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**SPECIAL ISSUE PAPER**

# Chronic ozone exposure affects leaf senescence of adult beech trees: a chlorophyll fluorescence approach

**Birgit Gielen[1,*], Markus Löw[2], Gaby Deckmyn[1], Ursula Metzger[2], Fabrice Franck[3], Christian Heerdt[4], Rainer Matyssek[2], Roland Valcke[5] and Reinhart Ceulemans[1]**

[1] *University of Antwerpen, Campus Drie Eiken, Department of Biology, Research Group of Plant and Vegetation Ecology, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

[2] *Technische Universität München, Ecophysiology of Plants, Am Hochanger 13, D-85354 Freising, Germany*

[3] *Université de Liège, Département des sciences de la vie/Biochimie végétale, BAT. B22 Photobiologie, Boulevard du Rectorat, 27, B-4000 Liège, Belgium*

[4] *Technische Universität München, Lehrstuhl für Ökoklimatologie, Am Hochanger 13, D-85354 Freising, Germany*

[5] *Hasselt University, Centre for Environmental Sciences, Dept. SBG, Laboratory of Molecular and Physical Plant Physiology, Agoralaan, Bldg D, B-3590 Diepenbeek, Belgium*

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## Abstract

**Accelerated leaf senescence is one of the harmful effects of elevated tropospheric ozone concentrations ([$O_3$]) on plants. The number of studies dealing with mature forest trees is scarce however. Therefore, five 66-year-old beech trees (*Fagus sylvatica* L.) have been exposed to twice-ambient (2×ambient) [$O_3$] levels by means of a free-air canopy $O_3$ exposure system. During the sixth year of exposure, the hypothesis of accelerated leaf senescence in 2×ambient [$O_3$] compared with ambient [$O_3$] trees was tested for both sun and shade leaves. Chlorophyll (chl) fluorescence was used to assess the photosynthetic quantum yield, and chl fluorescence images were processed to compare functional leaf homogeneity and the proportion of $O_3$-injured leaf area (stipples) under ambient and 2×ambient [$O_3$] regimes. Based on the analysis of chl fluorescence images, sun leaves of both ambient and 2×ambient [$O_3$] trees had apparently developed typical necrotic $O_3$ stipples during high $O_3$ episodes in summer, while accelerated senescence was only observed with sun leaves of 2×ambient [$O_3$] trees. This latter effect was indicated along with a faster decrease of photosynthetic quantum yield, but without evidence of changes in non-photochemical quenching. Overall, treatment effects were small and varied among trees. Therefore, compared with ambient [$O_3$], the consequence of the observed $O_3$-induced accelerated leaf senescence for the carbon budget is likely limited.**

Key words: Chlorophyll fluorescence imaging, cumulative ozone uptake, *Fagus sylvatica*, free-air exposure, image analysis, quantum yield of photosystem II, tropospheric ozone.

## Introduction

Tropospheric ozone ($O_3$) is considered an important air pollutant affecting forest trees (Sandermann *et al*., 1997). Among others, effects of $O_3$ on plants include reductions in photosynthesis, visible leaf injury and growth limitation (Matyssek and Sandermann, 2003). An overview of plant responses to $O_3$, in particular, the perception and signalling

* To whom correspondence should be addressed. E-mail: birgit.gielen@ua.ac.be

Abbreviations: Chl, chlorophyll *a*+*b*; *cci*, chlorophyll content index; DEPS, de-epoxidation state of the xanthophyll cycle pigments; $F_o$,$F_m$, minimum and maximum chl fluorescence, respectively; $F_s$, chl fluorescence at steady-state light intensity; $F_v/F_m$, maximum quantum yield of primary photochemistry of photosystem (PS) II=$(F_m-F_o)/F_m$; *PPFD*, photosynthetic photon flux density; PSII, photosystem II; $Q_A$, quinone A; $1-V_J$, the efficiency by which a trapped exciton can move an electron further than $Q_A^-$ into the electron transport chain; $\varphi_{Eo}$, product of $F_v/F_m$ and $(1-V_J)$, corresponding to the probability that an absorbed photon will move an electron into the electron transport chain; $\Phi_{PSII}$, effective quantum yield of electron transport through PSII calculated as $\Phi_{PSII} = (F'_m - F_s)/F'_m$, in which $F_s$ is the steady-state chl fluorescence at a certain *PPFD* and $F'_m$ the chl fluorescence at saturating light for a light-acclimated leaf.

of $O_3$ stress, has been provided by Baier *et al*. (2005) and Kangasjärvi *et al*. (2005).

Because closed chambers have often been used, the impact of increased $O_3$ on forest trees has mostly been restricted to studies on seedlings (Musselman and Hale, 1997). However, physiological differences between juvenile and adult trees (Wieser *et al*., 2003; Herbinger *et al*., 2005), resulting in a different response to $O_3$, have been observed (Matyssek *et al*., 2005; Nunn *et al*., 2005*a*). Scaling $O_3$ responses to mature trees and forests therefore suffers from serious limitations (Kolb and Matyssek, 2001). Progress has been made by the development of free-air exposure techniques of trees and forests under field conditions (Karnosky *et al*., 2001; Werner and Fabian, 2002). The present study was performed at 'Kranzberger Forst' near Freising, Germany where 66-year-old beech trees (*Fagus sylvatica* L.) were exposed to ambient [$O_3$] (= control) or twice-ambient [$O_3$] (2× ambient [$O_3$]) by means of free-air canopy fumigation (Nunn *et al*., 2002; Werner and Fabian, 2002).

Accelerated senescence has been widely reported as one of the harmful effects of $O_3$ on plants, including juvenile trees (Matyssek and Sandermann 2003). This phenomenon was also observed at the free-air $CO_2$+$O_3$ exposure site in northern Wisconsin (Aspen FACE) for aspen, aspen–birch, and aspen–maple stands (Karnosky *et al*., 2005). Autumnal leaf shedding determined at the tree canopy level was also consistently accelerated under 2× ambient [$O_3$] in *F. sylvatica* during the first three years (2000 through 2002) of the experiment at 'Kranzberger Forst' (Nunn *et al*., 2005*b*). Leaf senescence is an organized, genetically controlled process of nitrogen resorption and degradation of chlorophyll, Rubisco and proteins, involving decreasing photosynthesis (Smart, 1994; Noodén *et al*., 1997; Chandlee, 2001). Long-term exposure of *Populus tremuloides* to elevated tropospheric $O_3$ in the Aspen FACE facility caused up-regulation of senescence-associated genes (Gupta *et al*., 2005). A study on *Arabidopsis* showed that $O_3$-induced senescence involves many, although not all, of the genes associated with natural leaf senescence (Miller *et al*., 1999).

