

Review

# Photosynthetic behavior of woody species under high ozone exposure probed with the JIP-test: A review

Filippo Bussotti <sup>a,\*</sup>, Reto J. Strasser <sup>b</sup>, Marcus Schaub <sup>c</sup>

<sup>a</sup> Department of Plant Biology, University of Florence, Piazzale delle Cascine 28, I-50144 Florence, Italy

<sup>b</sup> Bioenergetics Laboratory, University of Geneva, CH-1254 Jussy-Geneva, Switzerland

<sup>c</sup> Swiss Federal Institute for Forest, Snow and Landscape Research WSL, CH-8903 Birmensdorf, Switzerland

Received 16 August 2006; accepted 20 August 2006

*Analytical techniques based on the direct fluorescence of chlorophyll a, allow us to discriminate species-specific physiological behavior in relation to ozone air pollution.*

## Abstract

Visible ozone symptoms on leaves are expressions of physiological mechanisms to cope with oxidative stresses. Often, the symptoms consist of stippling, which corresponds to localized cell death (hypersensitive response, HR), separated from healthy cells by a layer of callose. The HR strategy tends to protect the healthy cells and in most cases the efficiency of chlorophyll to trap energy is not affected. In other cases, the efficiency of leaves to produce biomass declines and the plant loses its photosynthetic apparatus replacing it with a new, more efficient one. Another strategy consists of the production of pigments (anthocyanins), and leaves become reddish. In these cases, the most significant physiological manifestation consists of the enhanced dissipation of energy. These different behavior patterns are reflected in the initial events of photosynthetic activity, and can be monitored with techniques based on the direct fluorescence of chlorophyll *a* in photosystem II, applying the JIP-test.

© 2006 Elsevier Ltd. All rights reserved.

**Keywords:** Chlorophyll *a* fluorescence; JIP-test; Oxidative stress; Ozone; Performance Index

## 1. Introduction

Photosynthesis is the most important physiological process affected, in sensitive plants, by the excess of ozone in the troposphere. The reduction of net photosynthesis ( $P_N$ ), following exposure to high levels of ozone, has been described in a number of scientific studies (Bortier et al., 2000; Schaub et al., 2003; Novak et al., 2005). It is a pre-visual manifestation, i.e. not strictly related to symptom development, but one which is especially noteworthy in symptomatic leaves (Zhang et al., 2001; Novak et al., 2003; 2005; Gravano et al., 2004). It

can be the result of several, very different physiological processes, as summarized below:

- (a) The alteration of chloroplasts, with the swelling of thylakoids and granulation of the stroma, was the first ultrastructural change typical of ozone damage described in literature (Selldén et al., 1996). This mechanism may lead to a progressive destruction of the chlorophyll, with the formation of “yellow patches” and early senescence phenomena.
- (b) The collapse of cells or group of cells (hypersensitive response, HR, see Schraudner et al., 1997; Wohlgemuth et al., 2002), which reduces the overall content of chlorophyll in the leaves. This response is genetically defined and is commonly triggered as a response to pathogen

\* Corresponding author. Tel.: +39 055 328 8369; fax: +39 055 360137.

E-mail address: [filippo.bussotti@unifi.it](mailto:filippo.bussotti@unifi.it) (F. Bussotti).

infections. The stippling is the more evident visual manifestation of the HR.

- (c) A third process leading to the reduction of  $P_N$  is the stomatal closure, which some plants enact to cope with high ozone levels (Minnocci et al., 1999).

The aim of this paper is to discuss the relationship between visible symptoms, ultrastructural responses and photosynthetic processes in woody plant species (detected by JIP-test) subjected to high concentrations of ozone, and their role of these responses in survival strategies. Two symptoms typologies will be analyzed: the hypersensitive response (HR) and the reddening. The majority of the examples (either the authors' direct observations or taken from the literature) refer to broadleaves. Among conifers, JIP-test was applied on *Pinus halepensis* (Manes et al., 2001), but the symptomatology of this species cannot be included in the series reported above (since these are cases of yellowing, i.e. *chlorotic mottling*).

## 2. The “JIP-test”

Among the techniques suitable for assessing stress conditions in terms of photosynthesis, the measurement of direct chlorophyll *a* (Chl *a*) fluorescence in photosystem II (PSII) and the “JIP-test analysis” (i.e., the analysis of the O-J-I-P polyphasic rising transient, see Strasser et al., 2000; for review see Strasser et al., 2004), combine several advantages: (i) easy and fast method, (ii) possibility of obtaining robust datasets, (iii) easy to repeat, (iv) easy to process data and report findings, (v) the instruments available on the market are relatively inexpensive and the measurement procedures are equally inexpensive.

The rising transients, measured on dark-adapted leaves, are induced by a red saturating light. On a logarithmic time scale, the rising transient from  $F_0$  ( $F$  at 50  $\mu$ s, when all the reaction centers of the PSII are open, i.e. when the primary acceptor quinone  $Q_A$  is fully oxidized) to  $F_P$  (where  $F_P = F_M$  under saturating excitation light, when the excitation intensity is high enough to ensure the closure of all reaction centers of PSII, i.e. the full reduction of all reaction centers) has a polyphasic behavior. Analysis of the transient took into consideration fluorescence values at 50 or 20  $\mu$ s ( $F_0$ , step 0), 100  $\mu$ s ( $F_{100}$ ), 300  $\mu$ s ( $F_{300}$ ), 2 ms (step J), 30 ms (step I) and maximal ( $F_M$ , step P).

