Ozone sensitivity and ethylenediurea protection in ash trees assessed by JIP chlorophyll *a* fluorescence transient analysis

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Abstract

The effect of ethylenediurea (EDU) was tested using the chlorophyll (Chl) *a* fluorescence transient analysis, performed with JIP-test, to assess ambient ozone (O_3) effects on photosynthesis of adult trees under natural conditions. Twelve adult European ash (*Fraxinus excelsior* L.) trees, known to be sensitive or tolerant to O_3, determined by presence symptomatic (S) or absence asymptomatic (AS) trees of foliar symptoms in previous years, were treated either with distilled water containing 450 g m^{-3} EDU or with distilled water. Once a month across the growing season [the accumulated exposure over a threshold of 40 nmol(O_3) mol^{-1} was 32.49 μmol mol^{-1} h^{-1}], Chl *a* fluorescence transients were measured *in vivo* on dark-adapted leaves of 1-year-old labeled shoots, from the lower crown part. Twenty-five parameters were calculated. The maximum quantum yield of primary photochemistry (φ_{Po} or F_v/F_m) did not differentiate between S- and AS-trees, while increased Chl content and de-excitation rates suggested compensation of O_3 injury in S-trees. Seasonal reductions in absorbing fluxes and increase in heat and fluorescence dissipation processes was due to leaf ageing and drought, the latter suggesting water deficit influenced Chl *a* fluorescence stronger than ambient O_3 exposure. AS-trees showed elevated probability of connectivity among photosystem 2 units, a mechanism to stimulate energy dissipation and reduce photo-oxidative injury. EDU prevented the inactivation of reaction centers. This slight effect does not warrant EDU as a tool to assess O_3 effects on photosynthesis, while the JIP-test is suggested for a quantitative assessment in adult trees.

Additional key words: *Fraxinus*; photosystem 2.

Introduction

The development of anthropogenic activities has led to an increase in global ground-level ozone (O_3) concentrations that adversely affect plant health (Bytnerowicz *et al*. 2007). Ozone severely damages the physiological and biochemical processes of trees (cf. He *et al*. 2007, Feng *et al*. 2008), primarily injuring the tissues of leaf mesophyll cells (Paoletti 2007). The photosynthetic apparatus is one of the primary targets of O_3 injury. Stomatal conductance, activity of photosynthetic enzymes, thylakoid membranes, rate of electron transport, and carbon assimilation per unit leaf area decline with exposure to O_3. Ozone further decreases carbon gain by reducing plant leaf area through accelerated senescence and in some cases increases respiratory demand for antioxidants and repair metabolism (Kangasjärvi *et al.* 1994, Pell *et al*. 1997, Schraudner *et al*. 1997). If O_3 exposure is long enough, species-specific chlorotic flecking, necrosis, or bronzing may gradually coalesce on the upper leaf surface (Paoletti 2007).

Quantitative assessments of O_3 effects on forest trees are still matter of uncertainty because the experimental techniques do not allow extrapolation to realistic conditions (Manning 2005). For logistic reasons, most of the information regarding O_3 effects on trees comes from experiments performed in indoor or outdoor chambers, where seedlings are exposed to controlled O_3 concentrations in unrealistic microclimatic conditions and for short periods (Paoletti 2007). The use of antioxidants

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eliminates O_3 as a factor of stress and allows the use of control trees exposed to O_3 under natural conditions (Manning 2005). Ethylenediurea (N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea; EDU) is one of the most successful and enduring antioxidant chemicals, although the protective mechanisms are still unclear and may be not the same in all plant species (Manning 2005). EDU has been used to prevent foliar O_3 injury and determine O_3 effects on growth of some woody plants (McClenahen 1979, Cathey and Heggestad 1982, Roberts *et al.* 1985, Roberts 1987, Long and Davis 1991, Ainsworth and Ashmore 1992, Ainsworth *et al.* 1996, Bortier *et al.* 2001, Manning *et al.* 2003, Paoletti *et al.* 2007a,b, 2008).

Another limitation to the quantitative assessment of O_3 effects on forest trees is the absence of an objective, simple, and possibly non-intrusive screening method. A promising approach is the analysis of chlorophyll (Chl) *a* fluorescence transients. This non-destructive method is widely used for monitoring and screening plant tolerance to environmental stresses and can provide large amounts of accurate data with a minimum of expertise and time (Clark *et al.* 2000). Irradiating a dark-adapted leaf results in characteristic changes in the intensity of Chl *a* fluorescence, known as the Kautsky effects (Kautsky and Hirsch 1931). The Kautsky transient shows a fast rise (1 s from an initial fluorescence, F_0 to a maximum fluorescence level, F_m) with a subsequent slower decline towards a steady state [several min]. Changes in the rising phase of the transient reflect changes in the primary reactions of photosynthesis (Krause and Weis 1991). The simplest and most immediate index, calculated from the rising phase of the transient, is the maximum quantum yield for primary photochemistry (F_v/F_m) (Kitajima and Butler 1975). Some studies reported that F_v/F_m remained nearly unchanged after O_3 exposure (Meinander *et al.* 1996, Gravano *et al.* 2004), while others suggested that the effect of O_3 on this parameter could be species-specific (Nussbaum *et al.* 2001, Paoletti *et al.* 2004, Bussotti *et al.* 2005, 2007a). Actually, F_v/F_m is a secondary response preceded by other metabolic disturbances and integrates information on different photosynthesis reactions, ranging from the photon absorption to the reduction of the electron acceptor pools, each of these being differently affected by O_3 (Nussbaum *et al.* 2001). The use of high-resolution time-solving direct fluorometers that enable data accumulation over several orders of magnitude of time (10 μs to 120 s) provides additional and more accurate information on the rising phase of the transient. In all oxygenic photosynthetic samples, the fast fluorescence rise transient, plotted on a logarithmic time scale, shows a sequence of phases, labeled as O (origin, initial fluorescence level, F_0), J (photochemical phase), I (intermediate), and P (peak, maximum fluorescence level, F_m, P=M in dark-adapted leaves; Strasser and Govindjee 1992, Strasser *et al.* 1995). The O-J-I-P polyphasic transient reflects the kinetics and heterogeneity involved in the filling up of the electron acceptor side of photosystem 2 (PS2), *i.e.* Q_A (primary electron acceptor of PS2), Q_B (secondary electron acceptor of PS2), and plastoquinone (PQ) pool, with electrons from the donor side of PS2 (Strasser and Govindjee 1992). It modifies its shape according to changes in environment (Srivastava and Strasser 1996, Lazár 1999, Clark *et al.* 2000). Strasser and Strasser (1995) introduced a quantitative analysis of the O-J-I-P transient, called JIP-test, by which the original O-J-I-P fluorescence measurements are translated into several biophysical and phenomenological expressions that quantify the stepwise flow of energy through PS2. Additionally, Strasser *et al.* (2000) introduced a new multi-parametric expression, derived by the JIP-test parameters, the so-called performance index (PI). Several authors have already demonstrated the advantage of using the JIP-test parameters to understand O_3 effect on plants (Meinander *et al.* 1996, Soja *et al.* 1998, Clark *et al.* 2000, Nussbaum *et al.* 2001, Gravano *et al.* 2004, Paoletti *et al.* 2004, Bussotti *et al.* 2005, 2007a).