Chlorophyll (chl) *a* fluorescence has frequently been used for studying leaf senescence (Jenkins *et al*., 1981; Bukhov, 1997; Rosenthal and Camm, 1997; Šesták and Šiffel, 1997; Lu and Zhang, 1998; Lu *et al*., 2001*a*, *b*). Disturbance of photosynthesis can readily be detected through chl fluorescence as a standard non-invasive tool for the quantification of stress impact on plants, prior even to the onset of visible leaf injury (Lichtenthaler and Miehé, 1997; Buschmann *et al*., 2000; Chaerle and Van Der Straeten, 2001; Chaerle *et al*., 2004). In crop plants, $O_3$ stress has been shown to affect the maximum ($F_v/F_m$) and effective ($\Phi_{PSII}$) quantum yield of PSII photochemistry negatively, to decrease the relative fraction of open PSII reaction centres (photochemical quenching coefficient, $q_P$), and to favour heat dissipation (non-photochemical quenching, *NPQ*; Carrasco-Rodriguez and del Valle-Tascon, 2001; Castagna *et al*., 2001; Calatayud *et al*., 2002*c*). Similar observations have been made in the case of seedlings of several tree species (Grams *et al*., 1999; Shavnin *et al*., 1999; Guidi *et al*., 2001; Ribas *et al*., 2005) although lack of response was reported as well (Maurer *et al*., 1997). Effects have been interpreted as a down-regulation of the linear electron transport to compensate for the $O_3$-induced reduction in the activity of the Calvin–Benson cycle (Reichenauer *et al*., 1997; Guidi *et al*., 2001). During previous years, 2×ambient [$O_3$] had decreased light-saturated $CO_2$ uptake rates of *F. sylvatica* trees of the present study, although results varied between years, and statistically significant effects on $F_v/F_m$ were not observed during summer (Herbinger *et al*., 2005; Nunn *et al*., 2005*b*; Löw *et al*., 2006).

It is hypothesized (i) that 2×ambient [$O_3$] caused accelerated leaf senescence during the sixth year of free-air $O_3$ fumigation in sequence in the 66-year-old *F. sylvatica* trees, (ii) that therefore the decline of $F_v/F_m$ relative to presenescent values was faster in leaves of 2×ambient [$O_3$] than in leaves of control *F. sylvatica* trees, and (iii) that these effects would differ between sun and shade leaves. Because both $O_3$ stress and leaf senescence result in a non-homogeneous distribution of $\Phi_{PSII}$ across the leaf, use was made of chl fluorescence imaging in addition to spot measurements of chl fluorescence to quantify the degree of photosynthetic leaf heterogeneity.

## Materials and methods

### *Experimental site and ozone fumigation*

The study was carried out at the field site 'Kranzberger Forst' near Freising, Germany (48°25′08" N, 11°39′41" E, 485 masl). Throughout six growing seasons (2000–2005), 60-year-old and up to 28 m high beech trees (closed canopy situation) were exposed to ambient [$O_3$] (=control) or twice-ambient [$O_3$] (2×ambient [$O_3$]) levels (five adjacent trees per treatment). The 2×ambient [$O_3$] regime was generated by a free-air canopy $O_3$ exposure system (Nunn *et al*., 2002; Werner and Fabian, 2002). To prevent acute $O_3$ injury, maximum [$O_3$] in the 2×ambient [$O_3$] regime was restricted to 150 nl $l^{-1}$. Hourly $O_3$ levels were monitored using five $O_3$ analysers (TML 8811; Teledyne Monitor Labs, Englewood, USA) at three heights (shade crown at 16 m, sun crown at 20 m, and above canopy at 30 m) under the ambient and 2×ambient [$O_3$] regime. The horizontal gradient was monitored by 120 passive samplers at three heights. From the hourly [$O_3$], cumulative [$O_3$] (SUM0) and AOT40 (accumulated ozone above a threshold of 40 nl $l^{-1}$, Fuhrer and Achermann, 1994) were calculated from day 100 onwards (approximate budbreak). $O_3$ uptake was simulated for the sun and shade crown with the mechanistic Anafore model (Deckmyn *et al*., 2006), which uses the Dewar stomatal model (Dewar, 2002) in combination with Farquhar's photosynthesis model (Farquhar *et al*., 1980) to simulate stomatal opening in response to the environment. The parameterization of the model for the years 2003 and 2004 was used (measured values of $V_{c_{max}}$, maximum rate of carboxylation, and $J_{max}$, ribulose-1,5-diphosphate-limited rate of electron transport, fitted to branch cuvette measurements of

stomatal opening and photosynthesis), as described in Deckmyn *et al*. (2007) and Op De Beeck *et al*. (2007). Measurements were made in September and October 2005; air temperature and global radiation in this period are presented in Fig. 1. Global radiation above the canopy was measured with a pyranometer (type CM 11; Kipp and Zonen, Delft, The Netherlands), and air temperature at 24 m height within the canopy with an aspirated psychrometer (model Assmann; Theiss, Göttingen, Germany). The annual sum of precipitation for 2005 was 821 mm, which can be considered as a normal value compared with previous years (Löw *et al*., 2006). Trees were thus not water-limited during our study. The $O_3$ regime at the site during 2005 up until the end of the measurement period in October is presented in Table 1. Scaffolding provided access to the shade and sun-exposed parts of tree crowns.

### *Assessment of senescent leaf area*

Before the onset of leaf senescence, one branch in the shade and sun crown (at 20 m and 25 m height, respectively) of each of nine (one 2×ambient [$O_3$] tree was not measured) study trees was enveloped by a net and revisited weekly to determine the proportion of senescent leaf area (as derived from the sum of shed and yellow leaves in proportion to total branch foliage). The time-course of senescence within the whole crown was monitored in parallel from the forest floor; findings were consistent at the individual branch and whole-crown level.