Table 1 summarizes the technical parameters of curves necessary for further elaborations, as well as the selection of the JIP-test parameters. The Performance Index, PI (Strasser et al., 2000; 2004), was calculated on an absorption basis (ABS), e.g. based on an equal amount of chlorophyll per sample.

$$PI_{ABS} = \frac{\gamma_{RC}}{1 - \gamma_{RC}} \cdot \frac{\phi_{P_0}}{1 - \phi_{P_0}} \cdot \frac{\Psi_0}{1 - \Psi_0} = \frac{RC}{ABS} \cdot \frac{\phi_{P_0}}{1 - \phi_{P_0}} \cdot \frac{\Psi_0}{1 - \Psi_0}$$

where  $\gamma_{RC}$  is the fraction of reaction-center (RC) chlorophylls relative to total chlorophyll:

$$\gamma_{RC} = Chl_{RC}/Chl_{total},$$

since

$$Chl_{total} = Chl_{antenna} + Chl_{RC},$$

then

$$\gamma_{RC}/(1 - \gamma_{RC}) = Chl_{RC}/Chl_{antenna} = RC/ABS.$$

$PI_{ABS}$  combines three parameters favorable to photosynthetic activity: (1) the density of reaction centers (expressed on an absorption basis RC/ABS); (2) the quantum yield of primary photochemistry  $\phi_{P_0} = TR_0/RC$ , and (3) the ability to feed electrons into the electron chain between PSII and PSI as  $\Psi_0 ET_0/TR_0$ .

The JIP-test has been elaborated both in terms of application (Strasser et al., 2000) and of theory (Strasser et al., 2004). It has been widely accepted and successfully used to analyze ozone-induced stress conditions in plants (see Table 2). The relative concepts were recently reviewed from Lazar (2006).

## 3. The experimental set-up

The findings reported here were mostly obtained in open-top chamber (OTC) experiments carried out in the cantonal forest nursery at Lattecaldo (Canton Ticino, southern Switzerland) and the regional forest nursery at Curno (Lombardy, northern Italy). The first OTC facility, managed by the WSL in Birmendorf, has been running since 1995 and has already yielded findings that have been published in several papers (VanderHeyden et al., 2001; Zhang et al., 2001; Novak et al., 2003, 2005). The second OTC facility was established in 2004 and the first results were released by Ballarin-Denti et al. (2005).

At both sites, ambient levels of ozone are sufficiently high to produce visible symptoms on a number of native and cultivated plants species. The OTC design consists of 4 plots treated with charcoal filtered air (CF), 4 plots treated with unfiltered air (NF) and 4 open plots exposed to ambient air (AA). At Curno site, for each treatment, 2 plots are subjected to a normal watering regime (to field capacity), and 2 are drought stressed (only rescue watering is carried out). Further observations were performed in a mountain area in Northern Italy (Moggio), subjected to high levels of ozone (Bussotti et al., 2005).

## 4. Photosynthetic responses determined with JIP-test

### 4.1. First events

The first photosynthetic events induced by ozone stress were observed in open-top chamber experiments at Lattecaldo and Curno. Early responses can be observed at the beginning of the growth season, a few days after full extension and maturation of the leaves, in complete absence of symptoms. In some cases (Schaub et al., 2003; Bussotti et al., 2004; Gravano

Table 1  
Explanation of the technical data of the O-J-I-P curves and the selected JIP-test parameters

Technical fluorescence parameters	
Area	Area between fluorescence curve and $F_M$
$T_{F_{max}}$	The time needed to reach $F_M$
$F_0$	$F_{50\mu s}$ , fluorescence intensity at 50 $\mu s$
$F_{100\mu s}$	Fluorescence intensity at 100 $\mu s$
$F_{300\mu s}$	Fluorescence intensity at 300 $\mu s$
$F_J$	Fluorescence intensity at the J-step (at 2 ms)
$F_I$	Fluorescence intensity at the I-step (at 30 ms)
$F_M$	Maximal fluorescence intensity
$F_V/F_0$	$(F_M - F_0)/F_0 = k_P/k_N$
$(\Delta V/\Delta t)_0$ or $M_0$	Slope of the normalized curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry (Srivastava et al., 1997). $M_0 = 4(F_{300} - F_0)/(F_M - F_0)$ expressed per 1 ms
$V_J$	Relative variable fluorescence at 2 ms. $V_J = (F_{2ms} - F_0)/(F_M - F_0)$
$V_I$	Relative variable fluorescence at 30 ms. $V_I = (F_{30ms} - F_0)/(F_M - F_0)$
$S_M$	Normalised Area. Measures the energy needed to close all reaction centers $Area/(F_M - F_0)$
N	Number of turnover of $Q_A$ . Indicates how many times $Q_A$ is reduced in the time-span from 0 to $T_{F_{max}}$ . $N = S_M \cdot M_0 \cdot (1/V_J)$
<i>Quantum efficiency or flux ratios</i>	
$\phi_{P_0}$ or $TR_0/ABS$	Trapping probability, or Quantum yield efficiency. Expresses the probability that an absorbed photon will be trapped by the PSII reaction center. $\phi_{P_0} = (F_M \cdot Area)/(F_M - F_0)F_0/F_M$
$\Psi_0$ or $ET_0/TR_0$	Expresses the probability that an exciton trapped by the PSII reaction center enters the electron transport chain. $\Psi_0 = 1 \cdot Area/(F_M - F_0)V_J$
<b>De-excitation constants</b>	
$k_P$	Photochemical de-excitation constant. $k_P = (ABS/CS)k_F[(1/F_0)Area/(F_M - F_0)(1/F_M)]$
$k_N$	Non-photochemical de-excitation constants, summing up $K_H$ (for heat dissipation) and $K_F$ (for fluorescence emission). $k_N = (ABS/CS)k_F(1/F_M)$
SumK	$k_P + k_N = (ABS/CS)k_F(1/F_0)$
<i>Specific fluxes or specific activities (expressed in arbitrary units)</i>	
ABS/RC	Effective antenna size of an active reaction center (RC). Expresses the total number of photons absorbed by Chl molecules of all RC divided by the total number of active RCs $ABS/RC = M_0(1/V_J)(1/\phi_{P_0})$
$TR_0/RC$	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the RC. $TR_0/RC = M_0(1/V_J)$
$ET_0/RC$	Electron transport in an active RC. $ET_0/RC = M_0(1/V_J)\Psi_0$
$DI_0/RC$	Effective dissipation in an active RC. $DI_0/RC = (ABS/RC) - (TR_0/RC)$
<i>Density of reaction centers</i>	
RC/CS <sub>0</sub>	Gives the number proportional to the active RCs to the cross-section of the measured sample $RC/CS_0 = \phi_{P_0}(V_J/M_0)F_0$
<b>Performance index</b>	
PI <sub>ABS</sub>	See text. $PI_{ABS} = (RC/ABS)[\phi_{P_0}/(1 - \phi_{P_0})] [\Psi_0/(1 - \Psi_0)]$

