



## Ozone stress in woody plants assessed with chlorophyll a fluorescence. A critical reassessment of existing data

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### ABSTRACT

This paper proposes an overall reassessment of results from fluorescence transient (FT) and modulated fluorescence (MF) analysis on tree species subjected to treatment with ozone. Findings from literature and open-top chamber experiments were used in this review, in order to identify damage mechanisms and repair/avoidance strategies. Main results are summarised below.

- (i) The most widely used parameter to assess the response to ozone treatment was the quantum yield of primary photochemistry in the dark-adapted state ( $\phi_{P_0}$ , or  $F_V/F_M$ ). This parameter proved to be very stable in stress and control conditions. Ozone-induced stress – and the related loss of photosynthetic efficiency and performance – was characterized by the change occurring in all parameters connected with the controlled dissipation: reduction of  $F_M$ ,  $F_V/F_M$ ,  $RC/CS_0$  (in the fluorescence transient analysis) and NPQ (in the MF analysis). This can be considered as a down-regulation mechanism aimed at lowering the electron supply as a consequence of a reduced demand from the Calvin cycle.
- (ii) The FT analysis revealed a change in the I–P region shape, indicating that events beyond PSI are affected. These events include a lesser density of PSI itself and the compromised ability of the end acceptors of electrons (ferredoxine, NADP<sup>+</sup>) and RuBP to manage effectively the flux of electrons. This behaviour may create an imbalance between electrons sent by the electron transport chain and those reaching the acceptors beyond PSI. Free electrons (those coming from PSI, but that do not reach the end acceptors) can activate the oxygen from fundamental to excited status, with production of ROS (Reactive Oxygen Species), thus inducing photo-oxidation processes of the cellular content.
- (iii) In many cases a temporarily enhanced efficiency of electron trapping and transport (expressed by the parameters  $F_V/F_M$ ,  $ET/TR$ ,  $PI_{ABS}$ ,  $pQ$ ) in PSII system has been observed. That efficiency has been connected to the triggering of repair processes, but when it is connected to a reduced end acceptor capacity in combination with reduced Calvin cycle energy demand lead to over-excitation of the photosynthetic apparatus and initiates response towards visible foliar injury.
- (iv) The behaviour of  $F_0$  can help us distinguish between different response strategies. The increase of  $F_0$  observed in some ozone-treated plant species is considered an expression of irreversible damage in PSII, whereas lowered values of this parameter may indicate the activation of PSII in the cells surrounding the damaged one, as part of a compensative process.

Future directions for the research in this field concern: (i) the possibility to combine fluorescence parameters with carbon assimilation and growth to support the study on critical levels and (ii) the analysis of the events concerning the activity of PSI and the events leading to the fixation of CO<sub>2</sub>, by using innovative technologies.

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### 1. Introduction

Analysis of the fluorescence properties of chlorophyll *a* (Chl *a*) in photosystem II (PSII) is an effective tool in the study of the physiological aspects of photosynthesis (Govindjee, 2004; Strasser et al., 2004), and has been extensively applied in plant stress investigations (Maxwell and Johnson, 2000; Adams and Demmig-Adams, 2004). Several approaches have been elaborated and proposed for basic and applied research (Roháček and Barták, 1999; Roháček, 2002; Baker and Rosenqvist, 2004; Schreiber, 2004; Strasser and Govindjee, 1992a,b; Strasser et al., 1995; Juneau et al., 2005).

The first study on the relationship between ozone stress and chlorophyll *a* fluorescence was published in 1978 (Schreiber et al., 1978) and concerned the comparison of fluorescence induction curves in bean leaves treated with ozone. Traditionally, the most widely used parameter that defines stress condition in a plant is the maximum quantum yield of primary photochemistry in the dark adapted state, i.e. the capacity of photosystem II (PSII) to trap the energy from the sun and reduce  $Q_A$  (Krause and Weis, 1991). The quantum yield is calculated as the ratio between the variable and the maximum fluorescence of a dark-adapted sample and is expressed in all cases as  $\varphi_{Po} = 1 - F_0/F_M = (F_M - F_0)/F_M = F_V/F_M$  (Paillotin, 1976).

Direct (fluorescence transient, FT) and modulated fluorescence (MF) analysis techniques propose a large number of parameters aimed at describing the functionality of PSII; these have been extensively applied to evaluate the responses of photosynthesis in woody plants subjected to ozone stress. The aim of the present review is to:

- assess the behaviour of  $F_V/F_M$  across several different tree species and experimental conditions, to verify common and consistent responses;
- compare the responses obtained with different parameters from different fluorescence techniques, to verify the existence of common patterns of the response indicators;
- investigate the behaviour of the different parameters from FT and MF and compare them with other findings reported by the authors (such as  $P_N$ ), to verify if it is possible to obtain a mechanistic and physiologically sound interpretation of the dynamics of ozone damage.

In other words chlorophyll *a* fluorescence should not merely be considered a technique that simply provides a response parameter, since it also enables us to explore the physiological processes following the exposure to ozone.

### 2. Chlorophyll a fluorescence parameters

Chlorophyll *a* fluorescence is a dissipation pathway for light energy absorbed by the antennae of PSII, and is dependent on the redox state of  $Q_A$ , the primary quinone acceptor of PSII. In dark adapted photosynthetic samples, the fluorescence intensity changes as a consequence of the application of a saturating light pulse: it rises from  $F_0$  (when all the reaction centres of PSII are open, i.e. when the primary acceptor quinone  $Q_A$  is fully oxidised) to  $F_M$  (when the excitation intensity is high enough to ensure the closure of all reaction centres of PSII, i.e. the full reduction of all reaction centres). After reaching  $F_M$ , and under saturating light conditions, the fluorescence signal decreases and within a few minutes reaches a stable level ( $F_S$  or  $F_T$ ). The variable fluorescence is calculated for each point at the time  $t$  intermediate between  $F_0$  and  $F_M$ , as  $F_V = F_t - F_0$ . The maximum variable fluorescence is  $F_V = F_M - F_0$ .

The rise from  $F_0$  to  $F_M$  of dark adapted samples is called “fluorescence transient” (FT). Plotted on a logarithmic time scale, FT shows a polyphasic behaviour (Strasser and Govindjee, 1992a,b;

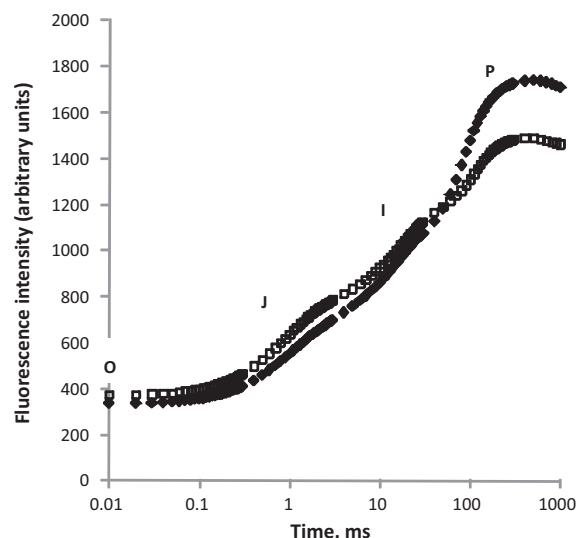


Fig. 1. Example of OJIP transients, plotted on logarithmic scale. The different steps at 0.05 ms (O), 2 ms (J), 30 ms (I) and maximal (P) are labelled. Full signs: not stressed sample; empty signs: stressed sample.