Gravitational trunk infusion of EDU prevents foliar O_3 injury and improves growth in sensitive ash (*Fraxinus excelsior* L.) trees (Paoletti *et al.* 2007a,b). Increase in ascorbate peroxidase, and decrease in apoplastic hydrogen peroxide and stomatal conductance were suggested to regulate EDU action in alleviating O_3 effects on ash growth, while no effect on net photosynthesis and the maximum quantum yield for primary photochemistry (F_v/F_m) were recorded (Paoletti *et al.* 2008).

We report here additional Chl *a* fluorescence results from the same field experiment when adult trees, considered to be either O_3-sensitive or tolerant, were gravitationally infused with EDU. The first aim was to investigate whether the Chl *a* fluorescence parameters change over time with O_3 sensitivity and EDU treatments of trees. The second aim was to validate the use of the antioxidant EDU and the JIP-test analysis as possible tools to quantitatively assess the effects of ambient O_3 on adult trees under natural conditions.

Materials and methods

Experimental conditions: The experimental site was located at the 0.34-km^2 ''Millerose'' park in Turin, Italy. The O_3 exposure index AOT40, calculated as Accumulated exposure Over a Threshold of 40 nmol O_3 mol^{-1} for daylight hours (ICP 2004), for the growing season (1 May–30 September, 2005) was 32.49 g m^{-3} h^{-1}, which is more than six times above the critical level of 5 g m^{-3} h^{-1} to protect forest trees (ICP 2004). The daily mean (±S.D) air temperature was 23.4±4.0, 24.8±3.1, 22.9±2.2, and 15.4±2.7 °C in June, July, August, and September, respectively. The daily mean relative humidity was 59.5±11.2, 53.5±10.6, 66.5±16.3, and 69.5±12.6 %. The

total precipitations were 6.6, 110.8, 58.6, and 124.6 mm, respectively (Paoletti *et al.* 2007b).

Six O_3-sensitive (S) and six O_3-tolerant (AS) adult ash trees, determined by presence or absence of foliar injury in previous years, were selected. The six O_3-tolerant trees had never shown foliar symptoms of O_3 injury in the previous years (Paoletti *et al.* 2007b). Bud break occurred in late April. At 3-week intervals from 31 May until 13 September 2005, six trees (three S and three AS) were treated by gravitational trunk infusion with distilled water containing 450 g m^{-3} EDU [(N-[2-(2-oxo-1-imidazolid-inyl)-ethyl]-N'-phenylurea)] and the other six trees were infused with distilled water. In order to get an even distribution of EDU inside the crown, two 2-cm-long holes on opposite sides were made at breast height. A 1-cm^3 pipette tip was inserted into the holes and connected to a commercially available 2 000 cm^3 infusion bag containing the correct volume of pure water or EDU solution. The bags were shaded with white paper and hung on the trunk at least 1 m above the holes. To prevent overflow outside the holes, flow was controlled by a Hoffman clamp. Details about infusion methodology, site characteristics, and EDU protection from O_3 visible injury are in Paoletti *et al.* (2007b). Details about growth and physiological and biochemical results are in Paoletti *et al.* (2007a, 2008).

Chlorophyll (Chl) *a* fluorescence measurements: Once a month from June to September, Chl *a* fluorescence transient was measured *in vivo* at midday, at ambient temperature with a direct fluorometer (Handy PEA, Plant Efficiency Analyser, *Hansatech Instruments*, Kings Lynn, UK). Measurements were carried out on the adaxial surface of apical leaflets, from the 4th to 6th leaf from the tip of four 1-year-old labeled shoots per tree, from the lower crown part. The shoots were terminal shoots of a lateral branch and were exposed to SW. Leaves were free of any symptoms. The selected leaves were subjected to a 50-min dark adaptation, which was sufficient to completely oxidise the RCs. The rising transient was induced by saturating red-actinic-radiation (1 500 μmol m^{-2} s^{-1}, peak at 650 nm, duration 1 s). Data acquisition was recorded from 10 μs (resolution time of the instrument's sensor) to 1 s after the onset of irradiation. The values of F_0 (approximated as florescence value at 10 μs) and F_m were recorded. F_0 is fluorescence measured when all RCs of PS2 are considered open, *i.e.* all the primary acceptors, quinone Q_A, are fully oxidized. F_m is the maximal fluorescence yield in the dark, when the excitation intensity is high enough to close all RCs of PS2, *i.e.* all the Q_A is fully reduced. In dark-adapted leaves, values of the maximum quantum yield for primary photochemistry (F_v/F_m, *i.e.* the maximal efficiency that an exciton is trapped by a RC of PS2) was calculated as $(F_m - F_0)/F_m$ (Strasser *et al.* 2004). The maximum quantum yield for primary photochemistry is equal to the quantity φ_{Po} or TR_0/ABS. F_v/F_m provides information about the processes which alter the potential quantum efficiency of PS2 and is used as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson 2000).

