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Responses to ozone on *Populus* "Oxford" clone in an open top chamber experiment assessed before sunrise and in full sunlight

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Abstract

The effects of ambient levels of ozone and summer drought were assessed on a poplar clone (*Populus maximowiczii* Henry X *P.* × *berolinensis* Dippel – Oxford clone) in an open top chamber experiment carried out at the Curno facilities (Northern Italy). Chlorophyll (Chl) *a* fluorescence parameters (from both modulated and direct fluorescence) were assessed at different hours of the day (predawn, morning, midday, afternoon, and evening), from June to August 2008. This paper compares the results from predawn (PD, before sunrise) and afternoon (AN, in full sunlight) measurements, in order to evaluate the role of high sunlight as a factor influencing responses to ozone stress. Sunlight affected the maximum quantum yield of primary photochemistry (decrease of F_v/F_m) thus indicating photoinhibition. The effective quantum yield (Φ_{PSII}) and the photochemical quenching (q_P) were enhanced in the afternoon with respect to the predawn, whereas the nonphotochemical quenching (NPQ) was reduced. The effect of ozone was detected with fluorescence on well watered plants in the first week of July, before the onset of visible symptoms. As far as F_v/F_m are concerned, the differences between ozone-treated and control plants were statistically significant in the predawn, but not in the afternoon. Ozone exerted only minor effects on drought exposed plants because of the reduced stomatal ozone uptake, but effects on the IP phase of the fluorescence transient were observed also in drought-stressed plants.

Additional key words: fluorescence transient; JIP-test; leaf injury; linear electron transport; modulated fluorescence; drought stress; photoinhibition.

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Abbreviations: ABS – absorption energy flux; AN – afternoon; AOT40 – accumulated ozone above the threshold of 40 ppb; CF – charcoal filtered chambers; D – nonwatered plants (dry); DM – dry mass; ET – energy flux for electron; F_0 – minimal fluorescence of the dark-adapted state; F_0' – minimal fluorescence of the light-adapted state; F_m – maximal fluorescence of the dark-adapted state; F_m' – maximal fluorescence of the light-adapted state; FM – fresh mass; F_v – total variable fluorescence ($F_m - F_0$); F_v/F_m (= φ_{Po}) – maximum quantum yield of primary photochemistry in the dark-adapted state; F_v'/F_m' – PSII maximum efficiency in the light-adapted state; g_s – stomatal conductance to water vapour; IP phase - [$\Delta V_{IP} = 1 - V_I$] – indicates the amplitude of the IP phase, *i.e.* the efficiency of electron transport around the PSI to reduce the final acceptors of the electron transport chain; J step – [$\Psi_{Eo} = 1 - V_J$], expresses the efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors; K band – relative variable fluorescence at 300 μs; L band – relative variable fluorescence at 100 μs; NF – not filtered chambers; NPQ – nonphotochemical quenching; OEC – oxygen-evolving complex; OTC – open top chamber; PAR – photosynthetically active radiation; PD – predawn; PI_{tot} – Performance Index total, *i.e.* the performance index for energy conservation from photons absorbed by PSII to the reduction flux of PSI end acceptors; P_N – net photosynthetic rate; PSI – photosystem I; PSII – photosystem II; q_P – photochemical quenching; R – rainfall; RC – reaction center; RE – energy flux for the reduction of end acceptors; RH – relative humidity; RWC – relative water content; SM – satured fresh mass; T – temperature; TR – trapping capacity; V_t – variable fluorescence at time t; W – well watered plants; Φ_{PSII} – actual quantum yield of PSII, or PSII operating efficiency; Ψ_w – water potential.

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Introduction

Open top chambers (OTCs) are widely used in studies addressing the physiological effects of air pollutants (namely ozone stress) on tree species (Novak *et al.* 2005, Gerosa *et al.* 2009, Calatayud *et al.* 2011). An advantage is that OTCs only partially modify the surrounding environment (chamber effect, *see* Clark *et al.* 2000), and plants are subjected to fluctuating ecological factors such as high sunlight radiations. High light is a very powerful factor influencing the whole-plant physiology (Bruce and Vasil'ev 2004), and it is able to modify plant response to ozone itself (Topa *et al.* 2001, Wei *et al.* 2004, Cascio *et al.* 2010). An exposure to high light, in fact, produces a photoinhibition, *i.e.* the deactivation of D1 protein within photosystem II (PSII) (Ohira *et al.* 2004). The photoinhibition can be interpreted as an injury (photodamage) or as a downregulation mechanism reducing electron flow when excessive light excitation cannot be dispelled photochemically. The photoinhibition also affects the oxygen evolving complex (OEC) activity (Takahashi and Murata 2008, Takahashi and Badger 2011). Recent research suggests that light absorption of the manganese cluster of OEC plays a crucial role in the photoinhibition (Tyystjärvi 2008). According to this hypothesis excitation of the oxygen evolving Mn cluster triggers a disturbance of electron transfer from the Mn cluster to P_{680}^{+}. The inactivation of OEC is associated with the release of a Mn ion from PSII to the lumen of thylakoids. The photoinhibition status of a leaf can be determined by Chl *a* fluorescence tests, and it is quantified based on the reduction of maximum quantum yield of primary photochemistry in a dark-adapted sample (φ_{Po}). This parameter expresses the probability that an electron captured by the PSII antenna will be transferred to the reaction centre (TR_0/ABS, *i.e.* trapping capacity compared to absorption), and it is expressed through the well known formula:

$$TR_0/ABS = \varphi_{Po} = [F_m - F_0]/F_m = F_v/F_m$$

In dark-adapted samples, F_0 expresses loss of energy through fluorescence occurring in the antenna, before the photon reaches the reaction centre; whereas F_m represents the maximum fluorescence intensity when all reaction centres are closed. F_v is the total variable fluorescence. The diurnal pattern of F_v/F_m with minimum values during the central hours of the day and maximum before sunrise is a well known phenomenon (Adams and Demmig-Adams 2004). The changes between predawn and midday indicate diurnal (circadian) acclimation to the continuously changing environment (Desotgiu *et al.* in press).