Strasser et al., 1995) (Fig. 1). The different steps of this polyphasic transient are labelled alphabetically. The analysis of FT is called the JIP-test (Strasser et al., 2000, 2004) and can be performed using instruments with a very fast capacity of data acquisition in the first second of illumination (direct fluorescence). The different phases of this process are reflected in the fluorescence rise as steps J (2 ms), I (30 ms) and P. The step with the highest fluorescence intensity is called P (peak). The JIP-test refers to a translation of the original data to biophysical parameters – all referring to time zero (onset of fluorescence induction) – that quantify PSII behaviour. In particular, in the present study we have considered the yields and flux ratios. They are: the maximum quantum yield of primary photochemistry ( $\varphi_{Po} = TR_0/ABS$ ), the efficiency with which a trapped exciton can move an electron into the electron transport chain from  $Q_A^-$  to the intersystem electron acceptors ( $\Psi_{Eo} = ET_0/TR_0$ ), the quantum yield of electron transport from  $Q_A^-$  to the PSI end electron acceptors ( $\varphi_{Ro} = RE_0/ABS$ ) (Smit et al., 2008; Tsimilli-Michael and Strasser, 2008; Strasser et al., 2010), the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end acceptors namely ferredoxine and  $NADP^+$  ( $\delta_{Ro} = RE_0/ET_0$ ). The performance indices (PI) are multiparametric expressions elaborated through the above-mentioned independent steps, all contributing to photosynthesis.  $PI_{ABS}$  is the potential performance index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors (Strasser et al., 2000),  $PI_{TOT}$  is the potential performance index for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors (Strasser et al., 2010). Different FT can be compared graphically by mean of the  $\Delta V$  and  $\Delta W$  curves, where  $\Delta V = (V_{treated} - V_{control})$  and  $\Delta W = (W_{treated} - W_{control})$  (see definitions in Table 1) (Strasser et al., 2004). These curves highlight the differences of the fluorescence signals at each time  $t$ , thus revealing the intermediate bands between the OJIP steps. The K-band is especially interesting: it appears at 100–300  $\mu s$  and indicates the breakdown, or reduced efficiency, of the oxygen evolving complex (Srivastava et al., 1997).

Differently from the fast kinetics (FT analysis), the MF acts especially on light adapted leaves, by using pulsed light with Pulse-Amplitude-Modulated (PAM) fluorimeters. Among the parameters that PAM instruments measure are the  $F_V/F_M$  ratio (Quantum yield efficiency of primary photochemistry); photochemical quenching,

**Table 1**  
Explanation of the fluorescence technical data, and of the parameters used in this text.

Technical fluorescence parameters	
$F_t$	Fluorescence emission from a dark-adapted leaf at the time $t$
$F'$	Fluorescence emission from a leaf adapted to actinic light
$F_0$	Minimal fluorescence from a dark adapted leaf
$F_0'$	Minimal fluorescence from a leaf adapted to actinic light
$F_M$	Maximal fluorescence from a dark adapted leaf
$F_M'$	Maximal fluorescence from a leaf adapted to actinic light
$F_v$	Variable fluorescence from a dark adapted leaf at the time $t$ . $F_v = F_t - F_0$
$F_V$	Maximal variable fluorescence from a dark adapted leaf. $F_V = F_M - F_0$
$F_v'$	Variable fluorescence from a leaf adapted to actinic light. $F_v' = F_M' - F_0'$
$F_T$ or $F_S$	Steady fluorescence from a leaf adapted to actinic light
$\varphi_{P_0} = TR_0/ABS = [F_M - F_0]/F_M = F_V/F_M$	Trapping probability, or maximum quantum yield of primary photochemistry of a dark adapted leaf. Expresses the probability that an absorbed photon will be trapped by the PSII reaction centre.
Fluorescence parameters from the transient analysis (OJIP fluorescence)	
$F_j$	Fluorescence intensity at the J-step (at 2 ms)
$F_I$	Fluorescence intensity at the I-step (at 30 ms)
$M_0$	Slope of the curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300\mu s} - F_0)/(F_M - F_0)$
$V_t$	Relative variable fluorescence at the time $t$ between $F_0$ and $F_M$ . $V_t = (F_t - F_0)/(F_M - F_0)$
$V_j$	Relative variable fluorescence at 2 ms. $V_j = (F_{2\text{ms}} - F_0)/(F_M - F_0)$
$V_I$	Relative variable fluorescence at 30 ms. $V_I = (F_{30\text{ms}} - F_0)/(F_M - F_0)$
$W_t$	Relative variable fluorescence at the time $t$ between $F_0$ and $F_j$ . $W_t = (F_t - F_0)/(F_j - F_0)$
$W_K$	Relative variable fluorescence at the band K (100–300 $\mu s$ ). It indicates the inactivation of the oxygen evolving complex. $W_K = (F_{100-300\mu s} - F_0)/(F_j - F_0)$
$\Psi_{E_0} = ET_0/TR_0$	Probability that a photon trapped by the PSII reaction centre enters the electron transport chain. $\Psi_{E_0} = 1 - V_j$
$\varphi_{E_0} = ET_0/ABS$	Quantum yield of electron transport. $\varphi_{E_0} = [1 - (F_0/F_M)]\Psi_{E_0}$
$\varphi_{R_0} = RE_0/ABS$	Quantum yield of an electron reaching the acceptor. $\delta_{R_0} = [1 - (F_0/F_M)](1 - V_I) = 1 - F_I/F_M$
$\delta_{R_0} = RE_0/ET_0$	Probability that an electron is transported from reduced PQ to the electron acceptor side of PSI. $\delta_{R_0} = (1 - V_I)/(1 - V_j) = (F_M - F_I)/(F_M - F_j)$
RC/CS <sub>0</sub>	Number of active RCs to one inactive RC for a PSII cross-section. $RC/CS_0 = \varphi_{P_0}(V_j/M_0)F_0$
PI <sub>ABS</sub>	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors. $PI_{ABS} = (RC/ABS)[\varphi_{P_0}/(1 - \varphi_{P_0})][\Psi_{E_0}/(1 - \Psi_{E_0})]$
PI <sub>TOT</sub>	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors. $PI_{TOT} = PI_{ABS} [\delta_{R_0}/(1 - \delta_{R_0})]$
Fluorescence parameters from modulated fluorescence	
$\Phi_{PSII}$	Actual quantum yield of PSII. $\Phi_{PSII} = (F_M' - F_T)/F_M'$
qP	Photochemical quenching. Expresses the proportion of the open PSII. $qP = (F_M' - F_T)/(F_M' - F_0')$
NPQ	Non photochemical quenching. Non photochemical dissipation of energy (heat) at antenna level. $NPQ = (F_M - F_M')/F_M'$

which is a measure for the redox state of  $Q_A$  and therefore a measure of the fraction of open or closed reaction centres before the saturating pulse; and non-photochemical quenching, which represents mainly increased heat-dissipation. Fluorescence quenching parameters are commonly employed to evaluate the photosynthetic efficiency of plants exposed to pollutants or other environmental stresses under steady state conditions (Schreiber et al., 1995; Brack and Frank, 1998; Naessens et al., 2000). In PAM fluorometers, the time resolution is limited by the modulation and demodulation frequencies. The set of parameters and formulae used here is presented in Table 1.

### 3. Data availability and analysis

The data presented in this review are quite heterogeneous. The studies reported in literature differed in terms of experimental conditions (open-top chambers vs. growth and fumigation chambers), ozone concentrations (ambient vs. enriched ozone concentrations), duration of the experiments (from instantaneous to long-term responses), tree species employed and measured leaves (for example, young vs. old leaves). The different experimental conditions are reported in Table 2. To make the results more comparable, in this review we have considered only the responses directly attributable to ozone, i.e. without considering the role of the environmental modifying factors, such as drought stress, high light, UV-B and so on, that can enhance or reduce the responses. All the experimental results refer to seedlings or young plantlets, and adult trees were not considered. When the original dataset was available (for exam-

ple, Gravano et al., 2004; Bussotti et al., 2007a; Cascio et al., 2010), the data were partially re-elaborated to obtain more homogeneous and comparable results. Some data coming from experiments still are unpublished. The ozone metric reported in Table 2 is the same used by each author.

### 4. The quantum yield of primary photochemistry ( $F_V/F_M$ ) and the extremes ( $F_0$ and $F_M$ )

The maximum quantum yield of primary photochemistry in dark adapted leaves ( $\varphi_{P_0} = [F_M - F_0]/F_M = F_V/F_M$ ) is the most widely used parameter to express the physiological condition of a plant, as assessed by fluorescence. Table 2 reports 78 experimental cases where  $F_V/F_M$  values were used to evaluate the effects of ozone on woody plants. In about 48% of them there were no significant differences between treated and control plants. Deciduous broadleaved trees represent the largest part of the cases of study and, among them, *Populus* clones and *Fagus sylvatica* were the most studied species. It is difficult to compare the sensitivity of individual species due to the heterogeneity of experimental conditions; however, where *Populus* and *Fagus* were assessed simultaneously (Bortier et al., 2000b; Bussotti et al., 2007a) the former were more responsive. Very few significant responses, or none, were observed in evergreen Mediterranean species (Manes et al., 1998; Paoletti et al., 2004; Ribas et al., 2005; Vitale et al., 2008) and in conifers (Mikkelsen and Ro-Poulsen, 1994; Manes et al., 2001). Other findings concern the sensitivity of leaves in different parts of the same plant crown (old, more sensitive, vs. young leaves, less sensi-

**Table 2**

Review of the studies concerning the effects of ozone on trees, where the quantum yield of primary photochemistry ( $F_V/F_M$ ) was used as response indicator. The experimental conditions are reported for each study. AOT40 = Accumulated ozone above the threshold of 40 ppb. Poplar (sensitive clone): Eridano; poplar (resistant clone): I-214. Nr = data not reported (i.e. the  $F_V/F_M$  values were not reported numerically, but only in a graph). Ns = difference not significant. \* = reduction in treated conditions, with difference significant with  $p$  level < 0.05; \*\* = reduction in treated conditions, with difference significant with  $p$  level < 0.01; \*\*\* = reduction in treated conditions, with difference significant with  $p$  level < 0.001. When the original dataset was available (for example, Gravano et al., 2004; Bussotti et al., 2007a,b; Cascio et al., 2010), the data were partially re-elaborated to make them comparable.