### *Pigment concentration*

In September, 20 randomly chosen leaves (10 in shade and 10 in sun crown each at 20 m and 25 m height, respectively) from each of the 10 study trees were labelled and used to quantify leaf relative greenness (chlorophyll content index, *cci*, with a CCM-200, 'Chlorophyll Content Meter', ADC BioScientific Ltd., Herts, UK) once in September (presenescent) and four times during a 3-week period in October. Each leaf was characterized by the mean of four 0.7 $cm^2$ measurements. *cci* was converted to total chlorophyll concentration using a relationship obtained from destructive measurements [chl *a*+*b* (μg $cm^{-2}$)=4.181+1.956 *cci*, $r^2$=0.81, $P$ <0.0001]. 9.9 $cm^2$ fresh leaf area was sampled from four sun leaves (+ additional yellow leaves only used for the *cci*-calibration curve) of each of the 10 trees, frozen in liquid nitrogen and stored at −80 °C until analysis. Leaf samples were extracted in 80% acetone in the presence of washed sea sand and $CaCO_3$ under dim light. The extracts were centrifuged for 5 min at 5000 *g*. A 50 μl volume of the supernatant was subjected to reverse phase HPLC analysis using a set-up comprising a model-616 pump, a model-717+ autosampler, and a model-996 online diode array spectrophotometer (Waters, Milford, MA, USA). A Nova Pak C18, 60A column (length 150 mm, pore size 4 μm) was used for separation. The solvent program was as described in Cardol *et al*. (2003). Acquisition and data treatment were performed using the Millenium software (Waters). Concentrations of individual pigments were determined using authentic references prepared by chromatography on silica gel thin-layer plates or purchased from DHI-Water and Environment (Horstholm, Denmark). The de-epoxidation state of the xanthophyll cycle pigments (DEPS) was calculated from violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) as (0.5A+Z)/(V+A+Z) (Demmig-Adams and Adams III, 1996).

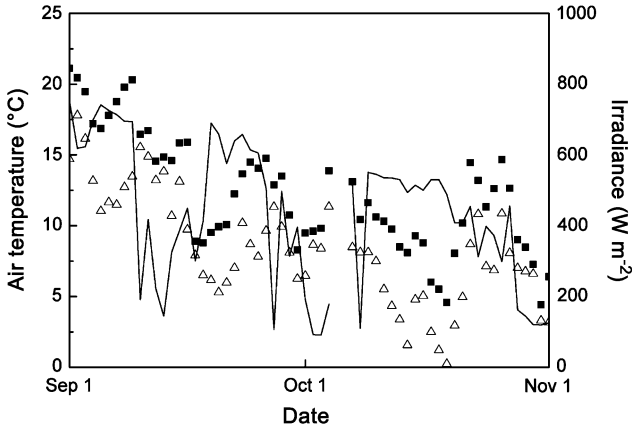


**Fig. 1.** Daily (24 h) mean (squares) and minimum (triangles) air temperature measured at canopy height, 24 m above ground, and daily maximum global radiation (line) for the period September–October 2005 at the research site.

### *Chlorophyll a fluorescence of dark-acclimated leaves*

Chl *a* fluorescence transients of same labelled (see above) dark-acclimated (30 min) shade and sun leaves each were measured *in situ* during early morning and at midday with a Plant Efficiency Analyser (PEA, Hansatech Ltd., King's Lynn, Norfolk, UK) as described earlier (Gielen *et al*., 2005). The following chl fluorescence variables were calculated (Strasser and Strasser, 1995; Strasser *et al*., 2000):

(i) $F_v/F_m$, the maximum quantum yield of primary photochemistry of photosystem (PS) II; $(F_m–F_o)/F_m$;

(ii) $1\text{-}V_J$, the efficiency by which a trapped exciton, having triggered the reduction of quinone A ($Q_A$), can move an electron further than $Q_A^-$ into the electron transport chain ($V_J=(F_{2ms}–F_o)/(F_m–F_o)$);

(iii) $\varphi_{Eo}$, the product of $F_v/F_m$ and $(1–V_J)$, corresponding to the probability that an absorbed photon will move an electron into the electron transport chain.

**Table 1.** *Ozone regimes at the site during the 2005 growing season until the start of October (day 274) and until the end of October (day 305)*

In addition, for October (representing the measurement period during leaf senescence), the mean instant $O_3$ flux is presented for sun and shade leaves. SUM0 is the sum of all $O_3$ concentrations, AOT40 is the accumulated exposure over a threshold of 40 nl $O_3$ $l^{-1}$, CU is cumulative $O_3$ uptake of sun leaves.

| Ozone | Day 274 | | | Day 305 | | | October (mean) |
|---|---|---|---|---|---|---|---|
| | SUM0 (μl $l^{-1}$ h) | AOT40 (μl $l^{-1}$ h) | CU (mmol $m^{-2}$) | SUM0 (μl $l^{-1}$ h) | AOT40 (μl $l^{-1}$ h) | CU (mmol $m^{-2}$) | Instant $O_3$ flux (μmol $m^{-2}$ $d^{-1}$) |
| Ambient [$O_3$] | 142.722 | 16.510 | 15.4 | 154.209 | 16.632 | 16.09 | 23.96 (sun)–3.0 (shade) |
| 2×ambient [$O_3$] | 227.477 | 62.290 | 24.3 | 244.930 | 64.392 | 25.22 | 43.39 (sun)–3.39 (shade) |

### *Chlorophyll* a *fluorescence of light-acclimated leaves*

The quantum yield of electron transport through PSII was calculated as $\Phi_{PSII} = (F'_m - F_s)/F'_m$ (Genty *et al*., 1989), where $F_s$ is the steady-state chl fluorescence at a given photosynthetic photon flux density (*PPFD*) and $F'_m$ represents chl fluorescence at a saturating flash of light of a light-acclimated leaf. $\Phi_{PSII}$ was measured with a MINI-PAM (Heinz-Walz, Effeltrich, Germany) at ambient *PPFD* on four sun leaves of each of six experimental trees with clear sun crown (three from ambient, three from 2×ambient [$O_3$]), at eight regular time intervals between 11.00 h and 17.00 h. These data were pooled together with midday measurements of another set of leaves of the same trees made on two additional days.