The JIP-test: The energy flux through the PS2 can be thought as an absorbed photon flux by antenna pigments (ABS, absorption flux) creating excited chlorophyll. Part of ABS is trapped by RCs (TR, trapping flux) to be converted to redox energy by reducing the primary electron acceptor Q_A to Q_A^- which is then re-oxidized to Q_A reducing the electron transport chain beyond Q_A^- (ET, electron transport flux) and leading ultimately to CO_2 fixation. Another part of excitation energy is dissipated in form of heat or fluorescence emission (DI, dissipated flux). As showed in Table 1, according to Strasser and Strasser (1995) and Force *et al.* (2003), O-J-I-P fluorescence values were used to calculate the stepwise flow of energy through PS2 at RC level (ABS/RC, TR_0/RC, ET_0/RC, and DI_0/RC) as well as at the level of a PS2 cross-section (CS) (ABS/CS_0, TR_0/CS_0, ET_0/CS_0, and DI_0/CS_0). The flux parameters are inter-related by probabilities that define: the maximum quantum yield for primary photochemistry (φ_{Po} or TR_0/ABS or F_v/F_m); electron transport (ψ_0 or ET_0/TR_0), *i.e.* the efficiency with which a trapped exciton, having triggered the reduction of Q_A to Q_A^-, can move an electron further than Q_A^- into the electron transport chain; quantum yield for electron transport (φ_{Eo} or ET_0/ABS), *i.e.* the probability that an absorbed photon moves an electron into the electron transport chain beyond Q_A^-; and exciton dissipation (φ_{Do} or $1 - TR_0/ABS$), *i.e.* the probability that an absorbed photon is dissipated. The JIP-test allows to determine: the density of RCs per excited CS (RC/CS_0), *i.e.* the number of active RCs to one inactive RC for a PS2 CS; the turnover number (N), *i.e.* the number of electrons flowing through the electron transport chain; the average relative variable fluorescence (B_{av}), *i.e.* the average fraction of close RCs during the time needed to complete the closure of all RCs; and the de-excitation constants (k_n and k_p), *i.e.* the rate of de-excitations through heat dissipation, fluorescence emission, and energy migration to PS1, and the rate of de-excitations through photochemical reactions, respectively. Additionally the three independent parameters RC/ABS, φ_{Po}, and ψ_0 were combined to calculate the performance index (PI), an index combining the three main functional and structural properties affecting the potential photosynthetic activity (Strasser *et al.* 2000): (*a*) density of RCs; (*b*) efficiency of light reactions (probability that an absorbed photon is used for a charge separation), and (*c*) a component related to forward electron transfer. The natural logarithm of PI gives the driving force (DF) (Table 1). DF can be defined as the total driving force for photosynthesis of the observed system, created by summing up the partial driving forces for each of the several energy bifurcations

in PS2. Analysis of the transient with the JIP-test took into consideration fluorescence values at 50 μs ($F_{0,}$), 100 μs (F_{100}), 300 μs (F_{300}), 2 ms (step J), 30 ms (step I), and maximal fluorescence (step P) and was performed with *Biolyzer 3.06* software (by Ronald Maldonado-Rodriguez, Bioenergetics Laboratory, Geneva, CH).

Extension of the JIP-test to include PS2 connectivity: The JIP-test was originally formulated with the assumption of no grouping between photosynthetic units, *i.e.* no exciton moves from neighboring pigment beds. For the unconnected units' version, the relative variable fluorescence at any time (V_t) equals the fraction of close RCs (B_t). Later on, the existence of grouping was detected (Strasser *et al.* 2004). A change in connectivity influences the behavior of RC parameters, so that the irradiation-induced changes in the RC parameters were in error when calculated with the unconnected unit version (Force *et al.* 2003). If units are energetically connected, the correlation of V_t and B_t is hyperbolic and represented by the equation $V_t = B_t/[1+C(1 - B_t)]$ (Strasser 1978). C is the curvature constant of the hyperbola and can be calculated as $C = [(W_E - W)/W \cdot (1 - W_E) \cdot V_j)]$, where $W = [(F_{100} - F_0)/(F_j - F_{50})]$ and $W_E = [1 - (F_j - F_{300})/(F_j - F_0)^{1/5}]$. Connectivity is accounted for by multiplying the RC parameters of the JIP-test (Table 1, ABS/RC, TR_0/RC, ET_0/RC, DI_0/RC) by the term (1+C) (Force *et al.* 2003, Strasser *et al.* 2004).

Table 1. Summary of the JIP-test parameters calculated using data extracted from the fast fluorescence transient. Parameter definitions are given in the text.

Basic symbols and terms: ABS = absorption flux; AS – asymptomatic; Chl – chlorophyll; CS – cross-section; DI = dissipated energy flux; ET = energy flux for electron transport; k_F = rate constant for fluorescence emission; PS2 CS cross section, constant; Q_A = primary electron acceptor of PS2; P_N – net photosynthetic rate; RC = reaction centre; S – symptomatic; TR = energy flux for trapping;.
Extracted fluorescence (F) parameters: F_t = F at time t; F_0 = F at 50 μs; F_{300} = F at 300 μs; F_j = F at 2 ms; F_i = F at 30 ms; F_m = maximal F; F_v = variable F at time t = $F_t - F_0$; t_{Fm} = time to reach F_m [ms].
Technical fluorescence parameters: Area: $\int(F_m - F_v)\,dt$; net rate of PS2 closure: $M_0 = (F_{300} - F_0)/F_m - F_0)$; relative F_v at the J-step: V_j: $(F_j - F_0)/(F_m - F_0)$; relative F_v at the I-step: $V_i = (F_i - —F_0)/(F_m - F_0)$.
Flux ratios of yields: maximum quantum yield of primary photochemistry: $\varphi_{P0} = TR_0/ABS = 1 - (F_0/F_m) = F_v/F_m$; electron transport probability: $\psi_0 = ET_0/TR_0 = (1 - V_j)$; quantum yield for electron transport: $\varphi_{E0} = ET_0/ABS = \varphi_{P0}\ \psi_0$; quantum yield for energy dissipation: $\varphi_{D0} = 1 - TR_0/ABS = 1 - \varphi_{Po} = F_0/F_m$.
Specific energy fluxes or specific activities: effective antenna size of an active RC: $ABS/RC = M_0\ (1/V_j)\ (1/\varphi_{Po})$; maximal trapping rate of PS2: $TR_0/RC = M_0\ (1/V_j)$; electron transport in an active RC: $ET_0/RC = M_0\ (1/V_j)\ \psi_0$; effective dissipation of an active RC: $DI_0/RC = (ABS/RC) - (TR_0/RC)$.
Phenomenological energy fluxes: number of photons absorbed by an excited PS2 CS: $ABS/CS_0 \approx F_0$; maximal trapping rate in a PS2 CS: $TR_0/CS_0 = (ABS/CS_0)\ \varphi_{P0}$; electron transport in a PS2 CS = $ET_0/CS_0 = (ABS/CS_0)\ \varphi_{P0}\ \psi_0$; dissipation in a PS2 CS: $DI_0/CS_0 = (ABS/CS_0) - (TR_0/CS_0)$.
Density of RCs: concentration of RCs per excited CS: $RC/CS_0 = \varphi_{P0}\ (V_j/M_0)\ (ABS/CS)$.
Complementary area and turnover number: normalised total complementary area: $S_m = Area/F_v$; time dependent turnover number of Q_A: $N = S_m\ M_0\ (1/V_j)$; average relative F_v: $B_{av} = 1 - (S_m/t_{Fm})$.
De-excitation constants: non-photochemical de-excitation constant: $k_n = (ABS/CS)\ k_F\ (1/F_m)$; photochemical de-excitation constants: $k_p = (ABS/CS)\ k_F\ [(1/F_0) - (1/F_m)]$; total of de-excitation constants: $SumK = k_P + k_n$.
Performance indexes and driving forces: performance index per absorption flux: $PI_{ABS} = (RC/ABS)\ [\varphi_{P0}/1 - \varphi_{P0})]\ [\psi_0/(1 - \psi_0)]$; performance index per CS: $PI_{CS} = (RC/CS_0)\ [\varphi_{P0}/1 - \varphi_{P0})]\ [\psi_0/(1 - \psi_0)]$; diving forces per absorption flux: $DF_{ABS} = In(PI_{ABS})$; driving forces per CS: $DF_{CS} = In(PI_{CS})$.