Reference	Experimental set-up	O <sub>3</sub> treatment	Duration	Plant species	$F_V/F_M$		$P$
					Control	Treated	
Samuelson and Edwards, 1993	Open top chambers	2 Ambient	Apr–Sept	<i>Quercus rubra</i>	0.76	0.76	Ns
Mikkelsen and Ro-Poulsen, 1994	Open top chambers	NF + O <sub>3</sub> 30 nl l <sup>-1</sup>	May–Oct	<i>Picea abies</i> – current year needles	Nr	Nr	Ns
				<i>Picea abies</i> – previous year needles	Nr	Nr	Ns
Mikkelsen, 1995	Open top chambers	NF + O <sub>3</sub> 30 nl l <sup>-1</sup>	Apr–Oct	<i>Fagus sylvatica</i> – Midday	0.729 ± 0.007	0.705 ± 0.008	*
				<i>Fagus sylvatica</i> – Afternoon	0.724 ± 0.006	0.699 ± 0.014	Ns
Farage, 1996	Controlled growth chambers	80 nl l <sup>-1</sup>	Mar–Sept	<i>Quercus robur</i>	0.833 ± 0.06	0.82 ± 0.08	Ns
Lippert et al., 1996	Phytotrone	1.5 Ambient	Apr–Sept	<i>Fagus sylvatica</i>	0.725 ± 0.031	0.701 ± 0.071	Ns
		2 Ambient		<i>Fagus sylvatica</i>	0.725 ± 0.031	0.697 ± 0.041	Ns
Zeuthen et al., 1997	Open top chambers	NF + O <sub>3</sub> 40 ppb	Jul–Oct	<i>Fagus sylvatica</i>	Nr	Nr	*
Reinchenauer et al., 1997	Open top chambers	NF + O <sub>3</sub> 50 ppb	May–Aug	<i>Populus nigra</i>	Nr	Nr	*
Nali et al., 1998	Controlled growth chambers	O <sub>3</sub> 150 ppb. 5 h	Single pulse	Poplar (Sensitive clone)	Nr	Nr	Ns
				Poplar (Resistant clone)	Nr	Nr	Ns
Manes et al., 1998	Controlled growth chambers	O <sub>3</sub> cum 3.6 ppmh	3 weeks	<i>Quercus ilex</i>	Nr	Nr	Ns
	Controlled growth chambers	150 ppb; 6 h d <sup>-1</sup>	13 days	<i>Quercus ilex</i>	Nr	Nr	*
Soldatini et al., 1998	Controlled growth chambers	61 nl l <sup>-1</sup> ; 5 h d <sup>-1</sup>	15 days	Poplar (Sensitive clone)	0.789 ± 0.011	0.713 ± 0.047	***
				Poplar (Resistant clone)	0.78 ± 0.042	0.653 ± 0.013	*
Lorenzini et al., 1999	Controlled growth chambers	61 nl l <sup>-1</sup> ; 5 h d l <sup>-1</sup>	15 days	Poplar (Sensitive) – Mature leaves	0.789	0.713	***
				Poplar (Sensitive) – Young leaves	0.792	0.771	Ns
				Poplar (Resistant) – Mature leaves	0.789	0.653	***
				Poplar (Resistant) – Young leaves	0.793	0.768	Ns
Grams et al., 1999	Phytotrone	2 Ambient	Jun–Aug	<i>Fagus sylvatica</i>	0.70 ± 0.01	0.58 ± 0.05	**
Shavnin et al., 1999	Field fumigation chambers	90 nl l <sup>-1</sup> ; h 07–21	Apr–Oct	<i>Betula pendula</i> , High Fertility	0.818 ± 0.008	0.775 ± 0.026	***
				<i>Betula pendula</i> , Low Fertility	0.802 ± 0.010	0.726 ± 0.056	***
Lütz et al., 2000	Phytotrone	2 Ambient	Jun–Sept	<i>Fagus sylvatica</i>	Nr	Nr	Ns
Bortier et al., 2000a	Open top chambers	AOT40 8.9 ppmh	Apr–Aug	<i>Fagus sylvatica</i>	Nr	Nr	**
Bortier et al., 2000b	Open top chambers	NF + O <sub>3</sub> 30 nl l <sup>-1</sup>	Jun–Aug	<i>Fagus sylvatica</i>	Nr	Nr	**
				<i>Populus nigra</i>	Nr	Nr	*
Bortier et al., 2001	Controlled growth chambers	O <sub>3</sub> episodes – 40 ppb	Jun–Sept	<i>Fagus sylvatica</i>	Nr	Nr	Ns
		O <sub>3</sub> episodes – 100 ppb		<i>Fagus sylvatica</i>	Nr	Nr	**
Guidi et al., 2001	Controlled growth chambers	61 nl l <sup>-1</sup> ; 5 h d <sup>-1</sup>	7 days	Poplar (Sensitive clone)	0.778	0.769	Ns
			15 days	Poplar (Sensitive clone)	0.807	0.79	*
			7 days	Poplar (Resistant clone)	0.785	0.695	*
			15 days	Poplar (Resistant clone)	0.805	0.795	Ns
Manes et al. (2002, unpublished)	Controlled growth chambers	350 ppb, 7 h d <sup>-1</sup>	4 days	<i>Quercus ilex</i>	0.816 ± 0.011	0.824 ± 0.009	Ns
Paoletti et al., 2004	Controlled growth chambers	55 and 110 nm mol <sup>-1</sup>	90 days	<i>Laurus nobilis</i> , 55 nm mol <sup>-1</sup>	Nr	Nr	Ns
				<i>Laurus nobilis</i> , 110 nm mol <sup>-1</sup>	Nr	Nr	Ns
				<i>Phillyrea latifolia</i> , 55 nm mol <sup>-1</sup>	Nr	Nr	Ns
				<i>Phillyrea latifolia</i> , 110 nm mol <sup>-1</sup>	Nr	Nr	Ns
				<i>Arbutus unedo</i> , 55 nm mol <sup>-1</sup>	Nr	Nr	Ns
				<i>Arbutus unedo</i> , 110 nm mol <sup>-1</sup>	Nr	Nr	Ns
Gravano et al., 2004	Open top chambers (Lattecaldo, CH)	AOT40 20.2 ppb h	Apr–Sept,	<i>Viburnum lantana</i>	0.807 ± 0.014	0.767 ± 0.029	**
				<i>Prunus avium</i>	0.775 ± 0.018	0.741 ± 0.051	*
				<i>Fraxinus excelsior</i>	0.780 ± 0.019	0.723 ± 0.08	***
Ribas et al., 2005	Open top chambers	NF + O <sub>3</sub> 40 ppb	2 years	<i>Quercus ilex</i>	0.802 ± 0.005	0.788 ± 0.005	Ns
				<i>Ceratonia siliqua</i>	0.746 ± 0.012	0.725 ± 0.012	Ns
Calatayud et al., 2006	Open top chambers	AOT40 13.9 to 36.2 ppm h	6–12 months	<i>Citrus unshiu</i> , 12 months	0.705	0.648	*
				<i>Citrus unshiu</i> , 6 Months	0.7	0.569	*

Table 2 (Continued)