### *Chlorophyll* a *fluorescence images*

From 8 October to 19 October, images of chl fluorescence at steady-state light intensity ($F_s$) were made during the midday hours of eight days on three to five sun leaves of six trees with clear sun crown (three from ambient, three from 2×ambient [$O_3$]), with a prototype portable chl fluorescence imaging system (FIS). The FIS prototype, developed at the laboratory of Molecular and Physical Plant Physiology (Hasselt University, Belgium) in collaboration with Maastricht Instruments consists of an excitation unit, a detection unit, and a control unit. The imaging unit is composed of a monochrome CCD camera module. Measurements were performed without preceding dark adaptation. For further details, see Gielen *et al*. (2005, 2006). Each leaf was destructively sampled, immediately fitted into the leaf clip of the system and the $F_s$-image was excited at a *PPFD* similar to environmental conditions, i.e. 250 μmol $m^{-2}$ $s^{-1}$ or 900 μmol $m^{-2}$ $s^{-1}$ in case of cloudy or sunny conditions, respectively. Image processing was performed with Matlab 7 using the Matlab Image Processing Toolbox (The MathWorks, Inc., Natick, USA) and common texture analysis techniques; the method is fully detailed in Gielen *et al*. (2006). Homogeneity, inertia, entropy, and energy are frequently used texture features initially proposed by Haralick *et al*. (1973), and homogeneity of the low-pass filtered version of the $F_s$-images was used in this study. On a 0 to 1 scale, images with low values are less homogeneous while an index of 1 corresponds to an image where all pixels have the same intensity. In addition, the number of pixels that had smaller values than the mean pixel intensity minus the standard deviation (or twice the standard deviation depending on the range of pixel intensities) of all pixel intensities within a leaf was calculated. By this, pixels belonging to zones in the leaf with rather low chl fluorescence intensities were covered. Such zones will be referred to as 'injured leaf area', which is largely determined by necrotic zones caused by $O_3$ stress ('stipples'). Preceding analysis, masking of the major veins was performed (Gielen *et al*., 2006).

### *Statistical analysis*

To test for the effects of [$O_3$], time and their interaction, analysis of variance and repeated measures ANOVA were performed with SAS (version 8.2, SAS Institute Inc., Cary, NC, USA) using the mixed procedure (Littell *et al*., 1996). In case of a significant time×$O_3$ interaction, *a posteriori* treatment comparison of means was performed with Bonferroni corrections for multiple comparisons. Tree was the unit of replication.

## Results

### *Senescent leaf area*

During the period of incipient leaf fall, leaf yellowing and shedding tended to be accelerated in the sun crowns under 2×ambient [$O_3$] (Fig. 2A). A similar trend was present in shade crowns during the entire period of leaf fall (Fig. 2B). However, only the time effect was significant (*P*-time <0.0001). Given the scatter in response amongst trees, statistical analysis did not yield significant $O_3$ effects in sun and shade crowns.

### *Leaf pigment concentrations*

Non-destructive assessments of chl with the CCM-200 were consistent with visual assessments of senescent leaf area at the branch level with only a significant *P*-time effect despite indication of accelerated leaf discoloration under 2×ambient [$O_3$] conditions (Fig. 2A, B). In the sun crown, the $O_3$ effect was not statistically significant because one 2×ambient [$O_3$] tree had dark-green leaves although leaves had millimetre-sized necrotic stipples in their laminas. In the shade crown of another tree leaves stayed green on the sampled branch for a longer time than on neighbouring branches with leaves of advanced visible senescence, underlining natural within and between-tree variability.

Total chl of sun leaves destructively sampled on 7 October was smaller under 2×ambient [$O_3$] than ambient [$O_3$] (Table 2; $P$=0.095), in the absence of differences in

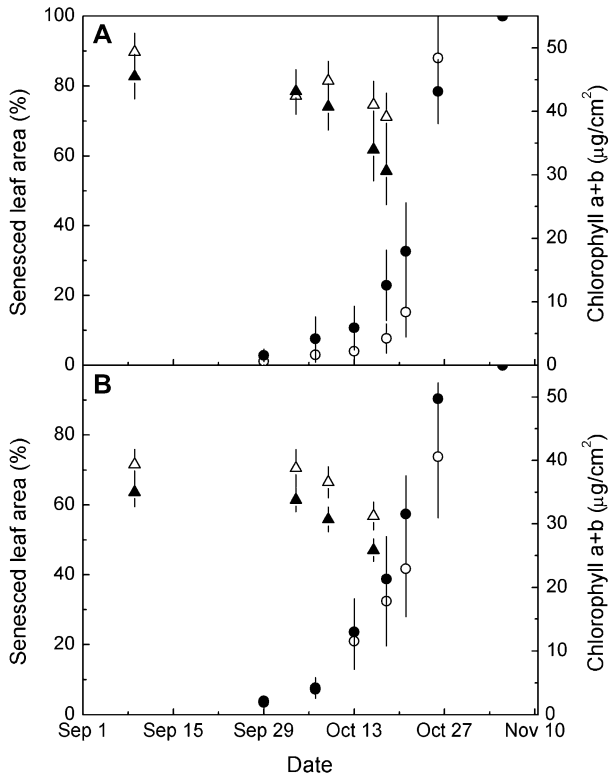


**Fig. 2.** Proportion of senesced (yellow and shed leaves) leaf area (circles) and total chlorophyll concentrations (triangles) of sun (A) and shade (B) canopy leaves of *Fagus sylvatica* trees exposed to ambient (open symbols) and 2×ambient (closed symbols) [$O_3$]. The mean (SE) of four/five trees per treatment is presented for autumn of the sixth year of free-air $O_3$ fumigation.