Statistical analysis: The statistical unit was the single leaf. Data were checked for normal distribution (Shapiro-Wilk W test) and homogeneity of variance (Levene's test). Effects of tree O_3-sensitivity, EDU-treatment and month of measurement were tested by analysis of variance (ANOVA). When ANOVA results were statistically significant, a Tukey HSD test was used to select the homogeneous groups of means within each variable. Tests of significance were made at a 95 % confidence level. Data were processed using *STATISTICA 6.0 Package for Windows* (*StatSoft*, Tulsa, UK).

Results

Dark-adapted leaves of *F. excelsior* exhibited a typical O-J-I-P Chl *a* fluorescence transient when irradiated with a saturating pulse (Fig. 1). In June, a marked difference existed between the O-J-I-P transient shapes in leaves of S-trees or in AS-trees: at all the transient steps, S-ashes showed higher fluorescence values (Fig. 1). The differences in O-J-I-P transient shapes between S- and AS-trees were reduced over the growing season (Fig. 1). Transient shapes were not affected by EDU treatments (Fig. 1).

ANOVA tests performed separately each month showed that F_0 and F_m values were significantly higher

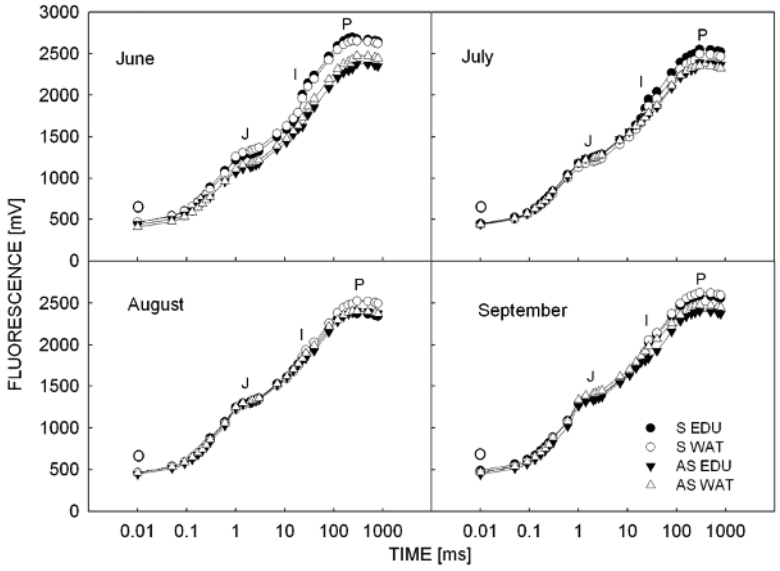

Fig. 1. Average O-J-I-P Chl *a* fluorescence transients (n=12) in asymptomatic (AS) and symptomatic (S) ash trees infused with water (WAT) or ethylenediurea (EDU) during the growing period (June-September).

Table 2. F values of three-way analysis of variance (n=192) for the effects of tree O_3-sensitivity (Sen), *i.e*. asymptomatic, AS or symptomatic, S; treatment (EDU), *i.e*. water or EDU-infused, and Month (Mon) *i.e*. June, July, August, or September on JIP-test parameters (PS2 unconnected version). For acronyms see Table 1. $^{*}p\leq0.05$; $^{**}p\leq0.01$; $^{***}p\leq0.001$; ns = $p>0.05$ (not significant); d.f. = degree of freedom. Significant values are printed in boldface.