Reference	Experimental set-up	O <sub>3</sub> treatment	Duration	Plant species	F <sub>V</sub> /F <sub>M</sub>		P
					Control	Treated	
Fares et al., 2006 Wittmann et al., 2007	Leaves growing in cuvette Controlled growth chambers	150 ppb; 11 h d <sup>-1</sup> 60, 120, 240 nl l <sup>-1</sup>	1 Month 10 weeks	<i>Populus alba</i>	0.77 ± 0.04	0.78 ± 0.01	Ns
				<i>Betula pendula</i> , 60 nl l <sup>-1</sup>	0.82 ± 0.02	0.81 ± 0.03	Ns
				<i>Betula pendula</i> , 120 nl l <sup>-1</sup>	0.82 ± 0.02	0.77 ± 0.03	***
				<i>Betula pendula</i> , 240 nl l <sup>-1</sup>	0.82 ± 0.02	0.77 ± 0.03	***
Calatayud et al., 2007	Open top chambers	NF + O <sub>3</sub> 30 ppb	May–Sept	<i>Acer campestre</i> , not sympt.	0.788 ± 0.005	0.787 ± 0.008	Ns
				<i>Acer campestre</i> , sympt.	0.788 ± 0.006	0.745 ± 0.015	*
				<i>Acer monspessulanum</i> , not sympt.	0.783 ± 0.007	0.791 ± 0.004	Ns
				<i>Acer monspessulanum</i> , sympt.	0.783 ± 0.008	0.742 ± 0.016	*
				<i>Acer opalus</i> , not sympt.	0.791 ± 0.007	0.776 ± 0.008	Ns
				<i>Acer opalus</i> , sympt.	0.791 ± 0.008	0.747 ± 0.011	*
				<i>Acer pseudoplatanus</i> , not sympt.	0.793 ± 0.003	0.781 ± 0.009	Ns
				<i>Acer pseudoplatanus</i> , sympt.	0.793 ± 0.004	0.739 ± 0.029	*
Contran and Paoletti, 2007	Controlled growth chambers	150 nmol mol <sup>-1</sup> 8 h d <sup>-1</sup>	35 days	<i>Fraxinus excelsior</i> , Central Italy	Nr	Nr	**
				<i>Fraxinus excelsior</i> , Northern Italy	Nr	Nr	***
				<i>Fraxinus ornus</i> , Central Italy	Nr	Nr	***
				<i>Fraxinus ornus</i> , Northern Italy	Nr	Nr	Ns
				<i>Ilex aquifolium</i>	Nr	Nr	*
				<i>Fagus sylvatica</i> clones	Nr	Nr	*
Ranford and Reiling, 2007 Paoletti et al., 2007 Bussotti et al., 2007a,b	Controlled growth chambers	70 nl l <sup>-1</sup> ; 7 h d <sup>-1</sup>	28 days	<i>Populus</i> "Oxford" clone	0.803 ± 0.009	0.761 ± 0.014	*
				<i>Quercus robur</i>	0.794 ± 0.028	0.763 ± 0.029	Ns
	Controlled growth chambers	O <sub>3</sub> 150 ppb. 5 h	14 days	<i>Fagus sylvatica</i>	0.755 ± 0.025	0.732 ± 0.057	Ns
				<i>Populus</i> "Oxford" clone	0.811 ± 0.006	0.769 ± 0.036	*
	Open top chambers Curno (North Italy)	AOT40 22.8, 2004 AOT40 17.4, 2005	Apr–Sept, Apr–Sept,	<i>Quercus robur</i>	0.790 ± 0.012	0.792 ± 0.025	Ns
				<i>Fagus sylvatica</i>	0.780 ± 0.014	0.744 ± 0.023	*
				Poplar (Sensitive clone)	0.843 ± 0.02	0.827 ± 0.04	Ns
				Poplar (Resistant clone)	0.833 ± 0.02	0.826 ± 0.03	Ns
Di Baccio et al., 2008	Controlled growth chambers	60 nl l <sup>-1</sup> ; 5 h d <sup>-1</sup>	15 days	<i>Quercus ilex</i>	0.847 ± 0.017	0.825 ± 0.019	Ns
				<i>Fraxinus ornus</i>	0.83 ± 0.01	0.78 ± 0.02	*
Vitale et al., 2008	Controlled growth chambers	O <sub>3</sub> 250 ppb. 4 h	4 weeks	<i>Quercus ilex</i>	0.847 ± 0.017	0.825 ± 0.019	Ns
Paoletti et al., 2009	Controlled growth chambers	150 ppb; 6 h d <sup>-1</sup>	7 weeks	<i>Fraxinus ornus</i>	0.83 ± 0.01	0.78 ± 0.02	*
Cascio et al., 2010	Open top chambers	AOT40 21.8 ppb h	Apr–Sept, 2004	<i>Fagus sylvatica</i>	0.737 ± 0.019	0.709 ± 0.03	*
Pellegrini et al. (in press)	Controlled growth chambers	120 nl l <sup>-1</sup> ; 5 h d <sup>-1</sup>	45 days	<i>Liriodendron tulipifera</i>	0.806 ± 0.01	0.742 ± 0.006	*
Nali et al. (unpublished)	Controlled growth chambers	120 nl l <sup>-1</sup> ; 5 h d <sup>-1</sup>	45 days	<i>Tilia americana</i>	0.81 ± 0.02	0.76 ± 0.03	Ns

**Table 3**  
Relative values (treated/control) of relevant parameters from OJIP fluorescence (see explanation in Table 1 re-elaborated from the experiments where the original datasets were available. Ns = difference not significant. \* = difference significant  $p$  level < 0.05; \*\* = difference significant  $p$  level < 0.01; \*\*\* = difference significant  $p$  level < 0.001.

Experiment	$F_M$	$F_0$	TR <sub>0</sub> /ABS	ET <sub>0</sub> /TR <sub>0</sub>	RC/CS <sub>0</sub>	PI <sub>ABS</sub>	RE <sub>0</sub> /ABS	RE <sub>0</sub> /E <sub>0</sub> T	PI <sub>TOT</sub>									
Gravano et al., 2004 (Lattecaldo 2001)																		
<i>Viburnum lantana</i>	0.81	****	0.97	Ns	0.95	**	0.85	***	0.83	**	0.48	***	0.88	*	1.06	Ns	0.61	*
<i>Prunus avium</i>	0.85	**	0.95	Ns	0.96	*	0.98	Ns	0.88	Ns	0.87	Ns	0.77	***	0.80	***	0.51	**
<i>Fraxinus excelsior</i>	0.67	***	0.74	***	0.93	***	1.00	Ns	0.72	***	0.70	*	0.72	***	0.77	***	0.47	***
Cascio et al., 2010 (Lattecaldo 2004)																		
<i>Fagus sylvatica</i>	0.90	Ns	0.99	Ns	0.96	*	0.95	Ns	0.91	Ns	0.71	Ns	0.81	**	0.86	Ns	0.56	*
Bussotti et al., 2007a,b; (Curno 2004)																		
<i>Populus</i> "Oxford"	0.87	**	1.05	Ns	0.95	*	0.83	*	0.80	**	0.40	*	0.64	***	0.82	Ns	0.28	Ns
<i>Quercus robur</i>	0.83	**	0.94	Ns	0.96	Ns	0.92	Ns	0.89	Ns	0.59	Ns	0.84	Ns	0.94	Ns	0.43	*
<i>Fagus sylvatica</i>	0.92	Ns	0.97	Ns	0.97	Ns	0.97	Ns	0.96	Ns	0.85	Ns	0.90	Ns	0.94	Ns	0.83	Ns
Bussotti et al., 2007a,b (Curno 2005)																		
<i>Populus</i> "Oxford"	0.77	***	0.89	Ns	0.95	*	0.88	***	0.79	*	0.49	***	0.70	***	0.79	**	0.36	***
<i>Quercus robur</i>	1.03	Ns	1.01	Ns	1.00	Ns	0.97	Ns	0.98	Ns	0.86	Ns	0.90	Ns	0.94	**	0.65	**
<i>Fagus sylvatica</i>	0.99	Ns	1.16	**	0.95	*	0.96	Ns	0.98	Ns	0.68	Ns	0.74	*	0.80	Ns	0.45	*
Manes et al., unpublished (Rome, 2002)																		
<i>Quercus ilex</i>	1.02	Ns	0.97	Ns	1.01		0.89	***	1.08	***	0.82	*	0.92	Ns	1.03	Ns	0.87	Ns

tive, Lorenzini et al., 1999) and the reversibility of photoinhibition ( $F_V/F_M$  depression, Mikkelsen and Ro-Poulsen, 1994; Nali et al., 1998). This reversibility is attributed to the degradation and turn-over of the D1 protein of PSII reaction centre (Aro et al., 1993).