et al., 2004; Novak et al., 2005) the early response consists of a depression of net photosynthesis ( $P_N$ ), but the results obtained through the JIP-test suggest more complex physiological considerations.

In *Fagus sylvatica*, *Quercus robur* (Curno, 2004) and *Populus nigra* (Lattecaldo, 2002; Curno, 2004) the first effect of ozone exposure (which can be observed both in unfiltered air and ambient air plots) consists of increased photosynthetic efficiency, expressed as  $PI_{ABS}$ , as compared to that observed in filtered air plots. That behavior seems to be caused in particular by the enhanced efficiency of the electron transport processes. The difference between plots exposed to ozone (NF and AA) and those exposed to approximately 50% ambient ozone concentrations (CF) is less pronounced during the

summer and decreases significantly in exposed plots towards the end of the growing season (Fig. 1). This behavior, however, was not consistently reconfirmed in the experiments carried out at Curno and Lattecaldo in later years.

Other species, such as *Viburnum lantana*, display the opposite behavior: the efficiency of the photosystems appears to be diminished starting from the very first phases of exposure to ambient ozone (Bussotti et al., 2004).

The increase in photosynthetic efficiency in relation to slight ozone exposures was reported also from Paoletti et al. (2004) in some Mediterranean evergreen species. Soja et al. (1998) observed the increase of the electron transport efficiency as early physiological response on *Malus domestica*. The increase in photosynthetic efficiency as a response to

Table 2  
Summary of the main results obtained from experimental studies applying the JIP-test to detect ozone stress

Reference	Species	Experiment	Treatment	Behavior of the ozone-treated plants (main results)
Soja et al., 1998	<i>Malus domestica</i>	OTC	AA + 25 nl L <sup>-1</sup> ; AA + 50 nl L <sup>-1</sup> , 5 days week <sup>-1</sup> , 8 h day <sup>-1</sup>	Decrease of N and RC/CS <sub>0</sub> . Slight exposure produces the increases of electron transport efficiency
Clark et al., 2000	<i>Fagus sylvatica</i>	OTC	50%, 85%, 100% of AA; 50% AA + O <sub>3</sub> 30 nl L <sup>-1</sup> per one growing season	Reduction of biomass correlated with visual symptoms development and decrease of performance index
Manes et al., 2001	<i>Pinus halepensis</i>	Fumigation chamber	300 ppb, per 6 h day <sup>-1</sup> , per 6 days week <sup>-1</sup> . Water stress was applied	Decrease of quantum yield efficiency ( $\phi P_0$ ) both for ozone and water stress
Nussbaum et al., 2001	<i>Bromus erectus</i> , <i>Centaurea jacea</i> , <i>Trisetum flavescens</i> , <i>Plantago lanceolata</i> , <i>Rumex obtusifolius</i> , <i>Trifolium pretense</i> , <i>Knautia arvensis</i>	OTC	AA ozone concentration; 1.5 and 2 times AA ozone concentration, throughout growing season	Decrease of variable fluorescence Fv:F <sub>0</sub> and RC/CS <sub>0</sub>
Gravano et al., 2004	<i>Fraxinus excelsior</i> , <i>Prunus avium</i> , <i>Viburnum lantana</i>	OTC (Lattecaldo, CH)	Charcoal filtered AA; non-filtered AA, through a growing season	Decrease of performance index in all species. The de-excitation constants (SumK) increased in <i>Fraxinus excelsior</i>
Paoletti et al., 2004	<i>Laurus nobilis</i> , <i>Arbutus unedo</i> , <i>Phillyrea latifolia</i>	Fumigation chamber	55 and 110 nl L <sup>-1</sup> per 90 days	Reduction of RC/CS <sub>0</sub> . Slight exposure increases the performance index
Bussotti et al., 2004	<i>Populus nigra</i> , <i>Viburnum lantana</i>	OTC (Lattecaldo, CH)	Charcoal filtered AA; non-filtered AA, throughout one growing season	Performance index was enhanced in young leaves of <i>Populus nigra</i> ; but was depressed in <i>Viburnum lantana</i>
Bussotti et al., 2005	<i>Ailanthus altissima</i> , <i>Fagus sylvatica</i> , <i>Fraxinus excelsior</i> , <i>Acer pseudoplatanus</i> , <i>Viburnum lantana</i>	Field observation	AOT 40 = 90 ppm.h	Decrease of performance index in all species with the diffusion of the symptoms. The de-excitation constants (SumK) increased in <i>Fraxinus excelsior</i> and <i>Acer pseudoplatanus</i> , but decreased in <i>Ailanthus altissima</i>
Ballarin-Denti et al., 2005	<i>Fagus sylvatica</i> , <i>Quercus robur</i> , <i>Populus nigra</i>	OTC (Curno, IT)	Charcoal filtered AA; non-filtered AA, through a growing season	At the beginning of the season the performance index was enhanced in all species. Later during the season, it declined quickly

oxidative stress factors has been described more generally by Liska et al. (2004) and Retuerto et al. (2004). It enables plants to respond to the need of metabolites, in order to feed the acclimatization process (detoxification and repair).