d.f.	Sen 1	EDU 1	Mon 3	Sen×EDU 1	Sen×Mon 3	EDU×Mon 3	Sen×EDU×Mom 3
F_0	**6.10***	0.34^{ns}	**3.34***	0.37^{ns}	1.68^{ns}	0.57^{ns}	1.69^{ns}
F_m	**8.01****	0.35^{ns}	1.17^{ns}	0.01^{ns}	0.59^{ns}	0.27^{ns}	0.32^{ns}
M_0	0.47^{ns}	0.73^{ns}	**4.30****	**4.57***	**3.34***	0.28^{ns}	0.41^{ns}
V_j	**16.00*****	1.15^{ns}	**23.51*****	0.49^{ns}	**3.95***	1.07^{ns}	0.64^{ns}
V_i	0.46^{ns}	**14.60*****	**5.46****	**8.32****	0.64^{ns}	0.47^{ns}	**3.49***
φ_{P0} (F_v/F_m)	0.80^{ns}	1.47^{ns}	**4.09****	0.71^{ns}	0.93^{ns}	0.23^{ns}	1.87^{ns}
ψ_0	**16.00*****	1.15^{ns}	**23.51*****	0.49^{ns}	**3.95***	1.07^{ns}	0.64^{ns}
φ_{E0}	**13.00*****	1.71^{ns}	**20.81*****	0.94^{ns}	**3.19***	0.42^{ns}	0.75^{ns}
φ_{D0}	0.84^{ns}	1.35^{ns}	**4.02****	0.70^{ns}	0.87^{ns}	0.23^{ns}	1.90^{ns}
ABS/RC	**15.90****	0.45^{ns}	**9.63*****	**10.20*****	1.75^{ns}	0.83^{ns}	0.78^{ns}
TR_0/RC	**21.10*****	1.07^{ns}	**14.20*****	**10.60****	1.31^{ns}	0.79^{ns}	0.21^{ns}
ET_0/RC	**37.80*****	1.65^{ns}	**34.20*****	3.89^{ns}	1.35^{ns}	1.30^{ns}	0.42^{ns}
DI_0/CS_0	2.58^{ns}	0.03^{ns}	**2.72***	**4.84***	1.71^{ns}	0.49^{ns}	2.24^{ns}
TR_0/CS_0	**8.44****	0.02^{ns}	1.36^{ns}	0.79^{ns}	1.36^{ns}	0.44^{ns}	0.34^{ns}
ET_0/CS_0	**21.20*****	0.06^{ns}	**3.92***	0.20^{ns}	1.13^{ns}	1.23^{ns}	0.72^{ns}
DI_0/CS_0	1.40^{ns}	0.41^{ns}	**3.32***	1.03^{ns}	2.01^{ns}	0.13^{ns}	2.43^{ns}
RC/CS_0	0.58^{ns}	**22.30*****	**6.28*****	**6.08***	0.82^{ns}	0.91^{ns}	2.08^{ns}
S_m	0.62^{ns}	2.03^{ns}	**7.11*****	1.68^{ns}	2.07^{ns}	0.57^{ns}	1.51^{ns}
N	1.23^{ns}	1.08^{ns}	2.06^{ns}	0.08^{ns}	**2.82***	0.79^{ns}	**2.81***
B_{av}	**4.50***	0.04^{ns}	5.55*	7.21**	0.43^{ns}	2.06^{ns}	1.04^{ns}
k_n	**7.11****	0.90^{ns}	1.01^{ns}	0.05^{ns}	0.32^{ns}	0.18^{ns}	0.28^{ns}
k_p	**4.18***	0.46^{ns}	**3.57***	0.95^{ns}	2.06^{ns}	0.73^{ns}	1.45^{ns}
SumK	**6.19***	0.09^{ns}	2.24^{ns}	1.53^{ns}	1.74^{ns}	0.60^{ns}	0.99^{ns}
PI_{ABS}	1.96^{ns}	0.57^{ns}	**7.56*****	2.36^{ns}	4.10**	0.21^{ns}	1.74^{ns}
PI_{CS}	**5.98***	0.30^{ns}	**6.32*****	1.64^{ns}	2.83*	0.49^{ns}	0.73^{ns}
DF_{ABS}	1.79^{ns}	0.41^{ns}	**7.79*****	1.98^{ns}	3.58*	0.35^{ns}	1.59^{ns}
DF_{CS}	**5.70***	0.41^{ns}	**7.12*****	1.64^{ns}	2.49*	0.58^{ns}	1.12^{ns}