One of the first events of ozone stress in plants concerns the reduction of photosynthesis rates, as consequence of the inactivation of the Rubisco (Dann and Pell 1989, Fontaine *et al*. 2003, Guidi *et al*. 2009) and the suppression of the Calvin-Benson cycle. The interaction between the high light (HL) and ozone stress is controversial. It is commonly assumed that shade leaves have a higher response to ozone as compared to light leaves (Topa *et al.* 2001, Wei *et al.* 2004). This behaviour has been explained with a greater reduction of the net photosynthesis (P_N) with respect to the stomatal conductance to water vapour (g_s) (Fredericksen *et al.* 1996). Foliar visible ozone symptoms, however, are generally lower in the inner parts of the crown and in shaded leaves (Novak *et al.* 2008, Gielen *et al.* 2007). Davison *et al*. (2003) consider sunlight an essential factor in triggering the pathway of anthocyanin synthesis, enhancing the related symptomatology. Guidi and Degl'Innocenti (2008) report that ozone enhances the photoinhibition processes induced by the high light, so amplifying the overall response.

The effect of ozone is influenced also by the water availability. Ozone exerts its harmful action primarily in well watered conditions. Plants subjected to a water shortage reduce their stomatal conductance and absorption of this pollutant, thus delaying the effects of ozone. An antagonistic effect between drought and ozone was described in the'90 (Pääkkönen *et al*. 1998) and confirmed in many papers (Matyssek *et al*. 2006).

Our hypothesis is that ozone contributes to the photoinhibition in the central hours of the day. Moreover, fluorescence parameters related to the linear electron transport (from the OEC to the reduction of the final acceptors at the PSI side) are enhanced by high light (Desotgiu *et al.*, in press), but depressed by the ozone treatment (Bussotti *et al.* 2011). Consequently all the differences (related to the photoinhibition and electron transport) between ozone-treated and control plants should be increased if the measurement of Chl *a* fluorescence is made during the central hours of the day (main hypothesis). Another specific hypothesis to be tested is that differences are not detectable in plants under water stress, which avoid the uptake of ozone by mean of the stomatal closure.

Materials and methods

Experimental setup: The experiment was conducted at the open top chambers (OTC) facilities at Curno (C.R.IN.ES., Centre of Research on Effects of Pollutants on Ecosystems, Lombardy, North Italy, 45°41' N, 9°37' E, elevation 245 m a.s.l), where the ozone levels are well above the UN-ECE thresholds (Gerosa *et al.* 2009). The experimental setup consisted of 6 open top chambers, 3 of which were flushed with charcoal filtered (CF) and 3 with ambient, nonfiltered air (NF). The OTCs were constructed according to Heagle *et al*. (1973). Each CF

chamber was equipped with 12 filters (1 × 1 m), containing 2.5 kg of powdered activated carbon (*Comlet, Cinisello Balsamo*, Milano, Italy). Ozone concentrations within each chamber, and outside, were continuously monitored with a *Dasibi 1108 RS* automatic analyzer (*Cologno Monzese*, Italy), *via* a solenoid valve switching system, which draws air from sampling points in the centre of each plot at a height of 90 cm. The environmental concentration of ozone (outside the chambers) was reduced minus 50% in the CF OTCcs, and minus 5% in the NF OTCs. Monthly mean ozone concentrations (under open field conditions) in the period June–August 2008 ranged from 38 (June) to 49 (July) ppb (parts per billion, 10^{-9}). The hourly maximum concentrations ranged from 117 (August) to 129 (July) ppb. The overall exposure to ozone in the period May–August 2008 was, in terms of AOT40 (accumulated ozone above the threshold of 40 ppb), 15,900 ppb h in the NF chambers and 1,300 in the CF ones (ppb h is the sum of the hourly values over the threshold of 40 ppb).

Plants: *Populus maximowiczii* Henry X *P.* × *berolinensis* Dippel – (Oxford clone, *see* Schreiner and Stout 1934) was used for this study. This clone has already been used in previous experiments due to its demonstrated ozone sensitivity (Marzuoli *et al.* 2009). The plants used were reproduced through cuttings. In early April, the cuttings about 20 cm long were placed in 5-l pots containing a commercial soil substrate, expanded clay and peat, in the ratio 3:1:0.5 (v/v). The plants were placed in the OTCs at about 8 weeks (early June). 12 plants were placed in each plot; of these, 6 were subjected to repeated (nondestructive) measurements during the experiment, while the remaining 6 were used for the (destructive) collection and testing of samples between the measurement dates. A sprinkler system was set up in each OTC, providing every plant with 150 ml of water a day. A subsample of 6 plants in each OTC was administered a further supply of water through a drip irrigation system, for a total of 750 ml per day. Therefore, within each OTC there were two irrigation regimes: W (well watered, full irrigation, sprinkler + drip) and D (dry, sprinkler only). Sampling was done during summer 2008 on the following dates: Date 1: 10 June; Date 2: 24 June; Date 3: 8 July; Date 4: 22 July; Date 5: 5 August. Table 1 shows the mean weather conditions during the experimental period.