In our experiments reported here the Performance Index values were not directly related to the net photosynthesis. Actually, the photosystem efficiency provides no direct information on P<sub>N</sub>, since electron flux is not necessarily used in carbon metabolism as it may also be re-routed to other biochemical pathways (Adams and Demming-Adams, 2004), such as reduction of O<sub>2</sub>, nitrite, sulfate, or thioredoxin.

#### 4.2. The common responses

In all cases, the development of ozone-induced symptoms led to decreased vitality of the leaf, as expressed by lower PI<sub>ABS</sub> index values. The depression of F<sub>M</sub> is also a common feature. Among other responses, Soja et al. (1998), Nussbaum et al. (2001), Manes et al. (2001) and Paoletti et al. (2004) observed a reduction in values of RC/CS<sub>0</sub> and  $\phi P_0$  although  $\phi P_0$

appeared to be very stable and its reduction is only appreciable when the level of foliar injury is very high. This suggests a reduced efficiency of processes linked to uptake of light energy, which can be interpreted as a strategy aimed at lowering the high excitation pressure in mesophyll cells. The decrease of the fraction of fully active RC<sub>s</sub> (Q<sub>A</sub> and Q<sub>B</sub> reducing centers) in favor of an increase of the fraction of heat sink centers (non Q<sub>A</sub> reducing centers or silent centers, see Strasser et al., 2004) is considered a down-regulation mechanism, to dissipate in a controlled way the excess of absorbed light. The 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. The de-excitation rate constant for non-photochemical events k<sub>N</sub> increases while the one for photochemical events k<sub>P</sub> decreases. This mechanism protects the photosystems from the risks of photo-oxidation and—unless other response processes are triggered—allows them to maintain their basal fluorescence intensity (F<sub>0</sub>) unaltered due to the relation F<sub>0</sub> = ABS k<sub>F</sub>/(k<sub>N</sub> + k<sub>P</sub>).

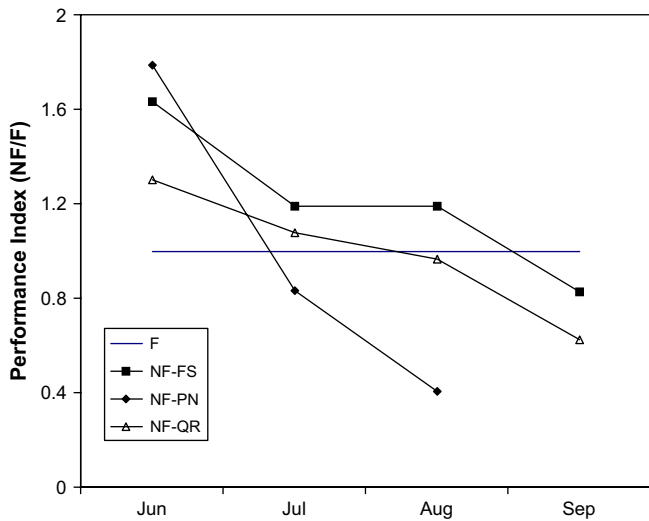


Fig. 1. Curno experiment, 2004. Behavior of the relative Performance Index in the plants grown in the non-filtered chambers (NF) respect to those grown in the filtered ones (F). FS, *Fagus sylvatica*; PN, *Populus nigra*; QR, *Quercus robur*. The observation dates were 9 June, 6 July, 3 August and 10 September. The exposition values were 2 ppm.h at the first observation (9 June) and 28 ppm.h at the latest observation (10 September).

Among the ozone-induced photosynthetic processes that can induce an excess of electron flux, we made the following observations:

- high concentrations of ozone depress stomatal apertures (Minnocci et al., 1999) and, as a consequence, the flow of carbon in the mesophyll, which is the target of the electron flux;
- electron flux from the two consecutive photosystems is not able to feed the dark reactions of the Calvin cycle because, in the photosynthetic apparatus, the first effect of the ROS (reactive oxygen substances, i.e. the secondary or tertiary products of ozone) impacts the RUBISCO, which is thus not able to fix carbon adequately (Gerant et al., 1996). Soja et al. (1998) revealed a higher level of ozone in carboxylation efficiency than in light utilization efficiency. In other words, the dark phase of photosynthesis is more sensitive to ozone than the luminous phase.