The extreme values  $F_0$  and  $F_M$  were not reported in most of the considered papers. When these data were available we observed that  $F_M$  is generally decreasing in treated plants (Soldatini et al., 1998; Calatayud et al., 2006; Gravano et al., 2004; Bussotti et al., 2007a), whereas  $F_0$  shows a more species-specific pattern. This parameter increased in ozone-treated poplars (Soldatini et al., 1998), *Betula pendula* (Shavnin et al., 1999; *F. sylvatica* (Bussotti et al., 2007a) and in symptomatic *Ailanthus altissima* leaves (Bussotti et al., 2005), but decreased in many other species and conditions, especially in relation to the onset and spread of foliar symptoms (Bussotti et al., 2005).

The extremes  $F_0$  and  $F_M$  are closely connected to the conformational parameters (Sum  $k$ ,  $k_p$  and  $k_N$ ). These parameters are based on the theory of energy fluxes in biomembranes (Sironval et al., 1981; Strasser, 1978,1981), and the expressions relating them to phenomenology are elaborated by Havaux et al. (1990,1991). The non-photochemical de-excitation constant ( $k_N$ ) sums up  $k_D$  (for heat dissipation),  $k_F$  (for fluorescence) and  $k_X$  (for energy migration to PSI, or spillover): [ $k_N = k_D + k_F + k_X$ ], and is calculated as [ $k_N = (ABS/CS)k_F(1/F_M)$ ].  $k_p$  expresses the ability to feed the electron chain and is calculated as [ $k_p = (ABS/CS)k_F\{(1/F_0) - (1/F_M)\}$ ]. Finally, the overall value of the photochemical and non-photochemical constants (Sum  $k$ ) expresses the ability to manage the electron fluxes avoiding damage from over-excitation, and is calculated as [ $k_N + k_p = (ABS/CS)k_F(1/F_0)$ ] (Havaux et al., 1990,1991).

The decrease of  $F_M$  is associated to the increase of the non-photochemical de-excitation constant ( $k_N$ ); and the decrease of  $F_0$  indicates an enhancement of the overall de-excitation constants (Sum  $k$ ). The lower  $F_0$  values observed in several plant species in relation to ozone exposure and/or foliar symptoms may indicate the activation of PSII in the cells surrounding the damaged one, within a compensative process (Layne and Flore, 1992). The increase of  $F_0$ , on the other hand, is considered an expression of irreversible damage in PSII (Krause, 1988) and indicates that heat dissipation ( $k_D$ ) occurs in an uncontrolled manner, producing an excess of excitation within the leaves.

## 5. The fluorescence transient analysis

The first paper reporting on the application of the JIP-test to study the action of ozone on trees was Soja et al. (1998) on *Malus*,

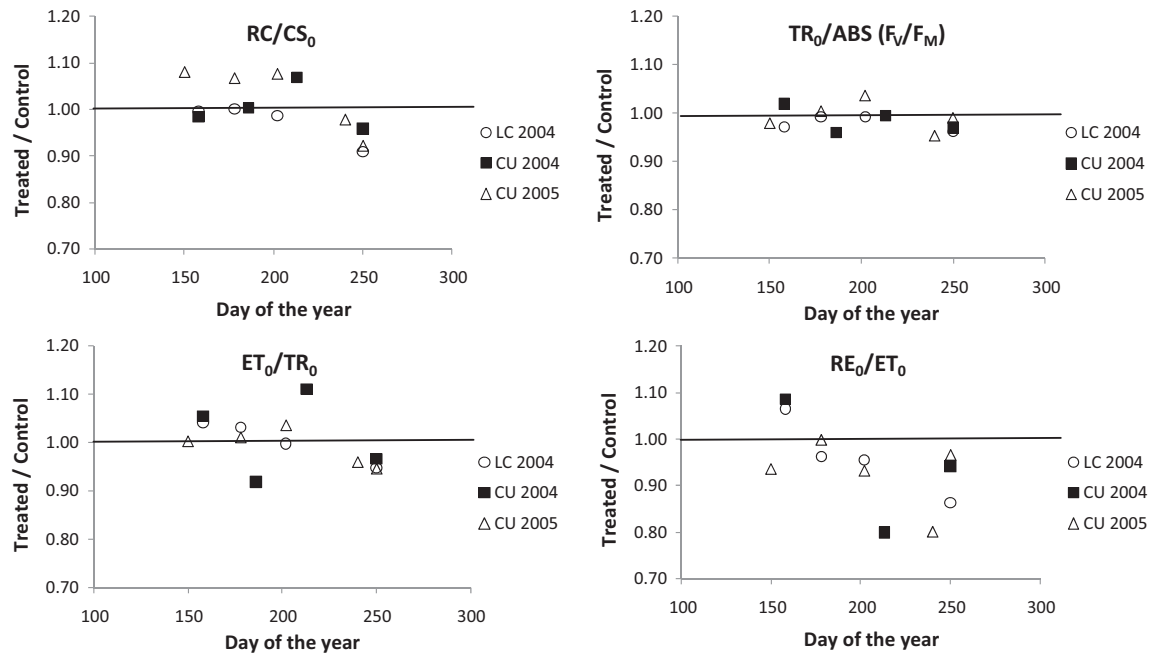
followed from Manes et al. (2001) on *Pinus halepensis*. Both studies underlined that the reduction of RC/CS<sub>0</sub> was the most suitable indicator of ozone stress. The same finding was confirmed by Nussbaum et al. (2001) for herbaceous species, and partially by Paoletti et al. (2004) for Mediterranean evergreen shrubs (only *Phillyrea latifolia* was sensitive for this parameter). The following paragraphs examine the effects of ozone on the different regions of the fluorescence induction curve, and their physiological meaning.

### 5.1. Single turn-over region

The first part of the transient curve (O–J) is called "single turn-over region" (Strasser et al., 2004). It expresses the photochemical events and represents a single event of reduction of  $Q_A$ . Lower  $\Psi_{E_0}$  (ET<sub>0</sub>/TR<sub>0</sub>) values in ozone-treated plants (Table 3), indicate the slowing down of the primary reduction of  $Q_A$ . This corresponds to an increase of relative variable fluorescence at the J-step (at ca. 2 ms) and is probably caused by accumulation of the reduced  $Q_A$  pool (Strasser et al., 1995; Lazár and Ilík, 1997). It is a consequence of decreased electron transport beyond  $Q_A^-$ , which limits the re-oxidation reaction (Haldimann and Strasser, 1999). Such behaviour can be shown graphically with the so-called  $\Delta V$  curves (Fig. 2A). In Fig. 2A the  $\Delta V_j$  peak, especially evident on the poplar sample, indicates the increased relative variable fluorescence at this step. Another important event in the single turn-over region in relation to ozone stress is the appearance of the so-called K band. The K band occurs in the fast region (100–300  $\mu$ s) of the transients and is caused by the breakdown, or the reduced efficiency, of the oxygen evolving complex (OEC). The K band is highlighted in the  $\Delta W$  curves and represents an early response to ozone stress. This behaviour was shown by Desotgiu et al. (2010) in *F. sylvatica* and in an ozone sensitive clone of *Populus* (cf. Fig. 2B).

### 5.2. The multiple turn-over region

The J–I–P region (thermal phase) of the fluorescence transient (and the related JIP-test parameters  $\varphi_{R_0} = RE_0/ABS$  and  $\delta_{R_0} = RE_0/ET_0$ ) reflects the velocity of ferredoxine reduction beyond the PSI (Schansker et al., 2003). The I–P phase is related to electron transfer through PSI, and the increase of the relative variable fluorescence is attributable to the induction of a traffic jam of electrons caused by a transient block at the acceptor side of PSI (inactive ferredoxin-NADP<sup>+</sup>-reductase (FNR) (Schansker et al., 2005). This part of the curve proved to be sensitive to ozone in most of the analyzed cases (see behaviour of relative parameters in Table 3)



**Fig. 2.** Temporal pattern (from May to September) of the JIP-test parameters contributing to  $PI_{TOT}$ . *Fagus sylvatica* seedling in different open-top chamber experiments: LC (Lattecaldo) 2004; CU (Curno) 2004 and 2005 (re-elaborated from Bussotti et al., 2007a,b; Cascio et al., 2010). The data are presented as relative values: Treated/Control.

and the appearance of the  $\Delta V_I$  peaks in the  $\Delta V$  curves has also been considered an early indicator of ozone stress (Desotgiu et al., 2010). The appearance of the  $\Delta V_I$  peaks is connected to a relative increase of the fluorescence signal at 20 ms ( $F_I$ ) in ozone-treated plants as compared to controls (see in Fig. 2A).