**Table 2.** *Chlorophyll* a+b *concentration and other leaf pigments of sun leaves of* Fagus sylvatica *trees exposed to ambient and 2×ambient [$O_3$]*

The mean (SE) of five trees per treatment is presented for autumn of the sixth year of free-air $O_3$ fumigation. DEPS, de-epoxidation status (see text for details). Xanthophyll represents the sum of violaxanthin, antheraxanthin, and zeaxanthin.

| | Ambient [$O_3$] | 2×ambient [$O_3$] |
|---|---|---|
| Chlorophyll *a+b* (μg $cm^{-2}$) | 48.57 (3.18) | 40.80 (2.65) |
| Chlorophyll *a/b* | 2.89 (0.11) | 2.97 (0.12) |
| Violaxanthin (mg $g^{-1}$ chl) | 25.74 (2.11) | 31.47 (2.65) |
| Antheraxanthin (mg $g^{-1}$ chl) | 4.93 (0.85) | 6.08 (2.23) |
| Zeaxanthin (mg $g^{-1}$ chl) | 5.90 (1.72) | 6.24 (2.33) |
| Lutein (mg $g^{-1}$ chl) | 93.41 (1.43) | 99.07 (3.99) |
| Neoxanthin (mg $g^{-1}$ chl) | 28.45 (0.42) | 28.92 (1.11) |
| Xanthophyll (mg $g^{-1}$ chl) | 36.56 (0.72) | 43.80 (4.42) |
| DEPS | 0.22 (0.05) | 0.18 (0.06) |

chl *a/b*, or in concentrations of other pigments relative to chl (Table 2) or expressed per unit leaf area (data not shown). The DEPS was not affected by 2×ambient [$O_3$].

### *Chlorophyll* a *fluorescence of dark-acclimated leaves*

Comparing, in mid-October, $F_v/F_m$ of sun leaves with that of early September indicated a distinct decrease (Fig. 3). Significant regressions were found under ambient [$O_3$] ($r^2$=0.156) and 2×ambient [$O_3$] ($r^2$=0.372). An analysis of covariance showed that regressions significantly differed from each other (*P*-lines=0.0036). The mean (±SE) relative decrease of $F_v/F_m$ between September and mid-October was 13.8±1.6% at ambient and 18.2±2.6% at 2×ambient [$O_3$]. The *P*-$O_3$ was 0.1813 including all trees, and 0.0095 excluding two trees as indicated in Fig. 4 (see legend and below). In shade leaves, the difference between October and September was −5.0±1.2% under ambient and −7.6±1.9% under 2×ambient [$O_3$] conditions; the effect was not significant (data not shown). The effect on $F_v/F_m$ in the sun crown was mainly due to a faster decrease of $F_m$ under 2×ambient [$O_3$] than ambient [$O_3$]. The decrease of $F_v/F_m$ between September and October [(Oct–Sep)/Sep] and between morning and midday [(pm–am)/am] measurements is demonstrated in Fig. 4 for individual trees. Except for two trees (Fig. 4), in sun leaves, midday levels of $F_v/F_m$ were typically lower compared with morning levels because of photoinhibition in the afternoon. Differences between midday and morning measurements therefore indicated two 2×ambient [$O_3$] trees of which leaves of the upper crown part had shade leaf characteristics, which apparently had resulted from an intense competition for light in the crowded stand canopy with the neighbouring trees. Therefore, subsequent measurements of chl fluorescence under light-acclimated conditions were restricted only to the trees with typical sun foliage. Consequently, measurements with the MINI-PAM and with the FIS were made in six out of the 10 trees. Figure 5 illustrates the decline of $\varphi_{Eo}$ through time with

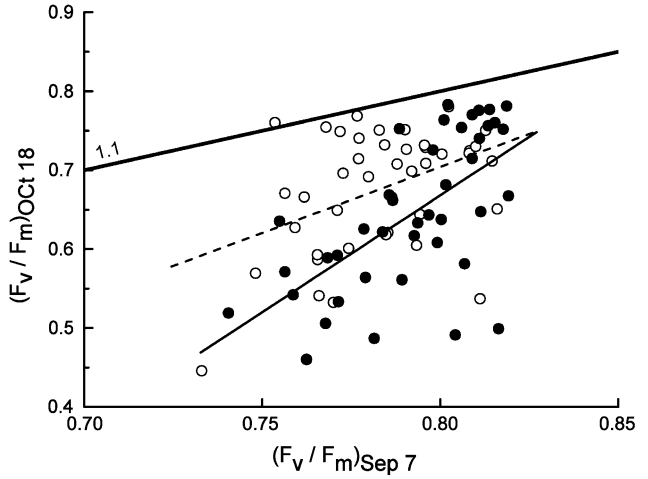


**Fig. 3.** Maximum quantum yield of primary photochemistry of photosystem II ($F_v/F_m$) for sun leaves of *Fagus sylvatica* trees exposed to ambient (open symbols) and 2×ambient (closed symbols) [$O_3$]. Values of mid-October are plotted versus presenescent values of September for several leaves of five trees per treatment. The 1:1 line is indicated as well as are regression lines for ambient (dashed line) and 2×ambient (full line) [$O_3$].

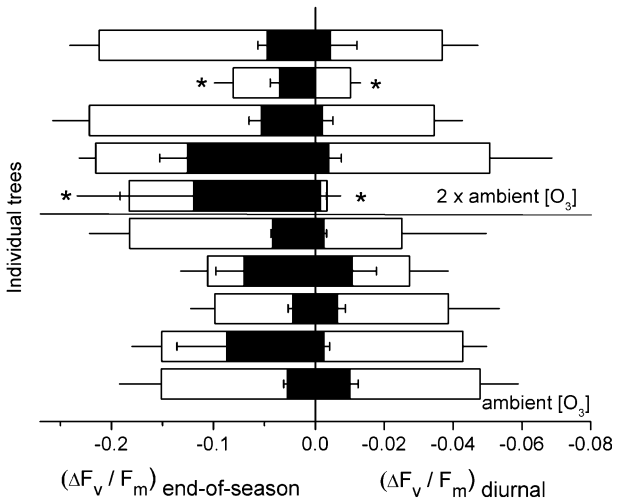


**Fig. 4.** Tree variability of the relative decrease in the maximum quantum yield of primary photochemistry of photosystem II ($\Delta F_v/F_m$) through time (left, senescent versus presenescent) and throughout the day (right, midday versus morning) for sun (open bars) and shade (black bars) canopy leaves of *Fagus sylvatica* trees exposed to ambient and 2×ambient [$O_3$]. Two trees of which the top-canopy-leaves had shade-leaf characteristics are indicated by an asterisk.