#### 4.3. The hypersensitive response

One of the most common and easily detectable responses is the Hypersensitive Response (HR), which leads to the programmed death of groups of cells of varying sizes (Schraudner et al., 1997; Wohlgemuth et al., 2002). The programmed cell death (pcd) is thus a strategy aimed at limiting the scope and spread of the infection; it is preceded by processes of de-generation of the cell content. Ozone-induced HR is characteristic by the fact that the first cells to be affected are the palisade mesophyll cells, which are not directly adjacent to the leaf's veins. Furthermore, the damage is initially restricted to isolated cells and expands as the process continues

(Wohlgemuth et al., 2002). HR is accompanied by oxidative burst phenomena (OB), i.e. the oxidative degeneration of cellular content. A collateral process is the accumulation of callose, which isolates the affected zones of the mesophyll (Gravano et al., 2003, 2004). The injured areas spread from the edges of pre-existing injuries. The collapse of single cells, or localized groups of cells, in many cases appears to be a strategy enabling the plant to circumscribe the damage, thus allowing other cells to function normally (Gravano et al., 2004; Bussotti et al., 2005). Thus, affected leaves can retain their detoxifying ability in the symplast (through the synthesis of antioxidant enzymes) and in the apoplast (by thickening the cell wall which is where most detoxifying processes are located) (Lyons et al., 2000).

For HR species, the parameters that best distinguish the different physiological behavior patterns are those linked to the de-excitation rate constants ( $k_P$ ,  $k_N$  and the sum of all constants  $\text{Sum}K = k_N + k_P$ ). These are chlorophyll conformation parameters and describe chlorophyll's efficiency in controlling processes of de-excitations in terms of heat and fluorescence emission and energy conservation in the photosynthetic electron transport. The value of the constants  $k_N$  and  $k_P$  is strictly connected to the value of  $F_0$ , which is also used as a measure for the chlorophyll concentration.

For some HR species, such as *Ailanthus altissima*, the  $F_0$  value increases with the appearance of the symptoms. That goes in parallel with irreversible damage to the photosystems (Krause, 1988). The overall value of the constants ( $\text{Sum}K$ ) diminished, especially due to the marked decrease in  $k_P$  values (Fig. 2A and E). The plants are probably enacting two distinct and converging responses: an HR combined with premature senescence of the leaf.

In *Acer pseudoplatanus* and *Fraxinus excelsior* (also HR species) the extent of symptoms is strictly correlated with a decrease of  $F_0$  and with an increase in the value of de-excitation constants for photochemical and non-photochemical events [ $F_0 = \text{ABS } k_P / (k_P + k_N)$ ] (Fig. 2B and F). The increase of the non-photochemical constant ( $k_N$ ) suggests that energy dissipation is a physiologically controlled process, whereas the increase of the photochemical constant ( $k_P$ ) indicates that the leaf tends to compensate for the loss of entire cells (and thus the overall quantity of chlorophyll) by increasing the efficiency of the remaining chlorophyll in unaffected cells. A similar behavior was also observed by Berger et al. (2004), using imaging fluorescence techniques, in relation to pathogen infection. They observed that the infected leaf area was surrounded by a circle where the photosynthetic efficiency was enhanced.

Yet, the behaviors of these two species differ as the active responses implemented. *Acer pseudoplatanus* develops a thickening of the mesophyll cell walls, thus increasing both the apoplast's detoxifying capacity and the mechanical resistance to collapse. Thickening of mesophyll cell walls, in particular, is considered to be a specific marker of ozone injury in several species (Günthard-Goerg et al., 1997, 2000; Vollenweider et al., 2003; Bussotti et al., 2005). The synthesis of antioxidants and of the cellulose compounds that make up the cell