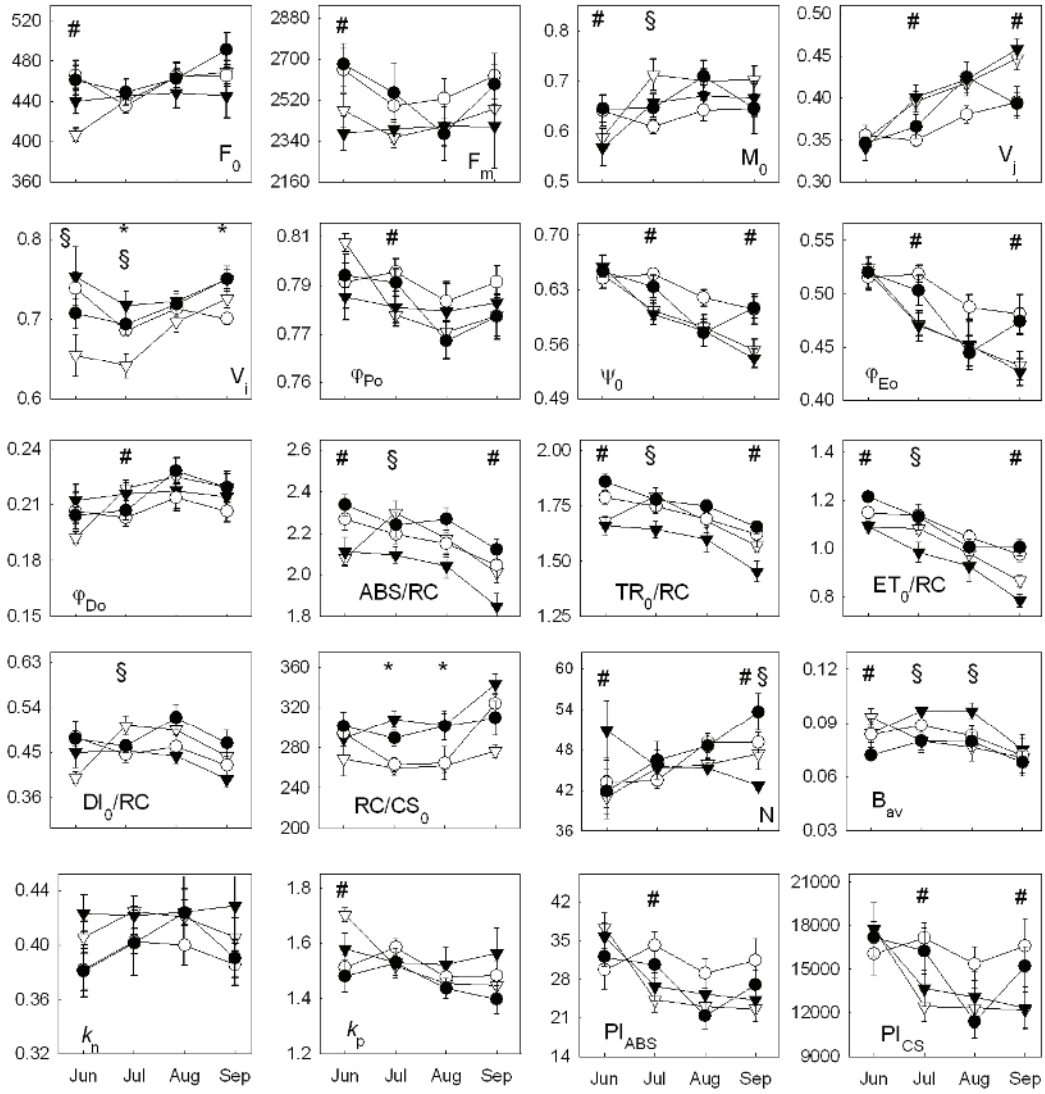

Fig. 2. Time course of JIP-test parameters in *F. excelsior* leaves. Means±S.E. [relative], (n=12). Acronyms are explained in Table 1. ANOVA test ($p \leq 0.05$), performed separately each month, are reported: #, significant effects of O_3-sensitivity (AS − asymptomatic or S − symptomatic), *, significant effects of treatment (water- or EDU-infused), and §, significant effects of O_3-sensitivity×treatment. ▽, asymptomatic water-infused trees; ▼, asymptomatic EDU-infused trees; ○, S water-infused trees; ●, S EDU-infused trees.

(+10 %) in S-ashes only in June (Fig. 2). In August and September, F_0 values increased, whereas F_m values remained constant (Table 2, Fig. 2). The highest values of M_0, V_j, and V_i were at the end of the season (Table 2, Fig. 2). In June, the initial slope M_0 was lower in AS-trees than in the S-ones. In July, in AS-trees M_0 was lower in EDU-infused trees, while in S-trees M_0 was lower in water-infused trees (Table 2, Fig. 2). In contrast, the relative variable fluorescence at the J-step V_j was lower in S-trees in July and September (Table 2, Fig. 2). AS-trees infused with EDU had higher V_i values than water-infused trees, except in August (Table 2, Fig. 2).

φ_{Po} (F_v/F_m) significantly decreased from June to August and, in July, was lower in AS-trees than in S-ones (Table 2, Fig. 2). Both ψ_0 and φ_{Eo} values were lower in AS- than in S-trees, with a significant effect in July and September, and decreased during the season (Table 2, Fig. 2). The ψ_0 and φ_{Eo} seasonal decline was stronger in AS-trees (ψ_0: −16 %, φ_{Eo}: −18 %) than in S-trees (ψ_0: −7 %, φ_{Eo}: −8 %) (Table 2, Fig. 2). φ_{Do} increased from June to August and in July was higher in the AS-trees than in the S-ones (Table 2, Fig. 2). None of these flux ratio parameters was affected by EDU treatments (Table 2, Fig 2).

All the energy fluxes through PS2 calculated with the unconnected JIP-test version at the RC level, as well as those at the CS level, changed during the season, with the exception of TR_0/CS_0 (Table 2). ABS/RC, TR_0/RC, ET_0/RC, and ET_0/CS_0 decreased over the season (Fig. 2). DI_0/RC and DI_0/CS_0 increased from June to August and decreased in September (Fig. 2). In June and September, S-trees showed higher ABS/RC, TR_0/RC, ET_0/RC,

Table 3. F values of three-way analysis of variance (n=192) for the effects of tree O_3-sensitivity, *i.e.* asymptomatic, AS or symptomatic, S; treatment (EDU), *i.e.* water or EDU-infused, and Month (Mon) *i.e.* June, July, August, or September on JIP-test parameters (PS2 connected version). C is the curvature constant of the hyperbola. For acronyms *see* Table 1.

d.f.	Sen 1	EDU 1	Mon 3	Sen×EDU 1	Sen×Mon 3	EDU×Mon 3	Sen×EDU×Mom 3
C	**7.71***	0.12^{ns}	1.53ns	1.39^{ns}	0.65^{ns}	2.44^{ns}	1.39^{ns}
ABS/RC	1.40^{ns}	0.51^{ns}	**2.84***	0.63^{ns}	0.45^{ns}	2.24^{ns}	0.67^{ns}
TR_0/RC	0.96^{ns}	0.67^{ns}	2.47^{ns}	0.15^{ns}	0.59^{ns}	2.16^{ns}	0.89^{ns}
ET_0/RC	0.26^{ns}	1.32^{ns}	**6.61*****	0.01^{ns}	1.46^{ns}	1.16^{ns}	0.99^{ns}
DI_0/RC	2.86^{ns}	0.03^{ns}	**4.81***	0.46^{ns}	0.53^{ns}	1.65^{ns}	1.26^{ns}

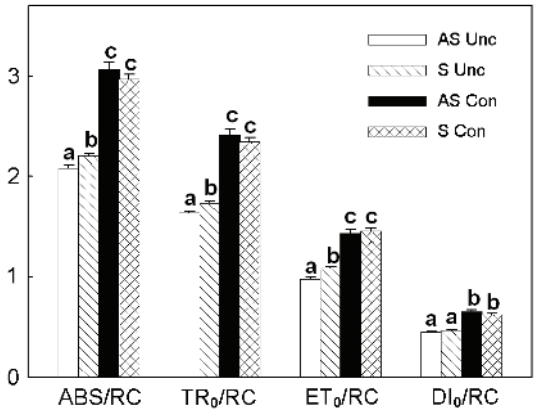

Fig. 3. Effective antenna size of an active RC (ABS/RC), maximal trapping rate of photosystem 2 (TR_0/RC), electron transport in an active RC (ET_0/RC), and effective dissipation of an active RC (DI_0/RC), calculated with the PS2 unconnected version (Unc) and PS2 connected version (Con) JIP-tests in leaves of asymptomatic (AS) and symptomatic (S) ash trees, without considering month and treatment as variables. Means±S.E. [relative], (n=12). *Different letters* indicate significant differences among bars within each parameter (Tukey HSD test, $p \leq 0.05$).

TR_0/CS_0, and ET_0/CS_0 values than AS-trees (Table 2). In July, O_3-sensitive trees infused with EDU had higher ABS/RC, TR_0/RC, ET_0/RC, and lower DI_0/RC than water-infused trees (Fig. 2). The flux ratios at PS2 cross-section level TR_0/CS_0 and ET_0/CS_0 were higher in O_3-sensitive trees than in the tolerant ones.