levels that were up to 20% lower under 2×ambient [$O_3$] compared with ambient [$O_3$]. In mid-October, $\varphi_{Eo}$ of sun leaves was 28.2±4.7% and 45.8±4.0% lower under ambient [$O_3$] and 2×ambient [$O_3$], respectively, than during early September. This effect was significant (*P*=0.021) including all trees. $\varphi_{Eo}$ being the product of $F_v/F_m$ and $(1–V_J)$ decreased along with $(1–V_J)$, which declined more distinctly through time under 2×ambient [$O_3$] than in ambient [$O_3$] (*P*=0.045). In the shade crown,

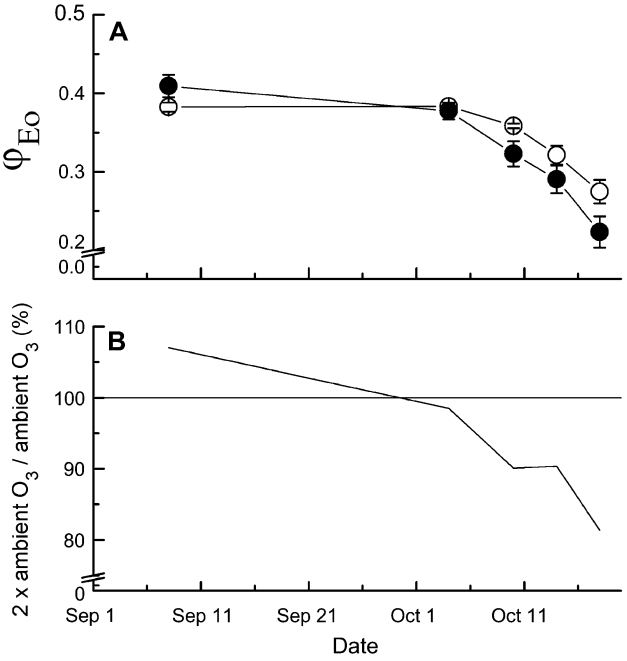


**Fig. 5.** Probability of absorbed photons to move electrons into the electron transport chain ($\varphi_{Eo}$) of sun leaves in *Fagus sylvatica* trees exposed to ambient (open symbols) and 2×ambient (closed symbols) [$O_3$]. The mean (SE) of five trees per treatment is presented for autumn of the sixth year of free-air $O_3$ fumigation. (A) Absolute values; (B) corresponding $O_3$ effect expressed as 2×ambient [$O_3$]/ambient [$O_3$].

$\varphi_{Eo}$ decreased by 29.7±2.3% and 38.1±3.5% between the first and last measurement ($P$=0.078) under ambient [$O_3$] and 2×ambient [$O_3$], respectively.

### *Chlorophyll* a *fluorescence of light-acclimated leaves*

In the *PPFD* range below 500 µmol $m^{-2}$ $s^{-1}$, $\Phi_{PSII}$ was consistently lower under 2×ambient [$O_3$] than ambient [$O_3$] (Fig. 6). Analysis of covariance demonstrated significant $O_3$ effects ($P$-$O_3$ <0.0001, $P$-*PPFD*×$O_3$= 0.0068).

### *Chlorophyll* a *fluorescence images*

Between 0% and 20% of the measured leaf area showed lowered levels of chl fluorescence as a consequence of $O_3$ impact (Fig. 7). The extent of $O_3$-injured leaf area was highly variable between leaves of the same tree in the absence of differences between ambient [$O_3$] and 2×ambient [$O_3$] trees ($P$=0.196, Fig. 7). The number of pixels characterizing injury in the leaves was negatively related (coefficient of determination $r^2$=0.259) to a measure of image homogeneity (Fig. 8A). This relationship was not different between $O_3$ treatments as neither the injured area, nor the homogeneity or other measures of leaf heterogeneity of the chl fluorescence images were affected by [$O_3$]. Chl fluorescence image homogeneity was only poorly related to chl and the relationship was neither significant at ambient [$O_3$] nor at 2×ambient [$O_3$]

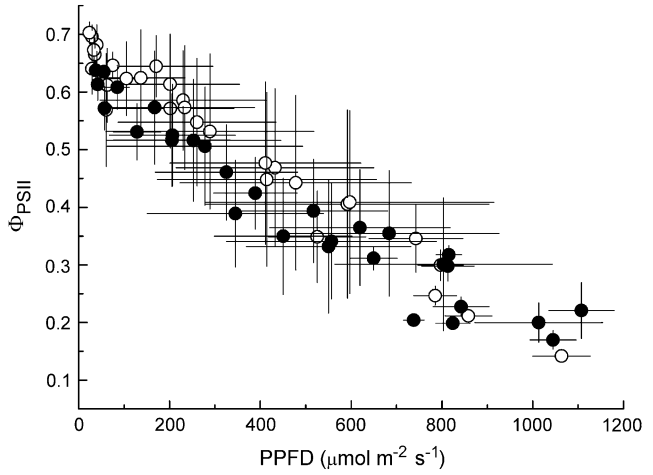


**Fig. 6.** Quantum yield of electron transport through photosystem II ($\Phi_{PSII}$) versus incident photosynthetic photon flux density (*PPFD*) of sun leaves of six *Fagus sylvatica* trees exposed to ambient (open symbols) or 2×ambient (closed symbols) [$O_3$]. The mean (SE) of at least four leaves is presented as combination of tree ($n$=3 per treatment)×time of day for autumn of the sixth year of free-air $O_3$ fumigation.

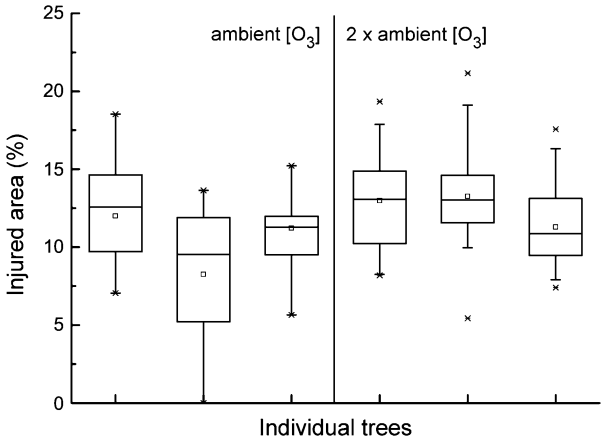


**Fig. 7.** Custom box and whisker plot of the proportion of pixels indicating injury within chlorophyll fluorescence images at steady-state light intensities of sun leaves of *Fagus sylvatica* trees exposed to ambient and 2×ambient [$O_3$]. The horizontal lines in the box denote the 25th, 50th and 75th percentile values. The error bars denote the 5th and 95th percentile values. The two symbols below the 5th percentile error bar denote the 0th and 1st percentile values. The two symbols above the 95th percentile error bar denote the 99th and 100th percentiles. The square symbol in the box denotes the mean.