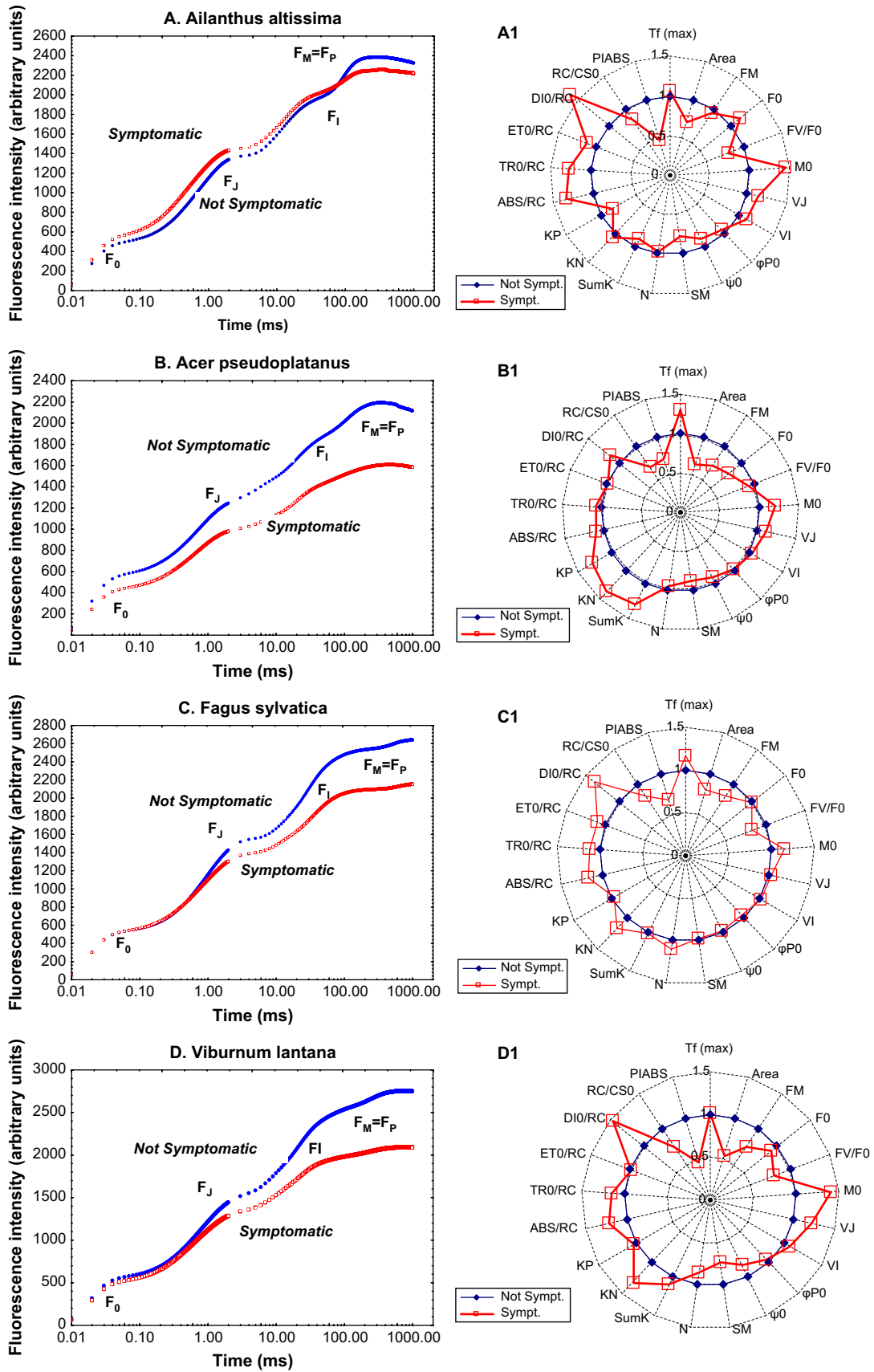


Fig. 2. (A, A1) *Ailanthus altissima*. (B, B1) *Acer pseudoplatanus*. (C, C1) *Fagus sylvatica*. (D, D1) *Viburnum lantana*. Plots at the left side: for each considered species the average behavior of the fluorescence transients is reported (mean of 15–20 leaves each transient), both for non-symptomatic and symptomatic leaves (in symptomatic leaves, symptoms were spread on about 40–60% of the leaf surface). Plots at the right side: for the same species is shown the JIP-test analysis by mean of “radar-plots” (each parameter is expressed by mean of the ratio “Symptomatic/Not Symptomatic”). The value of the Not Symptomatic sample is used as reference and set to unity.

wall implies an intense metabolic activity and requires efficient cell structures. However, the increased activity of individual cells occurs as the overall efficiency of the leaf as a whole diminishes, since the number of active cells is much reduced. This may explain the delayed appearance of symptoms.

*Fraxinus excelsior*, on the other hand, does not enact any particular defense mechanisms and tends to shed prematurely its affected leaves, replacing them with a new and more efficient crown (Gravano et al., 2004). This behavior has been described by Maurer and Matyssek (1997) and by Maurer et al. (1997) in *Betula pendula* as well, and is interpreted as being a typical compensation strategy enacted by plants growing on soils that do not present any nutritional limitations.

According to the observations reported by Vollenweider et al. (2003) and Bussotti et al. (2005), *Fagus sylvatica* has shown itself to be a weakly responding HR species in which foliar injury consists mainly of the oxidation of cellular contents (oxidative burst), with only a few cases of collapsed cells.  $F_0$  is unchanged in symptomatic leaves (Fig. 2C and G), whereas the value of  $k_N$  increases in relation to  $k_P$ , which remains unaltered. Further, the processes related to light capture ( $\phi_{P_0}$  and  $RC/CS_0$ ) are reduced in relation to those involved in electron transport ( $\Psi_0$ ,  $S_M$ ).

#### 4.4. Leaf reddening

Leaf reddening is a very early response to ozone stress. For the species in which it can occur, leaf reddening may be observed after just a few days of ozone exposure (Novak et al., 2003; Orendovici et al., 2003), even without any damage at ultrastructural level (Gravano et al., 2004). The reddening is caused by the accumulation of anthocyanins, which act as photoprotectors and antioxidants against light and other oxidative stresses such as drought and nutrient scarcity (Steyn et al., 2002; Neill and Gould, 2003). In the first case they are located mainly in the upper epidermal cells; in the latter, in the mesophyll cells. Furthermore, in several species, anthocyanins are associated with autumn senescence processes. In this case, they are in the mesophyll and their function is thought to be of photoprotection of the photosynthesis apparatus at a period in which environmental conditions make it especially unstable (Hoch et al., 2001).

The accumulation of anthocyanins can be considered as part of a strategy to preserve the chloroplasts against the action of the secondary or tertiary products of ozone (reactive oxygen substances, ROS), and to reduce the oxidative pressure caused by the concurrence of ozone with strong light radiation.

In the species observed by the authors (*Viburnum lantana* and *Prunus avium*, see Gravano et al., 2004), the leaf reddening was accompanied from processes of premature senescence. These were evidenced from an ultrastructural response consisting of the degeneration of parts of the chloroplasts. The photosynthetic behavior (JIP-test) of *Viburnum lantana* is shown in Fig. 2D and H. The main responses observed were: a slight reduction in the  $F_0$  value, the increase

of  $k_N$ , and the depression of processes involved in electron transport which lead to an increased dissipation of energy.

Leaf reddening was also observed in several herbaceous plants and shrubs, such as *Centaurea* sp. pl. and *Rubus* sp. pl. In these cases, an increase of the  $F_0$  value was observed, with a pattern of behavior that is basically analogous to that observed in *Ailanthus altissima*.

## 5. Conclusions

The patterns of behavior described above (both in terms of foliar symptom and of photosynthesis responses) cannot be considered species-specific, and plants cannot be classified accordingly. These patterns may vary, even on the same plant, in relation to ecological conditions, the physiological status of the plant, its age, etc. Even within the same leaf we may observe a combination of different symptoms occurring simultaneously (e.g. reddening plus HR, see Bussotti et al., 2005).