The density of RCs per excited PS2 cross section (RC/CS_0) did not change from June to August and increased in September (Table 2, Fig. 2). In July and August RC/CS_0 was significantly lower in water-infused ashes than in EDU-infused ones, with a higher reduction in AS-trees (−15 % *vs.* −5 % in S-trees).

N increased over the season in both S- and AS-trees infused with water, and remained constant in AS-trees infused with EDU (Table 2, Fig. 2). At the beginning of the season, in both the treatments, B_{av} values were higher in AS- than S-trees, while in July and August this was observed only in EDU-infused trees (Table 2, Fig. 2). The de-excitation constants k_p and k_n were higher in AS-trees than in the S-ones (Table 2, Fig. 2). k_p showed a decrease over the season, whereas k_n exhibited no significant change.

PI and DF were lower in AS-ashes when calculated per CS (Table 2, Fig. 2), while PI and DF calculated per ABS flux did not show significant differences respect to O_3 sensitivity of trees (Table 2). PI and DF seasonal decline was higher in AS- than S-ashes, although significant differences were observed only in July and September (Table 2, Fig. 2). PI and DF were not affected by EDU.

The connectivity parameter, C, was higher in AS-trees and was not affected by EDU or time (Table 3). Since the energy fluxes through PS2 at the RC level in the connected JIP-test version were calculated by multiplying the energy flux parameters of the unconnected version by (1+C), the differences observed in the unconnected version, *i.e.* lower ABS/RC, ET_0/RC, and ET_0/CS_0 in AS-trees than in S-trees, became not significant (Table 3, Fig. 3). While ABS/RC, ET_0/RC, and ET_0/CS_0 values decreased during the season even in the connected JIP-test version, TR_0/RC values did not (Table 3), according to the related CS parameter TR_0/CS. The flux ratio parameters calculated with the connected JIP version were not affected by EDU (Table 3).

Discussion

Seasonal effects: The analysis of 25 parameters (Table 2), calculated from the fast kinetics of fluorescence induction transient, revealed several biophysical changes during the growing season. The seasonal decline of electron transport probability (ψ_0), quantum yield for electron transport (φ_{Eo}), and specific fluxes (*i.e.* ET_0/RC and ET_0/CS_0 in both the JIP-test versions, and TR_0/RC only in the unconnected test version) was linked to the decrease of the effective antenna size of an active RC (ABS/RC), which is an expression of the average amount of absorbing Chl molecules per active RC (Krüger *et al.* 1997). The reduction of these parameters was probably due to the natural phenology of trees: from a juvenile (*i.e.* growth) to a mature (*i.e.* maintenance) state, photosyn-

thetic processes naturally decreased. This is also supported by a decrease in photochemical de-excitation constant (k_p) and increase in time dependent turnover number of Q_A (N). Also the dark processes, linked to the reduced efficiency in electron transport, seem to play an important role in the seasonal decline of fluxes. The reduction of ψ_0 and ET_0/RC, and the simultaneous increase of the kinetic parameters M_0, V_j, and V_i make us to hypothesise an accumulation of electrons in the transporters, both in the initial events of the transient and in the different steps of the curve. This is also supported by the decrease in the average redox state of Q_A^-/Q_A (B_{av}). The decrease in net photosynthetic rate (P_N) observed in our ashes trees over the season (Paoletti *et al.* 2008) caused a decreased demand for ATP and NADPH in the chloroplast and led to a redox back pressure on PS2. Thus, the primary photochemical reaction of PS2 was modified to down-regulate the linear photosynthetic electron transport. The accumulation of reduced Q_A finally resulted in an increase of excess energy, like all the dissipation parameters (φ_{Do}, DI_0/RC, and DI_0/CS) reveal. Additionally the seasonal variation in dissipation processes seemed also to be influenced by environmental condition, like water availability. Actually, *F. excelsior* trees displayed a progressive reduction in photochemical events (φ_{Po} or F_v/F_m) and an increase in dissipation processes (DI_0/RC and DI_0/CS), with the highest values in August, *i.e.* at the top of the summer drought period, and a recovery in September, *i.e.* after a raining period. As a result, the performance index and driving force, expressed per unit of photon absorbing flux and PS2 cross section, decreased during the season with the lowest values in August. Because RC/CS_0 increased in September, we hypothesize that in *F. excelsior* trees the dissipation of excess energy did not result in a photodamage to PS2, although the increase in F_0 may suggest it (Krause 1988). Actually a photodamage to PS2 should be connected with a decrease of fully active RCs and a concomitant increase of heat sink centers (non Q_A-reducing centers or silent centers, Strasser *et al.* 2004), a frequent down-regulation mechanism to dissipate the excess of absorbed photons in a controlled way (Soja *et al.* 1998, Gravano *et al.* 2004, Bussotti *et al.* 2005). Fluorescence responses over the growing season were not affected by EDU, while significant interactions with the tree O_3 sensitivity were detected.

Effects of tree ozone-sensitivity: Trees exhibit a wide range of inter- and intra-specific sensitivity to O_3 (Paludan-Muller *et al.* 1999, Paoletti *et al.* 2002, Oksanen 2003, Nali *et al.* 2004). This may be because plant responses to O_3 are affected by the interaction of various environmental conditions with several internal plant-specific factors, ranging from the molecular and cellular level to the whole-plant level (Lee 2000). *F. excelsior* is considered an O_3-sensitive species and responds to O_3 with a classic hypersensitive response, which leads to typical brown-purple stipples in symptomatic leaves (Pell *et al.* 1997, Contran and Paoletti 2007). Paoletti *et al.* (2008) pointed out that our S- and AS-ash trees showed several differences. S-trees took up more O_3, although they did not exhibit a marked reduction in shoot growth when compared to AS-trees. Actually S-trees, growing in an area of higher soil water content (soil moisture content of the upper soil was 17 % higher at the O_3-sensitive site than at the site of the insensitive trees; Paoletti *et al.* 2008), had higher P_N and stomatal conductance. Thus, in spite of improved carbon assimilation, the slight impairment of symptomatic leaf growth suggests that part of photosynthates was used to prevent or repair foliar damage rather than going toward growth. The growth induced by higher P_N was offset by lower growth from higher O_3 uptake (Paoletti *et al.* 2008). At the same time AS-ash trees had higher ascorbic acid pool and ascorbate peroxidase activity, one of the main detoxification mechanisms of O_3-derived reactive oxygen species (Conklin and Barth 2004). In contrast, the maximum quantum yield of primary photochemistry (calculated as F_v/F_m) was similar between S- and AS- trees (Paoletti *et al.* 2008), as confirmed by the calculation in the present study. The maximum quantum yield of primary photochemistry may remain nearly unchanged after O_3 exposure, as also found in three species with different O_3-sensitivity, *Fraxinus excelsior*, *Prunus avium*, and *Viburnum lantana* (Gravano *et al.* 2004).