Table 1. Monthly climatic features during the experimental period (year 2008). T_{mean} – mean temperature; T_{max} – maximal daily temperature;; RH – relative humidity, R – rainfall.

Month	T_{mean} [°C]	T_{max} [°C]	RH [%]	R [mm]
June	22.8	33.6	64.0	119.2
July	24.0	32.7	57.6	113.6
August	24.3	32.1	59.1	116.4
September	18.7	30.5	65.6	116.2

Water status measurements: The leaf water potential was determined by measuring at predawn (4 h) with a portable pressure chamber (*SKPM 1400, Sky Instruments Ltd*., Powys, UK). At each sampling date the water potential of 12 leaves (6 well watered and 6 dry, regardless the ozone treatment) was measured. Relative water content was measured on an additional sample of leaves (6 well watered and 6 dry) as:

$$RWC\ (\%) = [(FM - DM)/(SM - DM)] \times 100$$

where FM is the sample's fresh mass; DM is the dry mass, obtained after 72 h of drying in a 70°C oven; SM is the saturated fresh mass, obtained by keeping the leaves in the dark for 48 h, with their stem placed in a distilled water. The effect of the water treatment on stomatal closure was measured between 10 and 13 h on each sampling date, on those plants subjected to nondestructive sampling, using a dynamic diffusion porometer *AP-4* (*Delta-T Devices*, Cambridge, UK). Individual leaf measurements were carried out in ambient light with PAR values above 600 μmol m^{-2} s^{-1}. At the same hours, the net photosynthetic rate (P_N) was measured with a portable photosynthesis system (gas analyzer) *ADC – LCi* gas (*ADC BioScientific Ltd*., Hoddesdon, UK). The CO_2 concentration, temperature and PAR level into the chambers at the same time of measurements are shown in Table 2.

Fluorescence measurements and parameters: Chl *a* fluorescence transients of intact leaves were measured with a direct fluorimeter *HandyPEA* (*Hansatech Instruments*, Pentney – Norfolk, UK) on dark-adapted leaves (20 min). The rising fluorescence transients were induced by red light (peak at 650 nm) of 600 W m^{-2} provided by an array of three light emitting diodes; they were recorded for 1 s, starting from 20 μs after the onset of illumination, with 12-bit resolution.

The parameters concerning light-adapted leaves were assessed with a modulated fluorimeter (*FMS2 Hansatech Instruments*, Pentney–Norfolk, UK). After 20 min of dark adaptation, the minimal fluorescence F_0 was measured as the average of the fluorescence signal under a week pulse of the amber modulating radiation emitting diode over a 1.8-s period, and the maximal fluorescence F_m was measured after a saturating pulse (8,000 μmol m^{-2} s^{-1}) of 0.7 s. An actinic light (600 μmol m^{-2} s^{-1}) was then applied to obtain F_T (steady-state fluorescence yield). After that, a saturating pulse was applied for 0.7 s to obtaine F_m' (light-adapted maximal fluorescence). Minimal fluorescence of the light-adapted state (F_0') was then measured with a far red (FR) pulse [30 μmol(photon) m^{-2} s^{-1} at 720–6,730 nm] in the absence of the actinic light.

Direct fluorescence measurements were taken 5 times during the day: PD (predawn, at 5–6 h), MO (morning, at 9–10 h); MD (midday, from noon to 13 h); AN (afternoon, at 16–17 h); EV (evening, at 19–20 h). Modulated fluorescence measurements were taken twice during the

Table 2. Conditions in the chambers when gas-exchange parameters were measured (n = 36). CO_2 – carbon dioxide concentration in the cuvette; PAR – photosynthetically active radiation; $T_{chamber}$ – temperature in the chamber; T_{leaf} – temperature of the leaf. Mean ± SD. Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008.

	Date				
	1	2	3	4	5
CO_2 [ppm]	387 ± 15.1	398 ± 15.7	422 ± 1.04	372 ± 8.82	377 ± 5.26
PAR [μmol m^{-2} sec^{-1}]	1,156 ± 299	1,134 ± 369	627 ± 14	1,729 ± 156	1,594 ± 224
$T_{chamber}$ [°C]	36.7 ± 2.29	40.8 ± 2.06	42 ± 0.13	36.4 ± 1.34	40.7 ± 1.59
T_{leaf} [°C]	35.8 ± 2.30	41 ± 2.17	42.7 ± 0.18	36.7 ± 1.66	41.2 ± 1.80

day, at predawn and in the afternoon. At each time, one measurement per plant was carried out on a leaf from the basal part of the crown.

The fluorescence induction curve from F_0 to F_m in dark-adapted samples is called "fluorescence transient" (direct or prompt fluorescence, Strasser *et al*. 2000, 2004, 2010) and represents the "fast kinetics". Plotted on a logarithmic time scale, the fluorescence transient shows a polyphasic behaviour. The different steps of this polyphasic transient are labelled as: O (20 μm, in the JIP-test it represents F_0), J (2 ms), I (30 ms), and P (peak). This latter indicates the highest fluorescence intensity (F_m). The parameters considered were those connected to the different steps and bands of the polyphasic curve, each representing a different photochemical event.