Plants are well adapted to cope with oxidative stress agents in the environment (high light radiation and UV-B, drought, salinity, pest infection, etc.). The increased tropospheric ozone acts as an additional factor and plants respond with the same physiological strategies and mechanisms they developed under different conditions.

Visible symptoms, ultrastructural changes and physiological behavior show different manifestations in different species, thus describing species-specific strategies to address environmental oxidative pressure. Two different patterns of behavior can be identified: one based on compensation and the other based on conservation.

Compensatory patterns of behavior consist of the rapid abscission of symptomatic leaves (which are less efficient) and the subsequent replacement of the damaged portions of the crown with new and more efficient leaves. The species that adopt this behavior are fast-growing species, which do not invest their metabolism at all—or only to a limited extent—in defense mechanisms. This behavior is more frequent in species growing in eutrophic environments where environmental resources are not a factor limiting growth or the production of new leaves. An exception is the case of *Ailanthus altissima*, whose compensation behavior appears to be related to its capacity to reproduce rapidly through root suckers when under stress.

Conservatory patterns of behavior, on the other hand, are a characteristic feature of those species that enact defense strategies—such as the production and accumulation of anthocyanins, or cell wall thickening, etc.—in order to delay or avoid premature leaf loss. Usually, these are slow-growing species, in some cases ones that have adapted to more oligotrophic environments and which tend to defend their metabolic investment in the crown.

## References

- Adams III, W.W., Demming-Adams, B., 2004. Chlorophyll fluorescence as a tool to monitor plant response to environment. In: Papageorgiou, G.C., Govindjee (Eds.), *Chlorophyll Fluorescence: A Signature of*