(Fig. 8B). Leaves of both treatments were within similar ranges of homogeneity, although leaves of 2×ambient [$O_3$] were mostly concentrated within the lower part of the range of chl levels.

## Discussion

Free-air $O_3$ fumigation within a stand of adult *F. sylvatica* trees at 'Kranzberger Forst' appeared to favour accelerated

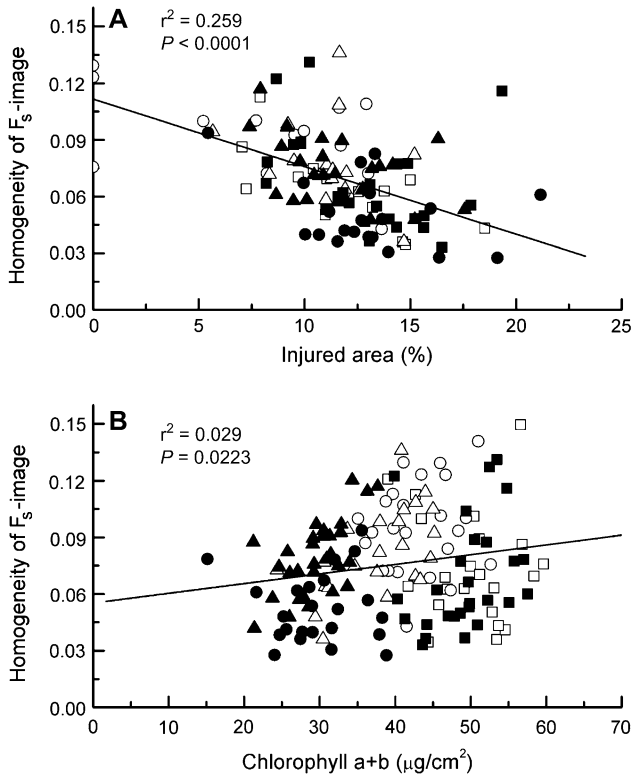


**Fig. 8.** Measure of homogeneity of chlorophyll fluorescence images at steady-state light intensities plotted versus the proportion of pixels indicating injury in such images (A) and plotted versus the total chlorophyll concentrations in these leaves (B) for the sun-exposed canopy of *Fagus sylvatica* trees exposed to ambient (open symbols) and 2×ambient (closed symbols) [$O_3$]. The $r^2$-values and *P*-values of the regression lines are indicated. Different symbols represent individual trees.

leaf fall (Fig. 2) during the autumn of 2005. This trend obtained from monitoring leaf fall and yellowing at the branch level agreed with independent and frequent assessments of the *cci* on another set of leaves of the same trees. The effect was, however, not statistically significant. Also previous years' leaf fall data from the same experiment consistently showed accelerated senescence, although not always significant (Nunn *et al*., 2005*b*; Matyssek *et al*., 2007). Yet, in agreement with small-scale studies in growth chambers, accelerated senescence as a consequence of chronic $O_3$ exposure has been reported previously for field-grown aspen, aspen-birch, and aspen-maple in a free-air fumigation experiment (Karnosky *et al*., 2005). There are several possible reasons for the lack of a significant effect of the 2×ambient [$O_3$] treatment on leaf senescence in this study. First, beech trees are not as sensitive to elevated $O_3$ levels compared with, for example, fast growing pioneer species. Bussotti *et al*. (2005) found that beech was among the least $O_3$-sensitive of five woody species. This may partly explain why the effect of the 2×ambient [$O_3$] treatment on leaf senescence was rather small. Second, given the limited number of replicates, inherent to this type of field studies, such a small effect is difficult to prove statistically. This is problematic for studying leaf senescence, which is prone to within- and between-tree variability. Micrometeorological conditions, competition between neighbouring trees and source–sink relationships between branches within the same tree crown contribute to this variability. Nevertheless, because the 10 study trees were representative for 'Kranzberger Forst' (Reiter *et al*., 2005), it is not very likely that the effect would have been statistically significant for a larger number of trees. Third, the shade leaves of the 2×ambient [$O_3$] treatment received less light (due to the larger trees in this treatment), resulting in a lower simulated stomatal conductance and therefore a lower ozone influx (Deckmyn *et al*., 2007). In this study, effects of 2×ambient [$O_3$] were less evident in shade leaves compared with sun leaves (see below). The variable proportion of shade versus sun leaves in adult and juvenile trees may be one of the reasons for differences in effects of elevated [$O_3$] on leaf senescence between this study and previous studies. Fourth, a complicating factor is, in addition, frequently high ambient [$O_3$] in summer which can induce $O_3$ injury to the ambient [$O_3$] trees, regarded as 'control' in this study. Seasonal $O_3$ exposure and cumulative $O_3$ uptake were high for both $O_3$ regimes (Table 1), and this is typical for the site. Control trees indeed showed stipples on their leaves resembling $O_3$ injury (Innes *et al*., 2001; Vollenweider and Günthardt-Goerg, 2005). In *Fagus sylvatica*, stipples, indicating the presence of necrotic areas, are associated with the localized degeneration of the cell contents (oxidative burst) (Vollenweider *et al*., 2003; Bussotti *et al*., 2005). Hence, $O_3$ treatment effects on the extent of injured leaf area were vague, and consistent with no $O_3$ treatment effect on chl fluorescence image homogeneity of $F_s$. Intuitively, one would expect lower homogeneity of chl fluorescence (indicating spatially variable photosynthetic yield) to occur within leaves of 2×ambient [$O_3$] because of necrotic stipples. As leaves from ambient [$O_3$] trees also developed these stipples, homogeneity was unaffected by the $O_3$ treatment. Measures of chl fluorescence image homogeneity were vaguely related to chl, although senescence in some parts of the leaf would also result in lower image homogeneity. Given the absence of differences in the range of homogeneity between $O_3$ treatments, Fig. 8 reveals that leaves of ambient [$O_3$] trees have higher chl levels than leaves of 2×ambient [$O_3$]. Therefore, accelerated leaf fall and yellowing were at least partly independent of $O_3$ stipples. One may conclude that ambient [$O_3$] trees, like 2× ambient [$O_3$] trees, may have developed $O_3$ stipples during high $O_3$ episodes in the summer, whereas autumn $O_3$ concentrations appeared to accelerate leaf yellowing only in the 2×ambient [$O_3$] trees. Because the stress response in plants is determined by the actual $O_3$ uptake through leaf stomata rather than by exposure (Matyssek *et al*., 2004) $O_3$ uptake was simulated with the mechanistic Anafore

