419	6.44±0.775	≤0.01
Chlorophyll b	15	3.50±0.121	3.27±0.121	≤0.01
	45	3.69±0.155	3.38±0.123	≤0.01
Chl a / Chl b	15	2.74	1.80	≤0.01
	45	2.31	2.01	≤0.01

Table 5. *Xanthophyll cycle pigments and carotenoids content (μg mg^{-1} FW) (mean±standard deviation) in leaves of Tilia americana plants exposed to 120 ppb O_3 for 45 consecutive days (5 h day^{-1}). For each parameter, the significance of differences following the t-Student's test is: significant, P≤0.01, P≤0.05; not significant P>0.05. DEPS = de-epoxidation index*

	Days	Control	Ozone	P
Neoxanthin	15	5.13 ± 0.554	4.40 ± 0.416	>0.05
	45	5.09 ± 0.194	3.84 ± 0.041	≤0.01
Lutein	15	8.11 ± 0.313	6.18 ± 0.447	≤0.05
	45	9.96 ± 1.002	4.33 ± 0.986	≤0.05
β-Carotene	15	7.63 ± 0.539	4.52 ± 0.966	≤0.05
	45	7.61 ± 0.444	5.15 ± 0.384	≤0.05
Violaxanthin	15	3.73 ± 0.109	2.97 ± 0.393	>0.05
	45	3.03 ± 0.177	2.01 ± 0.173	≤0.05
Anteraxanthin	15	2.27 ± 0.067	2.13 ± 0.065	>0.05
	45	2.81 ± 0.153	1.05 ± 0.009	≤0.05
DEPS	15	18.9 ± 0.68	23.3 ± 2.18	>0.05
	45	24.1 ± 0.38	15.8 ± 0.911	≤0.05

4. Discussion

The degree to which plants develop visible injury is commonly utilised as an indicator of their O_3 sensitivity. In these terms, in this experiment *T. americana* could be regarded as a tolerant species: at the end of exposure, plants did not showed any symptoms. This conclusion is supported by similar findings described in Bussotti et al. (2003) and in Novak et al. (2003) on *Tilia* genus: Authors showed that *T. cordata* did not show any foliar injury induced by O_3 and *T. platyphyllos* developed minute severe injury calculated as percent of injured leaves per plants, as well as the average of the symptomatic leaf area.

Although the literature concerning other tree species presents a wide range of photosynthetic responses to O_3 exposure, the physiological response of *Tilia* to this pollutant is unknown. Gas exchange measurements showed that in *T. americana* treated plants there was a significant reduction in CO_2 assimilation rate and in stomatal closure; the concomitant increase of C_i suggests that the reduction of CO_2 photoassimilation observed was attributable to mesophyll limitation and that stomatal response could be a secondary event following changes in mesophyll activity. This conclusion is supported by similar findings described in soybean by Fiscus et al. (1997), in poplar by Nali et al. (1998) and Noormets et al. (2001), in tulip tree by Pellegrini et al. (2011b) and in *Ginkgo biloba* by He et al. (2007), who showed that the variation in photoassimilation did not correlate to stomatal closure, but was attributable to an alteration in the activity of mesophyll. The analysis of photosynthetic response curves to increasing irradiance showed that a reduction in the light saturation point was observed only at the end of the treatment, according to the gas exchange parameters. At the end of our treatment, O_3 caused a slight but significant decrease in F_v/F_m. Similar minor changes in response to O_3 have been observed in a variety of species, such as wheat (Reichenauer et al., 1997), soybean (Sawada et al., 1990), lemon balm (Pellegrini et al., 2011a) and birch (Wittmann et al., 2007). This significant reduction of F_v/F_m was associated with an increase in F_0, indicating that an increased fraction of the excitation energy received by the light–harvesting pigment–proteins was not used to drive electron transport, but was re–emitted as fluorescence. Some Authors have interpreted this phenomenon as representing a partially closure of PSII reaction center or a disconnection of the peripheral antenna system from the reaction centre (Pellegrini et al., 2011b).

The significant increase in 1–*qP* values observed at the end of our treatment suggests that O_3 caused an increase in the reduction state of the PSII primary acceptor, producing a decrease in the fraction of open PSII (Krause and Weiss, 1991), which determined a lower possibility of electron transport from PSII to PSI. As a confirmation of this hypothesis, Φ_{PSII}, which is closely related with the quantum yield of non–cyclic electron transport, was reduced at the end of treatment. The explanation for the fraction of the PSII reaction centers that remain reduced could be related to slow re–oxidation of Q_A, producing some centers that become unable to carry out stable charge separation. The decreasing capacity of Q_A re–oxidation may be due to inhibition of Calvin cycle activity, as indicated by reduction in the CO_2 assimilation rate. Increase in 1–*qP* and the decrease in F_v/F_m ratio is known to be closely associated with photoinhibition (Ogren and Rosenqvist, 1992). PSII reaction centers can be protected from damage induced by an excess of photons if excitons are deactivated and dissipated as heat in inner antenna systems (Melis, 1999) and this determines the observed decrease in F_v/F_m ratio and in Φ_{PSII} in treated leaves. This is in agreement with results obtained by Calatayud et al. (2010) in *Viburnum lantana* exposed to O_3.

At the end of exposure, the decrease in CO_2 assimilation rates (that can be considered a primary photosynthetic reaction) was accompanied by a decline in total chlorophyll levels. This is similar to what observed in poplar (Reich, 1983), red spruce (Rebbeck et al., 1993), Norway spruce (Robinson and Wellburn, 1991), birch (Wittmann et al., 2007) and *G. biloba* (He et al., 2007). These pigments as well as β–carotene (Ranieri et al., 2000) concentrations have been found to decrease following both short and long periods of fumigation with O_3. The reduction in photosynthetic and accessory pigments has been reported as a consequence of O_3–induced early senescence (Mikkelsen et al., 1995). In particular, the decrease of β–carotene could be attributed to the role of this molecule as an antioxidant capable of scavenging the harmful singlet oxygen produced by the interaction of the triplet chlorophyll with O_2 (Ranieri et al., 2000). O_3 exposure did not activate the xanthophyll cycle. This has been confirmed by DEPS, which is generally used to evaluate the activation of the photoprotective mechanism of the xanthophyll cycle, which, in turn, through the de–epoxidation of viola– to anthera– and zeaxanthin, is able to dissipate the harmful excess of absorbed light energy, thus protecting the PSII reaction centers from photoinhibition.

5. Concluding Remarks

T. americana should be regarded as "tolerant" to O_3 in field observations, because at the end of a severe exposure, plants did not show any symptom. However under above reported circumstances, this species should be considered at least "middle tolerant/sensitive", if specific physiological parameters are evaluated. Reasons for this may include: (i) reduction of the photosynthetic capacity; (ii) changes in light reactions of photosynthesis and in the functioning or in the integrity of reaction centers; (iii) no activation of the xanthophyll cycle. These findings may be of practical concern in urban forestry.

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