- Photosynthesis. Advances in Photosynthesis and Respiration Series (Govindjee, Series Ed.). Springer, Dordrecht, pp. 583–604.
- Ballarin-Denti, A., Bussotti, F., Cascio, C., Desotgiu, R., Gerosa, G., Marzuoli, R., Strasser, R.J., Tagliaferri, A., 2005. An open-top chamber experiment at Curmo (Northern Italy): results of the first year of activity. Workshop: Critical levels of ozone: further applying and developing the flux-based concept. Obergurgl (Austria) 15–19 Nov. 2005. Background Documents.
- Berger, S., Papadopoulos, M., Schreiber, U., Kaiser, W., Roitsch, T., 2004. Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. *Physiologia Plantarum* 122, 419–428.
- Bortier, K., Ceulemans, R., de Temmerman, L., 2000. Effects of ozone exposure on growth and photosynthesis of beech seedlings (*Fagus sylvatica*). *New Phytologist* 146, 271–280.
- Bussotti, F., Strasser, R.J., Novak, K., Schaub, M., Cascio, C., 2004. Photosynthetic efficiency in *Populus nigra* and *Viburnum lantana* grown in open-top chambers. *Acta Physiologiae Plantarum* 26 (Suppl.), 243.
- Bussotti, F., Agati, G., Desotgiu, R., Matteini, P., Tani, C., 2005. Ozone foliar symptoms in woody plants assessed with ultrastructural and fluorescence analysis. *New Phytologist* 166, 941–955.
- Clark, A.J., Landolt, W., Bucher, J.B., Strasser, R.J., 2000. Beech (*Fagus sylvatica*) response to ozone exposure assessed with a chlorophyll *a* fluorescence performance index. *Environmental Pollution* 109, 501–507.
- Gerant, D., Podor, M., Grieu, P., Afif, D., Cornu, S., Morabito, D., Banvoy, J., Robin, C., Dizengremel, P., 1996. Carbon metabolism enzyme activities and carbon partitioning in *Pinus halepensis* Mill. exposed to mild drought and ozone. *Journal of Plant Physiology* 148, 142–147.
- Gravano, E., Giulietti, V., Desotgiu, R., Bussotti, F., Grossoni, P., Gerosa, G., Tani, C., 2003. Foliar response of an *Ailanthus altissima* clone in two sites with different levels of ozone-pollution. *Environmental Pollution* 121, 137–146.
- Gravano, E., Bussotti, F., Strasser, J.R., Schaub, M., Novak, K., Skelly, J., Tani, C., 2004. Ozone symptoms in leaves of woody plants in open top chambers: ultrastructural and physiological characteristics. *Physiologia Plantarum* 121, 620–633.
- Günthard-Goerg, M.S., McQuattie, Scheidegger, C., Rhiner, C., Matyssek, R., 1997. Ozone-induced cytochemical and ultrastructural changes in leaf mesophyll cell walls. *Canadian Journal of Forest Research* 27, 453–463.
- Günthard-Goerg, M.S., McQuattie, C.J., Maurer, S., Frey, B., 2000. Visible and microscopic injury in leaves of five deciduous tree species related to current critical ozone levels. *Environmental Pollution* 109, 489–500.
- Hoch, W.A., Zeldin, E.L., McCown, B.H., 2001. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology* 21, 1–8.
- Krause, G.H., 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. *Physiologia Plantarum* 74, 566–574.
- Lazar, D., 2006. The polyphasic chlorophyll *a* fluorescence rise measured under high intensity of exciting light. *Functional Plant Biology* 33, 9–30.
- Liska, A.J., Shevchenko, A., Pick, U., Katz, A., 2004. Enhanced photosynthesis and redox energy production contribute to salinity tolerance in *Dunaliella* as revealed by homology-based proteomics. *Plant Physiology* 136, 2806–2817.
- Lyons, T., Plöchl, M., Turcsáni, E., Barnes, J., 2000. Extracellular antioxidants: a protective screen against ozone? In: Agrawal, S.B., Agrawal, M. (Eds.), *Environmental Pollution and Plant Responses*. Lewis Publishers, Boca Raton, pp. 183–201.
- Manes, F., Donato, E., Vitale, M., 2001. Physiological response of *Pinus halepensis* needles under ozone and water stress conditions. *Physiologia Plantarum* 113, 249–257.
- Maurer, S., Matyssek, R., 1997. Nutrition and the ozone sensitivity of birch (*Betula pendula*). II. Carbon balance, water-use efficiency and nutritional status of the whole plant. *Trees* 12, 11–20.
- Maurer, S., Matyssek, R., Günthard-Goerg, M.S., Landolt, W., Einig, W., 1997. Nutrition and the ozone sensitivity of birch (*Betula pendula*). I. Responses at the leaf level. *Trees* 12, 1–10.
- Minnocci, A., Panicucci, A., Sebastiani, L., Lorenzini, G., Vitagliano, C., 1999. Physiological and morphological responses of olive plants to ozone exposure during a growing season. *Tree Physiology* 19, 391–397.
- Neill, S.O., Gould, K.S., 2003. Anthocyanins in leaves: light attenuators or antioxidants? *Functional Plant Biology* 30, 865–873.
- Novak, K., Skelly, J.M., Schaub, M., Kraeuchi, N., Hug, C., Landolt, W., Bleuler, P., 2003. Ozone air pollution and foliar injury development. *Environmental Pollution* 125, 41–52.
- Novak, K., Schaub, M., Fuhrer, J., Skelly, J.M., Hug, C., Landolt, W., Bleuler, P., Kräuchi, N., 2005. Seasonal trends in reduced leaf gas exchange and ozone-induced foliar injury in three ozone sensitive woody plants species. *Environmental Pollution* 136, 33–45.
- Nussbaum, S., Geissmann, M., Eggenberg, P., Strasser, R.J., Fuhrer, J., 2001. Ozone sensitivity in herbaceous species as assessed by direct and modulated chlorophyll fluorescence techniques. *Journal of Plant Physiology* 158, 757–766.
- Orendovici, T., Skelly, J.M., Ferdinand, J.A., Savage, J.E., Sanz, M.J., Smith, G.C., 2003. Response of native plants of northeastern United States and southern Spain to ozone exposure; determining exposure/response relationships. *Environmental Pollution* 125, 31–40.
- Paoletti, E., Bussotti, F., Della Rocca, G., Lorenzini, G., Nali, C., Strasser, R.J., 2004. Fluorescence transient in ozonated Mediterranean shrubs. *Phyton Annales Rei Botanicae* 44, 121–131.
- Retuerto, R., Fernandez-Lema, B., Roiloa, R., Obeso, J.R., 2004. Increased photosynthetic performance in holly trees infested by scale insects. *Functional Ecology* 18, 664–669.
- Schaub, M., Skelly, J.M., Steiner, K.C., Davis, D.D., Pennypacker, S.P., Zhang, J., Ferdinand, J.A., Savage, J.E., Stevenson, R.E., 2003. Physiological and foliar injury responses of *Prunus serotina*, *Fraxinus americana*, and *Acer rubrum* seedlings to varying soil moisture and ozone. *Environmental Pollution* 124, 307–320.
- Schraudner, M., Langebartels, C., Sandermann, H., 1997. Changes in the biochemical status of plant cells induced by the environmental pollutant ozone. *Physiologia Plantarum* 100, 274–280.
- Selldén, G., Sutinen, S., Skärby, L., 1996. Controlled ozone exposure and field observations in Fennoscandia. In: Sandermann, H., Wellburn, A.R., Heath, R.L. (Eds.), *Forest Decline and Ozone. A Comparison of Controlled Chamber and Field Experiments*. Ecological Studies 127. Springer, Berlin, pp. 249–276.
- Soja, G., Pfeifer, U., Soja, A.M., 1998. Photosynthetic parameters as early indicators of ozone injury in apple leaves. *Physiologia Plantarum* 104, 639–645.
- Steyn, W.J., Wand, S.J.E., Holcroft, D.M., Jacobs, G., 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist* 155, 349–361.
- Strasser, A., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus, M., Pathre, U., Mohanty, P. (Eds.), *Probing Photosynthesis: Mechanisms, Regulation and Adaptation*. Taylor and Francis, London, pp. 445–483.
- Strasser, A., Tsimilli-Michael, M., Srivastava, A., 2004. Analysis of the fluorescence transient. In: Papageorgiou, G.C., Govindjee (Eds.), *Chlorophyll Fluorescence: A Signature of Photosynthesis*. Advances in Photosynthesis and Respiration Series (Govindjee, Series Ed.). Springer, Dordrecht, pp. 321–362.
- VanderHeyden, D.J., Skelly, J.M., Innes, J.L., Hug, C., Zhang, J., Landolt, W., Bleuler, P., 2001. Ozone exposure thresholds and foliar injury on forest plants in Switzerland. *Environmental Pollution* 111, 321–331.
- Vollenweider, P., Ottiger, M., Günthard-Goerg, M.S., 2003. Validation of leaf ozone symptoms in natural vegetation using microscopical methods. *Environmental Pollution* 124, 101–118.
- Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., Weigel, H.J., Overmyer, K., Kangasjärvi, J., Sandermann, H., Langebartels, C., 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell and Environment* 25, 717–726.
- Zhang, J., Ferdinand, J.A., Vanderheyden, D.J., Skelly, J.M., Innes, J.L., 2001. Variation of gas exchange within native plant species of Switzerland and relationships with ozone injury: an open-top experiment. *Environmental Pollution* 113, 177–185.