model (Deckmyn *et al*., 2006). Average instant $O_3$ flux was 80% higher in sun leaves under 2×ambient [$O_3$] than ambient [$O_3$] during October and the AOT40 increased in October with 2.102 μl $l^{-1}$ h under 2×ambient [$O_3$] while only with 0.122 μl $l^{-1}$ h under ambient [$O_3$] (Table 1). Whether high summer ambient [$O_3$] have influenced the timing of senescence in the ambient [$O_3$] trees can not be tested in the field.

Progress is being made to improve our understanding of the biochemical and molecular processes underlying $O_3$-induced accelerated leaf senescence. Early studies of gene expression have indicated that $O_3$ elicits some of the same signals involved in natural senescence (Miller *et al*., 1999). Perception of ozone or reactive oxygen species from its degradation in the apoplast activates several signal transduction pathways, involving the plant hormones ethylene, abscisic acid, salicylic acid, and jasmonic acid, that regulate the responses of the cells to the increased oxidative load (see Kangasjärvi *et al*., 2005, for a recent review). Leaf injury and accelerated senescence of beech trees under 2×ambient [$O_3$] have indeed been linked to enhanced ethylene production (Nunn *et al*., 2005*b*).

Leaf fall and yellowing are at the cellular level accompanied by the dismantling of the photosynthetic apparatus so that chl fluorescence was analysed in this study to assess the status of the photosynthetic apparatus. A decrease of $F_v/F_m$ compared to the presenescent values of September is therefore an indication of leaf senescence. Both in the shade and sun crown, $F_v/F_m$ decreased during the autumn, however, the timing was not different between $O_3$ treatments in the shade crown. In the sun crown, $F_v/F_m$ decreased more clearly under 2×ambient [$O_3$] than ambient [$O_3$], mainly resulting from a more rapid decrease in $F_m$, indicating injury to PSII (Powles and Björkman, 1978; Kellomäki and Wang, 1997). Because ($1–V_J$) decreased more rapidly under 2×ambient [$O_3$] than ambient [$O_3$], the decrease in energy flow through PSII ($\varphi_{Eo}$) between September and mid-October was significantly larger at 2×ambient [$O_3$] (−46%) than ambient [$O_3$] (−28%; across all 10 trees in the analysis). This indicates an impairment of the electron flow after reduction of $Q_A^-$ in sun leaves under 2×ambient [$O_3$]. Thus, the trend of $O_3$-induced accelerated senescence observed by measuring leaf yellowing and leaf fall was accompanied by a significantly promoted decrease of photosynthetic efficiency. Apart from leaf senescence, also $O_3$ stress would impair $F_v/F_m$. Yet measurements of $F_v/F_m$ in September of 2005 (presenescent) had not revealed differences between the $O_3$ regimes, nor had measurements during the previous summers (Nunn *et al*., 2005*b*). Consequently, the present results reflect accelerated autumnal senescence under 2×ambient [$O_3$] rather than ambient $O_3$ stress. Findings were similar in the shade crown, although they were not statistically significant. In general, the response of shade crowns was less evident and $O_3$ stipples on shade leaves were not observed. This may be explained by the lower ozone influx (Table 1). Previously, Nunn *et al*. (2005*a*) and Vollenweider *et al*. (2003) suggested ozone-induced leaf injury to be enhanced under high light. It is as yet unclear whether shade protects leaves from ozone damage, since besides reducing the ozone flux, carbon available for repair and/or defence is also lower in shade leaves (Deckmyn *et al*., 2007). In fact, shade leaves have been reported to be $O_3$-sensitive because of light-limited defence and repair (Kolb and Matyssek, 2001; Matyssek and Sandermann, 2003). As a consequence of the changes in $F_v/F_m$ in the sun crown, $\Phi_{PSII}$ was lower at 2×ambient [$O_3$] than ambient [$O_3$], indicating lower electron transport rates at moderate light intensities (Fig. 6). An increase in *NPQ* due to $O_3$-stress was reported along with decreased $F_v/F_m$ (Soldatini *et al*., 1998; Grams *et al*., 1999; Shavnin *et al*., 1999; Guidi *et al*., 2001). However, Calatayud *et al*. (2002*a*, *b*, *d*) observed a decrease of *NPQ* in crop species, possibly due to damage in thylakoid membranes and lower rates of linear electron transport ($\Phi_{PSII}$) as a consequence of oxidative stress. Non-photochemical quenching is the feed-back regulatory mechanism by which photons absorbed in excess can be harmlessly dissipated as heat in the antenna complexes of PSII (Niyogi, 2000; Horton *et al*., 2005). This mechanism is correlated with the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin via the xanthophyll cycle (Demmig-Adams, 1990). Because the DEPS was not significantly affected by $O_3$ in this study, differences in *NPQ* are unlikely.

In conclusion, 2×ambient [$O_3$] promoted the decrease in photosynthetic efficiency, during the period of incipient leaf fall (hypothesis 2 accepted), at least in the sun crown of *F. sylvatica* trees. As leaves were still photosynthetically active in October (Löw *et al*., 2006), it could be concluded that this effect is relevant to the carbon budget of trees. However, although 2×ambient [$O_3$] favoured accelerated leaf fall, hypothesis 1 was not statistically accepted because of natural variability between trees. Moreover, the response of shade leaves, which are proportionally more important in large trees, was even smaller than that of sun leaves (hypothesis 3 accepted). Given the small differences observed between ambient [$O_3$] and 2×ambient [$O_3$] treatments, combined with high ambient [$O_3$], it is concluded that the accelerated senescence effect of 2×ambient [$O_3$] on the carbon budget of adult *F. sylvatica* trees is limited, but this should be investigated further.

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