Besides the slowing down of ferredoxine reduction,  $O_3$  stress also produces the inactivation of Rubisco. This effect was demonstrated in potato plants (Dann and Pell, 1989), and then reproduced in experimental conditions (open-top chambers and growth chambers) on tree species, such as *Populus* clones (Brendley and Pell, 1998) and *P. halepensis* Mill. (Fontaine et al., 2003; Inclan et al., 2005). These alterations, occurring beyond the PSI, can explain the decrease of net photosynthesis as an early response to ozone (see also Gravano et al., 2004; Novak et al., 2005; Calatayud et al., 2007).

### 5.3. The performance indices and density of reaction centres

The overall ozone stress conditions are described by the performance indices ( $PI_{ABS}$  and  $PI_{TOT}$ ). Clark et al. (2000) have suggested  $PI_{ABS}$  is a predictor for growth in *F. sylvatica* trees which are exposed to ozone fumigations. Most of the considered papers refer to  $PI_{ABS}$ , since  $PI_{TOT}$  was only introduced recently (Strasser et al., 2010).  $PI_{ABS}$  variations in ozone-treated plants are often related to variations of  $RC/CS_0$  (Bussotti et al., 2007b).  $RC/CS_0$  expresses the density of fully active RCs ( $Q_A$  and  $Q_B$  reducing centres) in relation to the inactive ones (non  $Q_A$  reducing centres or silent centres, see Strasser et al., 2004). The silent centres act as “heat sinks” and are considered part of a down-regulation mechanism, which dissipates the excess of absorbed energy in a controlled way. PSII thus switches from a process converting light energy into biochemical energy storage to an energy conversion process that transforms absorbed light energy into heat dissipation.  $RC/CS_0$  is closely related to  $\varphi_{P_0}$ . This latter parameter represents the average of the actual trapping capacity of the active PSII units (active RCs) and the inactive ones (silent centres,  $\varphi_{P_0} = 0$ ) (Strasser et al., 2010). The correlation between  $RC/CS_0$  and  $PI_{ABS}$  has been confirmed in earlier papers on ozone stress, but not in drought studies (Gravano et al., 2004; Oukarroum et al., 2007; Bussotti et al., 2007b).  $RC/CS_0$  was one of the most sensitive

parameters for ozone stress effects (Soja et al., 1998; Nussbaum et al., 2001; Manes et al., 2001; Paoletti et al., 2004).  $PI_{TOT}$  was recalculated from previous papers. Comparing the behaviour of the two  $PI$  ( $PI_{ABS}$  and  $PI_{TOT}$ ), the findings reported in Table 3 indicate that in most cases  $PI_{TOT}$  is affected by an ozone treatment stronger than  $PI_{ABS}$ . This confirms the importance of the J–I–P region (and the related parameter  $\delta_{R_0} = RE_0/ET_0$ ) of FT in the response of plants to ozone stress. Two important exception are represented from *Viburnum lantana* (Gravano et al., 2004) and *Quercus ilex* (Manes, unpublished). In the first case the most important changes have been documented in the O–J region, whereas the little response of *Q. ilex* confirm the low ozone sensitivity of Mediterranean sclerophyllous species (see also Bussotti and Gerosa, 2002; Calatayud et al., 2010).

**Table 4**

Results of the multivariate statistic (PCA – Principal Component Analysis, Equamax rotation). For the details of experiments, see Table 2. Where used the data (relativized as treated/control) coming from the datasets of the experiments at the open-top chamber experiments (Gravano et al., 2004; Bussotti et al., 2007a,b; Cascio et al., 2010). In bold are evidenced the loadings over 0.6. The selection of the principal factors was based on those with eigenvalues greater than 1.

	Fact 1	Fact 2	Fact 3
Day of the year	<b>−0.732</b>	0.270	−0.224
AOT40	<b>−0.609</b>	0.141	−0.197
$F_M$	<b>0.818</b>	−0.460	0.036
$F_0$	0.067	<b>−0.945</b>	−0.034
$\varphi_{P_0}$ ( $TR_0/ABS$ )	<b>0.884</b>	0.007	0.119
$\psi_{E_0}$ ( $ET_0/TR_0$ )	<b>0.833</b>	0.186	0.090
$RC/CS_0$	<b>0.864</b>	−0.389	−0.076
$PI_{ABS}$	<b>0.890</b>	0.187	−0.162
$\varphi_{R_0}$ ( $RE_0/ABS$ )	0.464	0.211	−0.217
$\delta_{R_0}$ ( $RE_0/ET_0$ )	0.243	0.208	<b>0.792</b>
$PI_{TOT}$	0.024	−0.129	<b>0.837</b>
Exp. Var.	5.562	1.578	1.522
Tot. Prop.	0.463	0.131	0.127

**Table 5**  
 Values of the some parameters from the modulated fluorescence studies (see explanation in Table 1. For the details of experiments, see Table 2. Nr= data not reported (i.e. the  $F_V/F_M$  values were not reported numerically, but only in a graph). (+) and (–) indicates if the difference respect to the control is an increment or a reduction. Ns= difference not significant. \* = difference significant with  $p$  level < 0.05; \*\* = difference significant with  $p$  level < 0.01; \*\*\* = difference significant with  $p$  level < 0.001.

Reference	Plant species	NPQ			qp			ΦPSII		
		Contr	Treat		Contr	Treat		Contr	Treat	
Nali et al., 1998	Poplar (Sensitive clone)	0.47	0.54	Ns	0.6	0.58	Ns	0.39	0.41	Ns
	Poplar (Resistant clone)	0.39	0.49	*	0.68	0.63	Ns	0.47	0.4	Ns
Soldatini et al., 1998	Poplar (Sensitive clone)	0.50 ± 0.01	0.53 ± 0.01	*	0.82 ± 0.01	0.74 ± 0.06	**	0.55 ± 0.01	0.41 ± 0.06	*
	Poplar (Resistant clone)	0.30 ± 0.00	0.47 ± 0.07	*	0.76 ± 0.00	0.33 ± 0.01	*	0.53 ± 0.00	0.18 ± 0.01	*
Lorenzini et al., 1999	Poplar (Sensitive) – Mature leaves	Nr	Nr	(+) *	Nr	Nr	(–) *	Nr	Nr	(–) *
	Poplar (Sensitive) – Young leaves	Nr	Nr	Ns	Nr	Nr	Ns	Nr	Nr	Ns
	Poplar (Resistant) – Mature leaves	Nr	Nr	(+) *	Nr	Nr	(–) *	Nr	Nr	(–) *
	Poplar (Resistant) – Young leaves	Nr	Nr		Nr	Nr		Nr	Nr	
Grams et al., 1999a	<i>Fagus sylvatica</i>							0.48 ± 0.03	0.52 ± 0.02	
	<i>Fagus sylvatica</i>	Nr	Nr	Ns	Nr	Nr	Ns	Nr	Nr	Ns
Bortier et al., 2001	<i>Fagus sylvatica</i>	Nr	Nr	(+) *	Nr	Nr	Ns	Nr	Nr	Ns
	<i>Fagus sylvatica</i>									
Guidi et al., 2001	Poplar (Sensitive clone), 7 d	0.62	0.55	Ns	0.85	0.86	Ns	0.5	0.55	Ns
	Poplar (Sensitive clone), 15 d	0.45	0.34	*	0.83	0.89	Ns	0.58	0.63	Ns
	Poplar (Resistant clone), 7 d	0.36	0.57	*	0.86	0.83	Ns	0.61	0.47	*
	Poplar (Resistant clone), 15 d	0.36	0.53	*	0.79	0.66	*	0.59	0.44	*
Calatayud et al., 2006	Citrus unshiu, 12 months				0.85	0.72	*	0.49	0.36	Ns
	Citrus unshiu, 6 months				0.83	0.8	Ns	0.51	0.47	*
Wittmann et al., 2007	<i>Betula pendula</i> , 60 nl l <sup>-1</sup>	1.18 ± 0.03	1.18 ± 0.26	Ns						
	<i>Betula pendula</i> , 120 nl l <sup>-1</sup>	1.18 ± 0.03	1.46 ± 0.30	*						
	<i>Betula pendula</i> , 240 nl l <sup>-1</sup>	1.18 ± 0.05	1.89 ± 0.36	***						
Calatayud et al., 2007	<i>Acer campestre</i> , not sympt.	1.48 ± 0.29	1.53 ± 0.21	Ns	0.69 ± 0.04	0.67 ± 0.02	Ns	0.34 ± 0.03	0.32 ± 0.02	Ns
	<i>Acer campestre</i> , sympt.	1.48 ± 0.29	2.29 ± 0.24	Ns	0.69 ± 0.04	0.54 ± 0.03	*	0.34 ± 0.03	0.232 ± 0.01	*
	<i>Acer monspessulanum</i> , not sympt.	1.44 ± 0.26	1.43 ± 0.14	Ns	0.73 ± 0.03	0.69 ± 0.02	Ns	0.33 ± 0.03	0.33 ± 0.02	Ns
	<i>Acer monspessulanum</i> , sympt.	1.44 ± 0.26	1.99 ± 0.31	Ns	0.73 ± 0.03	0.62 ± 0.03	Ns	0.33 ± 0.03	0.26 ± 0.02	Ns
	<i>Acer opalus</i> , not sympt.	1.07 ± 0.07	1.46 ± 0.04	*	0.75 ± 0.01	0.68 ± 0.01	*	0.38 ± 0.01	0.31 ± 0.00	*
	<i>Acer opalus</i> , sympt.	1.07 ± 0.07	2.00 ± 0.23	*	0.75 ± 0.01	0.62 ± 0.02	*	0.38 ± 0.01	0.25 ± 0.02	*
	<i>Acer pseudoplatanus</i> , not sympt.	0.48 ± 0.04	1.06 ± 0.17	*	0.83 ± 0.015	0.72 ± 0.01	*	0.538 ± 0.01	0.38 ± 0.02	*
	<i>Acer pseudoplatanus</i> , sympt.	0.48 ± 0.04	1.64 ± 0.33	*	0.83 ± 0.015	0.628 ± 0.04	*	0.538 ± 0.01	0.30 ± 0.03	*
	<i>Quercus ilex</i>	0.181 ± 0.065	0.203 ± 0.07	Ns	0.819 ± 0.065	0.797 ± 0.07	Ns	0.766 ± 0.060	0.743 ± 0.067	Ns
Pellegrini et al. (in press)	<i>Liriodendron tulipifera</i>	0.83 ± 0.00	0.82 ± 0.00	Ns	0.51 ± 0.01	0.78 ± 0.01	*	0.43 ± 0.03	0.45 ± 0.01	Ns
Nali et al. (unpublished)	<i>Tilia americana</i>	0.71 ± 0.00	0.74 ± 0.00	Ns	0.64 ± 0.02	0.77 ± 0.01	*	0.45 ± 0.01	0.30 ± 0.03	*