L band: $V_{0K}100 = (F_{100\mu s} - F_0)/(F_{300} - F_0)$. Relative variable fluorescence at 100 μs (transients normalized between F_0 and F_K), It expresses the energetic cooperativity of the tripartite system core antenna – LHCII – reaction centre (Strasser 1978, Stirbet *et al.* 1998, Strasser and Stirbet 2001);

K band: $V_{0J}300 = (F_{300\mu s} - F_0)/(F_J - F_0)$. Relative variable fluorescence at 300 μs (transient normalized between F_0 and F_K). It expresses the breakdown of the OEC (Srivastava *et al.* 1997);

J step: $\Psi_{Eo} = ET_0/TR_0 = 1 - V_J = 1 - [(F_{2ms} - F_0)/(F_m - F_0)]$. It expresses the efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors. V_J indicates the relative variable fluorescence at 2 ms (transients normalized between F_0 and F_m);

IP phase: $\Delta V_{I\text{-}P} = 1 - V_I = (F_M - F_{30ms})/(F_m - F_0)$ (Oukarroum *et al*. 2009). It indicates the amplitude of the I-P phase, *i.e*. the efficiency of the electron transport around the PSI to reduce the final acceptors of the ETC (electron transport chain), *i.e*. ferredoxin and NADP. V_I indicates the relative variable fluorescence at 30 ms (transients normalized between F_0 and F_m);

Performance Index total: PI_{tot} is the performance index for an energy conservation from photons absorbed by PSII to the reduction flux (RE) of PSI end acceptors. It is multiparametric expression that combines four parameters favorable to the photosynthetic activity: (*1*) the density of reaction centers; (*2*) the quantum yield of primary photochemistry; (*3*) the ability to feed electrons into the electron chain between PSII and; (*4*) the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors (Strasser *et al*. 2004, 2010) (*see* Appendix for details).

The parameters of modulated fluorescence used in the present paper are (Maxwell and Johnson 2000, Roháček 2002, Schreiber 2004, Baker 2008): $\Phi_{PSII} = (F_m' - F_T)/F_m' = F_q'/F_m'$; F_v'/F_m'; $q_P = (F_m' - F_T)/(F_m' - F_0')$; and $NPQ = (F_m - F_m')/F_m'$.

Statistics: The experiment was designed as "split plot", with ozone (nonfiltered – NF and charcoal-filtered – CF chambers) and water treatment (well watered, W and dry, D) as fixed factors. Ozone was the main treatment. A GLM (general linear model) was applied to evaluate the effect of ozone in W and D plants. Differences between predawn and afternoon assessment were evaluated by mean of the Student *t*-test for paired samples. All statistical routines were performed with the software *Statistica 7.1* (*Statsoft 2001*, Tulsa, OK, USA).

Results

The water treatment (W *vs*. D plants) severely affected g_s (Table 3) and water potential (Ψ_w). Only a small reduction was observed on RWC (Table 3). Visible ozone symptoms, consisting in widespread interveinal brownings which later degenerated into broad necrotic patches, appeared only on plants grown in the NF-W chambers, after sampling date 3 (8 July 2008). By sampling date 4 (22 July 2008), symptomatic leaves began to shed. Plants grown under dry conditions (D) did not display these symptoms.

The value of F_v/F_m was always lower during the central hours of the day than at predawn measurement. L band and K band displayed the opposite trend. The amplitude of the IP phase reached its highest value in the middle of the day in the dry treatment (Table 4). Among the modulated fluorescence parameters (Table 5), Φ_{PSII}

Table 3. Water status and gas-exchange parameters at the different dates, in watered (W) and dry (D) plants. Mean ± SD. Ψw_{PD} – water potential at predawn (n = 6); RWC – relative water content; (n = 6); g_s – stomatal conductance to water vapour (n = 18); P_N – net photosynthetic rate (n = 18). Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008.

		Date 1	2	3	4	5
Ψw_{PD} [MPa]	W	-	–0.11 ± 0.01	–0.13 ± 0.02	–0.17 ± 0.04	-
	D	-	–0.46 ± 0.07	–0.45 ± 0.13	–0.30 ± 0.12	-
RWC [%]	W	95.9 ± 1.2	96.9 ± 0.3	95.5 ± 3.1	91.1 ± 1.6	96.7 ± 1.9
	D	90.5 ± 6.9	95.3 ± 2.5	91.6 ± 2.1	89 ± 0.1	97.8 ± 1.9
g_s [mmol(H_2O) m^{-2} s^{-1}]	W	578 ± 112	357 ± 411	-	540 ± 179	576 ± 240
	D	588 ± 138	11 ± 8	-	93 ± 155	191 ± 224
P_N [µmol(CO_2) m^{-2} s^{-1}]	W	14.1 ± 2.6	16.1 ± 2.9	15.7 ± 3.1	11.7 ± 2.1	10.7 ± 4.1
	D	15.7 ± 3.1	3.24 ± 2.8	2.68 ± 1.9	3.01 ± 0.5	6.8 ± 4.9

Table 4. Direct fluorescence (JIP-test) parameters of dark-adapted leaves, at each measurement date and hour. Data are expressed as mean ± SD (n = 6). Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008. PD – predawn; AN – afternoon; NF – nonfiltered chambers; CF – charcoal-filtered chamber; D – dry (not watered); W – watered. JIP-test parameters: *see* Fig.1. Quantities are expressed as dimensionless ratios (PI_{tot} is expressed as arbitrary units).