The present more detailed analysis reveals intra-specific sensitivity to O_3 in the Chl *a* fluorescence kinetics of *F. excelsior* trees, confirming that JIP-test analysis is a good tool to quantitatively assess the effects of ambient O_3 on photosynthesis of adult trees under natural conditions (Gravano *et al.* 2004, Bussotti *et al.* 2007b). O_3 effects on Chl *a* transient fluorescence of Mediterranean tolerant species are offset by counter-reactions, which lead to over-compensation (Paoletti *et al.* 2004). Our results suggest that over-compensation mechanisms prevent or repair damage to the photosynthetic machinery of S-ash trees. The higher non-photochemical constant (k_n) suggests that energy dissipation in the AS-trees was a more physiologically-controlled process than in S-trees (Bussotti *et al.* 2007b). The higher F_0 and maximal fluorescence emission (F_m), and the lower photochemical constant (k_p) in S-trees indicate processes able to disturb Chl efficiency (Strasser *et al.* 2004). According to lower P_N (Paoletti *et al.* 2008), and thus decreased demand for ATP and NADPH, AS-trees showed lower absorbing (ABS), trapping (TR_0), and electron transport (ET_0) specific and phenomenological fluxes (calculated with the unconnected JIP-test version), electron transport probability (ψ_0), and quantum yield for electron transport (φ_{Eo}), and higher accumulation of electron transporters in the slow events of the fluorescence curve (V_i) and in the average redox state of Q_A^-/Q_A (B_{av}). This implied a reduction in the performance index (PI), which is a common response to

O_3 exposure (Clark *et al.* 2000). Meinander *et al.* (1996) found nearly no change in the quantum yield for electron transport (φ_{Eo}) of Scots pines exposed to elevated O_3 or/and CO_2 in open top chambers, although the absorption, trapping, or electron transport per cross-section increased considerably in all samples.

The influence of tree O_3 sensitivity on several JIP-test parameters changed during the season. AS-trees showed a higher seasonal reduction in electron transport probability (ψ_0), quantum yield for electron transport (φ_{Eo}), and performance index (PI), and a lower rise in turnover number of Q_A (N) than S-trees. In August, after a period of relatively high temperatures and low precipitations (Paoletti *et al.* 2007a), Chl *a* fluorescence parameters did not change with O_3 sensitivity with the exception of the average redox state of Q_A^-/Q_A (B_{av}). Water stress can influence photosynthesis and Chl *a* fluorescence (Manes *et al.* 2001). Water deficit was likely stronger than O_3 and reduced the intra-specific differences in O_3 sensitivity among *F. excelsior* trees.

All the previous studies of O_3 effect on Chl *a* fluorescence applied the unconnected JIP-test version (Meinander *et al.* 1996, Soja *et al.* 1998, Clark *et al.* 2000, Nussbaum *et al.* 2001, Gravano *et al.* 2004, Paoletti *et al.* 2004, Bussotti *et al.* 2005, 2007a). We found higher probability of connectivity (C) in AS-trees, suggesting that the photosynthetic units came physically closer to one another. This stimulates the mechanisms of energy dissipation (Strasser *et al.* 2004) and reduces photo-oxidative stress injury. As the typical patterns of fluorescence kinetics are influenced by the connectivity between PS2 units (Stirbert *et al.* 1998), the connected JIP-test version showed similar absorbing, trapping, and electron transport specific and phenomenological fluxes in S- and AS-trees, in contrast with what calculated with the unconnected JIP-test version. Since the connectivity in the JIP-test can influence the results, it should be considered in future studies of O_3 effect on Chl *a* fluorescence transient.

EDU effects: Gravitational infusion of EDU protects S-ash trees from O_3 injury without affecting carbon assimilation (Paoletti *et al.* 2008), as confirmed by the few differences in JIP-test parameters between water- and EDU-infused trees. The main EDU effect was a reduced inactivation of RCs, as RC/CS_0 was significantly higher in EDU-infused trees, in particular in AS-trees than in water-infused trees. A reduction in the density of active RCs is a common response to O_3 (Soja *et al.* 1998, Manes *et al.* 2001, Nussbaum *et al.* 2001, Gravano *et al.* 2004). According to reduced RC/CS_0, V_i was higher in EDU-infused trees, mainly in AS-trees, indicating an accumulation of electron transporters underway (Krause 1988). The intermediate step I is due to the existence of fast and slow reducing PQ pool-centers, as well as to the different redox states of the RCs of PS2 which reduces the PQ pools (Srivastava *et al.* 1997). This accumulation of reduced PQ pools (Q_A^-/Q_B^- and $Q_A^-/Q_B^{=}$) in EDU-infused trees may be due to the higher density of active RCs of PS2. Actually EDU-infused and water-infused trees had similar P_N (Paoletti *et al.* 2008), suggesting the absence of a redox back pressure on PS2 in EDU-infused trees.

A protective effect of EDU was observed in July, but only in symptomatic trees: EDU-infused trees showed higher specific energy fluxes (ABS/RC, TR_0/RC, DI_0/RC) than water-infused trees. Probably this effect of EDU protection was detected only in S-trees because some mechanisms of EDU protection are activated only when O_3 injury is present. Such limited EDU protection against O_3 effects on PS2 functioning may be explained by the fact that in *F. excelsior* the first effects of O_3 on the photosynthetic apparatus are not on the primary photochemical reactions regulated by PS2, but rather on the sites at which electrons are utilized, *i.e.* the dark phase connected to the Calvin cycle (Gravano *et al.* 2004).

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