#### 5.4. Relationships between parameters and ozone exposure

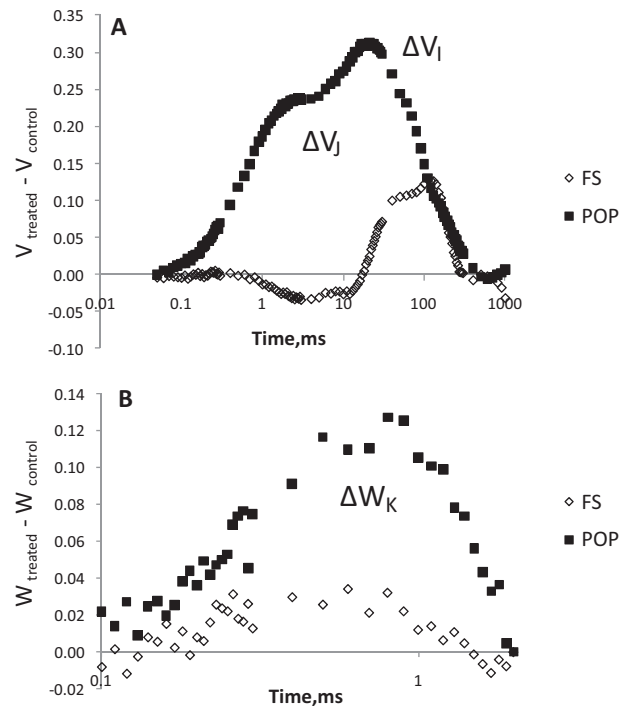
The findings of the OTC experiments at Curno (Bussotti et al., 2007a) and Lattecaldo (Gravano et al., 2004; Cascio et al., 2010) have been relativized (i.e. expressed as treated/control ratio) and analyzed using PCA (Principal Components Analysis) in order to find multivariate relationships among the JIP-test selected parameters and with the ozone exposure (expressed as AOT40). The results, reported in Table 4, show that most parameters expressing PSII functionality, and related to  $PI_{ABS}$ , were interconnected in Factor 1. They are correlated to the day of the year (Julian Day) and, weakly, to ozone exposure. This behaviour underscores the importance of the ageing factor in the physiological response to ozone.  $F_0$  is in Factor 2;  $\delta_{RO}$  ( $RE_0/ET$ ) and  $PI_{TOT}$  form a separate cluster in Factor 3.

#### 6. Results from modulated fluorescence

In ozone effect studies, modulated fluorescence parameters analysis gave additional insight. In many cases (Soldatini et al., 1998; Lorenzini et al., 1999) ozone exposed plants responded with an increase of NPQ (non-photochemical quenching) and a decrease of PQ (photochemical quenching). Bortier et al. (2000a,b) did not find any variation between controls and ozone exposed samples. Quantum yield parameters in light-adapted leaves (quantum yield of primary photochemistry in the light-adapted steady state  $\varphi_{Ps}' = 1 - F_S'/F_M' = (F_M' - F_S')/F_M' = \Delta F'/F_M'$ ) (Paillotin, 1976), proved to be more sensitive than the maximum quantum yield in the dark-adapted state (Calatayud et al., 2007). Similar behaviour (greater responsiveness in light-adapted conditions) was also detected by Lu and Zhang (1999) in relation to drought (Table 5).

#### 7. Temporal trends

The temporal pattern of some selected JIP-test relative parameters (ozone-treated/control), concerning the behaviour of *F. sylvatica* in three different open-top chamber experiments, is shown in Fig. 3. The maximum quantum yield of primary photochemistry ( $\varphi_{Po}$ ) was the least variable parameter, whereas  $RE_0/ABS$  declined markedly during the season. Many parameters ( $\varphi_{Po} = TR_0/ABS$ ,  $\Psi_{E_0} = ET_0/TR_0$ ,  $RC/CS_0$ ) showed a certain stability in the first part of the treatment season, but dropped suddenly in late summer. In the first part of the growing season it was not unusual to find a temporarily enhanced efficiency of electron capture and transport, and this effect was especially evident on  $\varphi_{Po}$  ( $TR_0/ABS$ ),  $\Psi_{E_0}$  ( $ET_0/TR_0$ ) and  $PI_{ABS}$  (Mikkelsen and Ro-Poulsen, 1994; Lippert et al., 1996; Bortier et al., 2000b; Bussotti et al., 2007a,b; Pellegrini et al., in press; Nali et al., unpublished). Plant responses, in terms of reduced photosynthetic efficiency, started to become significant only towards the later part of the season, with a drop in photosynthetic efficiency in August, when the cumulated ozone exposure/uptake rose above two thirds of its final value. A “threshold” effect may explain this behaviour (Marzuoli et al., 2009). In our opinion, however, it is to be interpreted as a consequence of changes in leaf physiology, including early senescence processes and lowered detoxifying capacity (Polle et al., 2001; Marzuoli et al., 2009). When leaves are fully efficient they are capable of opposing the harmful action of ozone; but the effect of ozone becomes predominant at the time when their efficiency declines. The monthly behaviour of  $PI_{ABS}$  was similar to the “stress curve” reported by Lichtentaler (1998). After an initial, slightly harmful effect (May), there was a fast recovery in June (eustress phase), followed by a stabilization phase (July). As the stress action continued, a rapid loss of efficiency (distress) occurred in August and September.



**Fig. 3.** Early responses to ozone of *Fagus sylvatica* (FS) and an ozone-sensitive poplar clone (“Oxford” clone, POP), assessed with the fluorescence transient (FT) analysis. The data come from an open-top chamber experiment (Curno 2005, see Bussotti et al., 2007a,b; Desotgiu et al., 2010). FT were measured at the begin of the growth season, when the ozone exposure (AOT40) was 5.78 ppm h. (A)  $\Delta V$  curves, obtained by subtraction ( $V_{treated} - V_{control}$ ) of the original fluorescence transients, normalized between  $F_0$  and  $F_M$  and multiplied per 4.  $\Delta V_j$  and  $\Delta V_i$  peaks are evidenced.  $\Delta V_j$  peaks are caused by accumulation of the reduced  $Q_A$  pool (Strasser et al., 1995; Lazár and Ilík, 1997) as a consequence of decreased electron transport beyond  $Q_A$ .  $\Delta V_i$  peaks are caused by the increase of the relative variable fluorescence in the I-P phase, attributable to the induction of a traffic jam of electrons caused by a transient block at the acceptor side of PSI/Schansker et al., 2005). (B)  $\Delta W$  curves, obtained by subtraction ( $W_{treated} - W_{control}$ ) of the original fluorescence transients, normalized between  $F_0$  and  $F_j$  and multiplied per 4. The  $\Delta V_{Wj}$  peak (about 300  $\mu$ s) is evidenced, indicating a loss of efficiency of the oxygen evolving complex (Srivastava et al., 1997) in the ozone treated samples respect to the non treated ones.