Date	Time	Ozone	Water	F_v/F_m	L band	K band	J step	IP phase	PI_{tot}
1	PD	NF	D	0.820 ± 0.003	0.191 ± 0.015	0.307 ± 0.020	0.586 ± 0.008	0.240 ± 0.001	29.90 ± 1.52
			W	0.818 ± 0.009	0.177 ± 0.021	0.319 ± 0.028	0.579 ± 0.020	0.257 ± 0.018	32.58 ± 7.53
		CF	D	0.825 ± 0.011	0.177 ± 0.010	0.303 ± 0.028	0.617 ± 0.013	0.259 ± 0.027	39.16 ± 13.10
			W	0.817 ± 0.005	0.182 ± 0.001	0.297 ± 0.005	0.595 ± 0.018	0.281 ± 0.009	40.66 ± 3.99
	AN	NF	D	0.779 ± 0.01	0.187 ± 0.006	0.345 ± 0.027	0.494 ± 0.073	0.213 ± 0.032	16.18 ± 7.96
			W	0.771 ± 0.020	0.188 ± 0.005	0.369 ± 0.020	0.427 ± 0.091	0.214 ± 0.017	14.42 ± 3.51
		CF	D	0.778 ± 0.014	0.196 ± 0.019	0.348 ± 0.028	0.544 ± 0.086	0.204 ± 0.043	15.45 ± 6.03
			W	0.776 ± 0.010	0.192 ± 0.018	0.343 ± 0.021	0.498 ± 0.091	0.203 ± 0.016	14.16 ± 1.88
2	PD	NF	D	0.824 ± 0.011	0.191 ± 0.005	0.316 ± 0.019	0.566 ± 0.029	0.186 ± 0.024	19.95 ± 4.83
			W	0.820 ± 0.010	0.186 ± 0.014	0.346 ± 0.018	0.554 ± 0.010	0.186 ± 0.017	17.36 ± 4.65
		CF	D	0.827 ± 0.012	0.180 ± 0.008	0.312 ± 0.044	0.576 ± 0.041	0.185 ± 0.042	22.59 ± 9.90
			W	0.825 ± 0.017	0.174 ± 0.013	0.325 ± 0.029	0.586 ± 0.022	0.216 ± 0.025	25.55 ± 6.92
	AN	NF	D	0.715 ± 0.043	0.212 ± 0.017	0.431 ± 0.031	0.501 ± 0.102	0.283 ± 0.032	15.78 ± 5.99
			W	0.715 ± 0.015	0.210 ± 0.008	0.444 ± 0.023	0.461 ± 0.029	0.253 ± 0.037	11.45 ± 4.63
		CF	D	0.743 ± 0.051	0.207 ± 0.008	0.414 ± 0.044	0.503 ± 0.121	0.280 ± 0.062	29.21 ± 17.00
			W	0.739 ± 0.042	0.200 ± 0.008	0.415 ± 0.021	0.513 ± 0.081	0.265 ± 0.028	15.84 ± 6.26
3	PD	NF	D	0.831 ± 0.005	0.176 ± 0.005	0.335 ± 0.027	0.567 ± 0.033	0.199 ± 0.030	22.96 ± 10.33
			W	0.783 ± 0.013	0.200 ± 0.007	0.408 ± 0.020	0.491 ± 0.022	0.163 ± 0.013	8.50 ± 1.85
		CF	D	0.834 ± 0.007	0.179 ± 0.003	0.309 ± 0.023	0.583 ± 0.017	0.207 ± 0.010	26.45 ± 4.72
			W	0.808 ± 0.011	0.185 ± 0.010	0.365 ± 0.029	0.536 ± 0.022	0.186 ± 0.024	14.93 ± 4.92
	AN	NF	D	0.784 ± 0.037	0.190 ± 0.016	0.373 ± 0.027	0.516 ± 0.144	0.216 ± 0.058	18.74 ± 11.41
			W	0.733 ± 0.020	0.212 ± 0.013	0.452 ± 0.042	0.476 ± 0.028	0.173 ± 0.034	5.98 ± 1.63
		CF	D	0.782 ± 0.026	0.190 ± 0.012	0.352 ± 0.032	0.501 ± 0.127	0.246 ± 0.063	24.73 ± 16.36
			W	0.754 ± 0.024	0.200 ± 0.015	0.426 ± 0.040	0.497 ± 0.052	0.179 ± 0.021	7.91 ± 2.14
4	PD	NF	D	0.838 ± 0.010	0.173 ± 0.006	0.314 ± 0.025	0.543 ± 0.030	0.229 ± 0.018	31.09 ± 7.77
			W	0.754 ± 0.026	0.211 ± 0.015	0.465 ± 0.062	0.413 ± 0.032	0.175 ± 0.016	6.98 ± 2.99
		CF	D	0.827 ± 0.020	0.170 ± 0.014	0.327 ± 0.056	0.536 ± 0.045	0.223 ± 0.033	27.99 ± 13.32
			W	0.806 ± 0.012	0.188 ± 0.009	0.409 ± 0.021	0.476 ± 0.016	0.208 ± 0.010	14.69 ± 2.67
	AN	NF	D	0.772 ± 0.018	0.198 ± 0.011	0.418 ± 0.042	0.499 ± 0.051	0.217 ± 0.011	12.34 ± 2.36
			W	0.708 ± 0.011	0.226 ± 0.019	0.504 ± 0.081	0.443 ± 0.021	0.152 ± 0.020	3.74 ± 1.37
		CF	D	0.750 ± 0.029	0.198 ± 0.010	0.403 ± 0.016	0.499 ± 0.038	0.237 ± 0.014	12.92 ± 1.83
			W	0.747 ± 0.017	0.213 ± 0.011	0.474 ± 0.026	0.478 ± 0.038	0.173 ± 0.014	6.17 ± 0.74
5	PD	NF	D	0.824 ± 0.011	0.186 ± 0.008	0.328 ± 0.041	0.604 ± 0.031	0.268 ± 0.039	37.33 ± 10.71
			W	0.767 ± 0.050	0.221 ± 0.042	0.483 ± 0.093	0.487 ± 0.051	0.188 ± 0.054	10.42 ± 7.56
		CF	D	0.840 ± 0.005	0.183 ± 0.015	0.280 ± 0.017	0.659 ± 0.018	0.315 ± 0.022	70.47 ± 8.30