Source: Desotgiu et al., 2010, modified.

#### 8. General discussion and conclusions

According to the results presented in this paper, the primary effect of ozone action on the photosynthetic apparatus is to be found in events beyond PSI, including a reduced density of PSI itself and the decreased ability of the final acceptors of electrons (ferredoxine and  $NADP^+$ ) and RuBP to manage the flux of electrons effectively. Consequently the Calvin–Benson cycle is not fed. This behaviour may create an imbalance between electrons sent by the ETC (electron transport chain) and those reaching the acceptors beyond PSI. “Free” electrons (those coming from PSI, but that do not reach the end acceptors) can activate oxygen (from fundamental to excited status: Powles, 1984; Krieger-Liszskay, 2005) with production of ROS, thus inducing photo-oxidation processes of the cellular content. According to Hald et al. (2008), if the flow of electrons through the electron transport chain exceeds the capacity of metabolism to consume the reductant produced, then potentially harmful side reactions are liable to occur. Foliar symptoms are thought to be connected to the excess of excitation produced by the concomitant action of ozone and high light (Davison et al., 2003; Cascio et al., 2010). In ozone resistant cultivars of *Phaseolus* (Guidi et al., 2009) the foliar symptoms are prevented by mean of the lowering of  $F_V/F_M$ . It is a down-regulation mechanism that restore the equilibrium between the electrons

sent by the ETC and those consumed by the final acceptors and RuBP.

The photosystems enhance the controlled dissipation of energy at antenna level to reduce the proportion of absorbed energy used in photochemistry and the risks of over-excitation related to the flux of electrons. This behaviour is proved by the change occurring in all parameters connected with the controlled dissipation: reduction of  $F_M$ ,  $F_V/F_M$ ,  $RC/CS_0$  (in the fluorescence transient analysis) and NPQ (in the MF analysis). Usually, this is considered a down regulation process, enacted in order to acclimate the intensity of electron flux to the needs of reduced net photosynthesis (Calatayud et al., 2007; Guidi et al., 2001). The early onset of the K-band may also be interpreted as an active action aimed at lowering electron flux by regulating the activity of the OEC. Overall, these mechanisms can be considered a “protective” strategy, a notion supported by the fact that “resistant” poplar clones showed a greater  $F_V/F_M$  reduction than sensitive ones (Guidi et al., 2001).

In many cases net photosynthesis declined earlier and faster than the quantum yield of primary photochemistry  $\varphi_{P_0}$  (Gravano et al., 2004; Bussotti et al., 2007a). According to Adams and Demmig-Adams (2004) PSII efficiency provides no direct information on net photosynthesis ( $P_N$ ). Electron flux is not necessarily used in carbon metabolism, since it may also be re-routed to other biochemical pathways, such as reduction of oxygen ( $O_2$ , Mehler reaction), or nitrite ( $NO_2$ ), or sulphate ( $SO_4$ ), or thioredoxin, or photorespiration. In optimal conditions of water (field capacity), light (saturating radiation) and  $CO_2$  availability, the flux of electrons is primarily directed to carbon reduction. Genty et al. (1989) found a very strong correlation between the actual quantum yield of PSII in light adapted state ( $\Phi_{PSII} = F_V/F_M'$ ) and the quantum yield of  $CO_2$  fixation ( $\Phi_{CO_2}$ ) in absence of photorespiration. This behaviour only rarely occurs in the field because stress conditions provoke changes in photorespiration and in the pseudocyclic electron transport (Mehler reaction) (Fryer et al., 1998). Relationships between “dark fluorescence” and gas exchange parameters are more difficult to determine, since the  $CO_2$ -assimilation rate can also be regulated by modifications after Rubisco. These modifications cannot be recognised by dark-adapted state measurements, i.e. when Rubisco is inactive. It is well known that dark fluorescence can reveal the potential (not actual) photosynthesis efficiency. However, there is evidence that the decline of dark parameters such  $F_V/F_M$  and/or  $PI_{ABS}$  may be connected to a decline of  $P_N$  (Van Heerden et al., 2003; Albert et al., 2008; Albert et al., in press), since in favourable ecological conditions “potential” and “actual” efficiency tend to be related. As a consequence, the reduction of  $F_V/F_M$  and/or  $PI_{ABS}$  appears to reflect growth reduction, although this statement is still controversial and not definitively demonstrated. Clark et al. (2000) found a close correlation between the performance index ( $PI_{ABS}$ ) and biomass loss in *F. sylvatica* exposed to ozone, and a similar correlation has been reported by Pollastrini et al. (2010) in an ozone-sensitive poplar clone. Contradictory results were reported in the experiments of Bortier et al. (2000a,b, 2001), where a reduction of  $F_V/F_M$  did not correspond, in all cases, to an equivalent reduction of growth. In our opinion there is a large variation in the photosynthetic efficiency within the crown of a same plant, and compensative processes may occur, in order to recover from the loss of photosynthesis in the damaged leaves. Recently, the introduction of RE parameters ( $\varphi_{R_0} = RE/ABS$  and  $\delta_{R_0} = RE/ET$ ), and the related parameter  $PI_{TOT}$ , have provided more insight to the discussion about the relationships between dark fluorescence and gas exchange. In a previous study on ozone stress (Cascio et al., 2010) we found a close correlation between  $\varphi_{R_0}$  ( $RE/ABS$ ) and  $P_N$  in high light conditions. Our hypotheses—to explain this behaviour—are: (i) the overall potential efficiency ( $PI_{TOT}$ ) of ozone treated plants was to a large extent determined by the shape of the I–P region of the FT (see also Table 3); (ii) the J–I–P region expresses a relative

abundance of PSI as compared to PSII (Schansker et al., 2003, 2005) and a high PSI/PSII ratio allows for a quick reduction of ferredoxine and, consequently, potential high  $P_N$  rates.

In some instances, a temporarily enhanced efficiency of electron capture and transport in PSII was observed in ozone exposed plants (enhanced values of  $F_V/F_M$ ,  $ET/TR$ ,  $PI_{ABS}$ ,  $qP$ , see Mikkelsen and Ro-Poulsen, 1994; Lippert et al., 1996; Bortier et al., 2000b; Bussotti et al., 2007a,b; Pellegrini et al., in press; Nali et al., unpublished). The maintenance (or the enhancing) of photosynthetic efficiency in stressful conditions may be interpreted in terms of compensation and acclimation, enacted by plants to recover from the damage induced by ozone (Bussotti et al., 2007b). When that enhanced efficiency is connected to a reduced end acceptor capacity in combination with reduced Calvin cycle, energy demand lead to over-excitation of the photosynthetic apparatus and initiates response towards visible foliar injury.

The behaviour of  $F_0$  can help us distinguish between different response strategies. Many plant species protect their leaves by isolating the damaged cells with a protective callose layer (Bussotti et al., 2005), whereas the healthy cells remain undamaged and maintain (or enhance) their photosynthetic efficiency. In these cases  $F_0$  remains unchanged or diminishes its value (the reduction is also connected to the breakdown of chlorophyll in the injured cells), leading to the increase of the controlled de-excitation pathways ( $Sum k = 1/F_0$ ). In other cases we can observe the opposite behaviour, with the increase of  $F_0$  in leaves damaged by ozone. These species, which behave by shedding the injured leaves and replacing them with new leaf emissions, are considered more ozone-sensitive. This alleged sensitivity, however, concerns only the individual leaves and not the whole plant, since the dynamics of foliar demography allow the plant to retain an effective photosynthetic apparatus at all times.

A careful analysis of the chlorophyll fluorescence properties, by combining different approaches and methods, is able to give insights about damage, repair and avoidance strategies of plants subjected to ozone stress. Future directions for research in this field: (i) the possibility of combining fluorescence parameters with carbon assimilation and growth, to support the study on critical levels (Musselman and Lefohn, 2007), (ii) the analysis of events relating to the activity of PSI and events leading to the fixation of  $CO_2$ , using innovative technologies (cf., for ex., Strasser et al., 2010).

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