Table 4 continues on the next page.

Table 4 (continued)

Date	Time	Ozone	Water	F_v/F_m	L band	K band	J step	IP phase	PI_{tot}
5			W	0.813 ± 0.016	0.190 ± 0.012	0.434 ± 0.035	0.531 ± 0.015	0.220 ± 0.028	17.18 ± 5.42
	AN	NF	D	0.756 ± 0.035	0.219 ± 0.024	0.403 ± 0.040	0.597 ± 0.091	0.310 ± 0.058	28.78 ± 16.61
			W	0.682 ± 0.083	0.247 ± 0.023	0.562 ± 0.073	0.473 ± 0.087	0.172 ± 0.028	4.52 ± 3.24
		CF	D	0.778 ± 0.033	0.199 ± 0.011	0.333 ± 0.036	0.602 ± 0.098	0.381 ± 0.047	63.09 ± 18.33
			W	0.744 ± 0.054	0.223 ± 0.022	0.519 ± 0.043	0.495 ± 0.075	0.204 ± 0.036	8.46 ± 3.70

Table 5. Modulated fluorescence parameters of light-adapted leaves, at each measurement date and hour. Data are expressed as mean ± SD (n = 6). Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008. PD – predawn; AN – afternoon; NF – nonfiltered chambers; CF – charcoal-filtered chamber; D – dry (nonwatered); W – well watered. Modulated fluorescence parameters: *see* Fig.1. Quantities are expressed as dimensionless ratios.

Date	Time	Ozone	Water	F_v'/F_m'	Φ_{PSII}	q_P	NPQ
1	PD	NF	D	0.669 ± 0.054	0.501 ± 0.100	0.746 ± 0.090	1.936 ± 0.917
			W	0.651 ± 0.006	0.519 ± 0.001	0.798 ± 0.010	2.064 ± 0.084
		CF	D	0.698 ± 0.036	0.556 ± 0.068	0.795 ± 0.055	1.912 ± 0.531
			W	0.675 ± 0.031	0.498 ± 0.054	0.738 ± 0.081	1.676 ± 0.043
	AN	NF	D	0.662 ± 0.051	0.554 ± 0.068	0.835 ± 0.038	1.442 ± 0.660
			W	0.641 ± 0.053	0.526 ± 0.070	0.819 ± 0.059	1.684 ± 0.601
		CF	D	0.663 ± 0.055	0.561 ± 0.083	0.843 ± 0.058	1.502 ± 0.543
			W	0.624 ± 0.062	0.527 ± 0.057	0.844 ± 0.007	1.947 ± 0.637
2	PD	NF	D	0.641 ± 0.048	0.476 ± 0.068	0.741 ± 0.049	2.108 ± 0.458
			W	0.695 ± 0.037	0.583 ± 0.041	0.838 ± 0.018	1.657 ± 0.596
		CF	D	0.675 ± 0.051	0.525 ± 0.105	0.773 ± 0.101	1.797 ± 0.397
			W	0.672 ± 0.070	0.545 ± 0.084	0.808 ± 0.047	2.012 ± 0.882
	AN	NF	D	0.707 ± 0.022	0.619 ± 0.024	0.875 ± 0.007	1.081 ± 0.477
			W	0.676 ± 0.024	0.563 ± 0.051	0.832 ± 0.054	1.347 ± 0.457
		CF	D	0.643 ± 0.116	0.537 ± 0.127	0.828 ± 0.052	1.370 ± 0.837
			W	0.590 ± 0.047	0.484 ± 0.050	0.820 ± 0.028	1.991 ± 0.097
3	PD	NF	D	0.62 ± 0.040	0.471 ± 0.076	0.748 ± 0.071	2.669 ± 0.495
			W	0.556 ± 0.027	0.299 ± 0.030	0.538 ± 0.043	2.513 ± 0.846
		CF	D	0.618 ± 0.030	0.466 ± 0.068	0.751 ± 0.073	2.591 ± 0.287
			W	0.593 ± 0.034	0.387 ± 0.079	0.650 ± 0.099	3.192 ± 0.357
	AN	NF	D	0.641 ± 0.049	0.523 ± 0.085	0.813 ± 0.070	2.055 ± 0.482
			W	0.570 ± 0.046	0.396 ± 0.073	0.691 ± 0.077	2.062 ± 0.451
		CF	D	0.639 ± 0.068	0.533 ± 0.096	0.830 ± 0.065	1.692 ± 0.346
			W	0.614 ± 0.068	0.471 ± 0.084	0.763 ± 0.050	1.960 ± 0.644
4	PD	NF	D	0.565 ± 0.014	0.246 ± 0.068	0.433 ± 0.112	2.407 ± 0.113
			W	0.575 ± 0.012	0.165 ± 0.051	0.289 ± 0.096	1.864 ± 0.169
		CF	D	0.607 ± 0.011	0.289 ± 0.069	0.475 ± 0.109	2.283 ± 0.415
			W	0.555 ± 0.012	0.254 ± 0.050	0.455 ± 0.082	2.731 ± 0.360
	AN	NF	D	0.721 ± 0.019	0.626 ± 0.040	0.868 ± 0.032	1.198 ± 0.424
			W	0.547 ± 0.020	0.369 ± 0.024	0.675 ± 0.055	1.728 ± 0.301
		CF	D	0.661 ± 0.029	0.565 ± 0.022	0.855 ± 0.004	1.066 ± 0.361
			W	0.653 ± 0.033	0.540 ± 0.041	0.827 ± 0.022	1.289 ± 0.330
5	PD	NF	D	0.668 ± 0.019	0.517 ± 0.038	0.773 ± 0.035	2.157 ± 0.737
			W	0.597 ± 0.028	0.387 ± 0.076	0.647 ± 0.111	2.346 ± 0.538
		CF	D	0.664 ± 0.045	0.547 ± 0.061	0.822 ± 0.039	2.080 ± 0.329
			W	0.616 ± 0.066	0.454 ± 0.077	0.735 ± 0.050	2.696 ± 0.652
	AN	NF	D	0.697 ± 0.014	0.605 ± 0.020	0.868 ± 0.010	1.298 ± 0.137
			W	0.618 ± 0.013	0.442 ± 0.029	0.715 ± 0.031	1.071 ± 0.425
		CF	D	0.705 ± 0.004	0.627 ± 0.002	0.889 ± 0.007	1.183 ± 0.026
			W	0.632 ± 0.036	0.524 ± 0.036	0.829 ± 0.027	1.816 ± 0.737

Table 6. Effect of sunlight at the different dates and experimental conditions (CF – charcoal filtered; NF – nonfiltered). Analysis of differences between afternoon (AN) and predawn (PD) measurements, with Student *t*-test for paired samples ($n = 6$ each date; for all dates $n = 30$). Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008. *Arrows* (↓) indicate that the value of the given parameter at AN is lower than at PD. *Arrows* (↑) indicate that the value of the given parameter at AN is greater than at PD. For explication of parameters *see* Fig. 1. Differences are assumed to be significant when $p<0.05$ (***in bold***). Differences with $p<0.1$ are reported as indicative of a tendency. ns – not significant.

Date		F_v/F_m		L band		K band		J step		IP phase		PI_{tot}		F_v'/F_m'		Φ_{PSII}		q_P		NPQ	
Well watered																					
1	CF	**<0.05**	↓	ns		ns		ns		**<0.05**	↓	**<0.01**	↓	ns		ns		ns		ns	
	NF	**<0.01**	↓	ns		<0.1	↑	<0.1	↓	ns		**<0.01**	↓	ns		ns		ns		ns	
2	CF	**<0.01**	↓	**<0.05**	↑	**<0.01**	↑	ns		ns		ns		ns		ns		ns		ns	
	NF	**<0.001**	↓	**<0.05**	↑	**<0.01**	↑	ns		ns		ns		ns		ns		ns		ns	
3	CF	**<0.01**	↓	**<0.01**	↑	**<0.001**	↑	ns		ns		ns		ns		ns		ns		ns	
	NF	**<0.05**	↓	ns		**<0.05**	↑	ns		ns		ns		ns		ns		ns		ns	
4	CF	**<0.001**	↓	**<0.05**	↑	ns		ns		**<0.05**	↓	ns		**<0.001**	↑	**<0.001**	↑	**<0.01**	↑	**<0.001**	↓
	NF	**<0.05**	↓	ns		ns		ns		ns		ns		ns		**<0.01**	↑	**<0.001**	↑	<0.1	↓
5	CF	<0.1	↓	ns		**<0.05**	↑	ns		ns		ns		ns		ns		ns		ns	
	NF	<0.1	↓	ns		**<0.05**	↑	ns		ns		ns		ns		ns		ns		ns	
All dates	CF	**<0.01**	↓	**<0.01**	↑	**<0.01**	↑	ns		ns		<0.1	↓	ns		**<0.05**	↑	**<0.01**	↑	**<0.01**	↓
	NF	**<0.05**	↓	**<0.05**	↑	**<0.05**	↑	**<0.01**	↓	ns		ns		ns		**<0.05**	↑	**<0.01**	↑	**<0.05**	↓
Dry																					
1	CF	**<0.01**	↓	ns		ns		ns		**<0.1**	↓	**<0.001**	↓	ns		ns		ns		ns	
	NF	**<0.05**	↓	ns		ns		ns		ns		ns		ns		ns		ns		ns	
2	CF	**<0.01**	↓	**<0.01**	↑	**<0.001**	↑	ns		**<0.01**	↑	ns		ns		ns		ns		ns	
	NF	**<0.001**	↓	<0.1	↑	**<0.001**	↑	ns		**<0.01**	↑	ns		ns		ns		ns		ns	
3	CF	ns		**<0.05**	↑	ns		ns		**<0.001**	↑	ns		ns		ns		ns		ns	
	NF	ns		<0.1	↑	<0.1	↑	ns		**<0.01**	↑	ns		ns		ns		ns		ns	
4	CF	**<0.001**	↓	**<0.01**	↑	<0.1	↑	ns		ns		**<0.01**	↓	<0.1	↑	**<0.001**	↑	**<0.001**	↑	**<0.01**	↓
	NF	**<0.001**	↓	**<0.05**	↑	<0.01	↑	ns		ns		**<0.001**	↓	**<0.001**	↑	**<0.001**	↑	**<0.001**	↑	**<0.01**	↓
5	CF	ns		ns		ns		ns		**<0.05**	↑	ns		ns		ns		ns		ns	
	NF	<0.1	↓	**<0.05**		ns		ns		ns		ns		ns		ns		ns		ns	
All dates	CF	**<0.01**	↓	**<0.01**	↑	**<0.01**	↑	**<0.01**	↓	ns		**<0.01**	↓	ns		**<0.05**	↑	**<0.01**	↑	**<0.01**	↓
	NF	**<0.05**	↓	**<0.05**	↑	**<0.05**	↑	**<0.01**	↓	ns		**<0.001**	↓	**<0.01**	↑	**<0.001**	↑	**<0.001**	↑	**<0.01**	↓

Table 7. Effect of ozone at the different dates and hours of the day (PD – predawn; AN – afternoon). Analysis of the differences between nonfiltered (NF) and charcoal-filtered (CF) samples, by applying a GLM ($n = 6$ each date; for all dates $n = 30$). Dates: 1 – 10 Jun; 2 – 24 Jun; 3 – 8 Jul; 4 – 22 Jul; 5 – 4 Aug 2008. *Arrows* (↓) indicate that the value of the given parameter in NF is lower than in CF chambers. *Arrows* (↑) indicate that the value of the given parameter in NF is greater than in CF chambers. For explication of parameters *see* Fig. 1. Differences are assumed to be significant when $p<0.05$ (***in bold***). Differences with $p<0.1$ are reported as indicative of a tendency. ns – not significant.

Date		F_v/F_m	L band	K band	J step	IP phase	PI_{tot}	F_v'/F_m'	Φ_{PSII}	q_P	NPQ
Well watered											
1	PD	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AN	ns	ns	**<0.05** ↑	ns	<0.1 ↓	ns	ns	ns	ns	ns
2	PD	ns	<0.1 ↑	**<0.05** ↑	ns	**<0.05** ↓	<0.1 ↓	ns	ns	ns	ns
	AN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
3	PD	**<0.01** ↓	<0.1 ↑	**<0.05** ↑	**<0.01** ↓	ns	<0.1	ns	ns	ns	ns
	AN	ns	<0.1 ↑	ns	ns	ns	**<0.01** ↓	ns	ns	ns	ns
4	PD	**<0.05** ↓	**<0.05** ↑	<0.1 ↑	**<0.05** ↓	**<0.05** ↓	**<0.001** ↓	**<0.05** ↓	ns	ns	ns
	AN	ns	**<0.05** ↑	ns	ns	ns	**<0.05** ↓	<0.1 ↓	**<0.01** ↓	**<0.05** ↓	ns
5	PD	<0.1 ↓	ns	ns	**<0.05** ↓	**<0.05** ↓	ns	ns	ns	ns	ns
	AN	<0.1 ↓	ns	ns	ns	ns	**<0.05** ↓	ns	ns	<0.1 ↓	ns
All dates	PD	**<0.05** ↓	**<0.01**	**<0.05**	ns	ns		ns	ns	ns	**<0.05** ↓
Dry	AN	**<0.001** ↓	**<0.05**	<0.1 ↑	**<0.05** ↓	ns	ns	ns	ns	ns	ns
1	PD	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
2	PD	ns	ns	ns	ns	ns	<0.1 ↓	ns	ns	ns	ns
	AN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
3	PD	ns	ns	**<0.05** ↑	ns	ns	ns	ns	ns	ns	ns
	AN	ns	ns	ns	ns	**<0.05** ↓	**<0.05** ↓	ns	ns	ns	ns
4	PD	<0.1 ↓	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AN	<0.1 ↓	ns	ns	ns	**<0.05** ↓	ns	ns	ns	ns	ns
5	PD	ns	ns	ns	**<0.05** ↓	ns	**<0.01** ↓	ns	<0.1 ↓	**<0.05** ↓	**<0.01** ↑
	AN	ns	**<0.05** ↑	ns	ns	<0.1 ↓	ns	ns	ns	**<0.1** ↓	ns
All dates	PD	ns	ns	ns	ns	ns	**<0.05** ↓	ns	ns	ns	ns
	AN	ns	ns	ns	**<0.05** ↓	ns	**<0.05** ↓	ns	ns	ns	ns

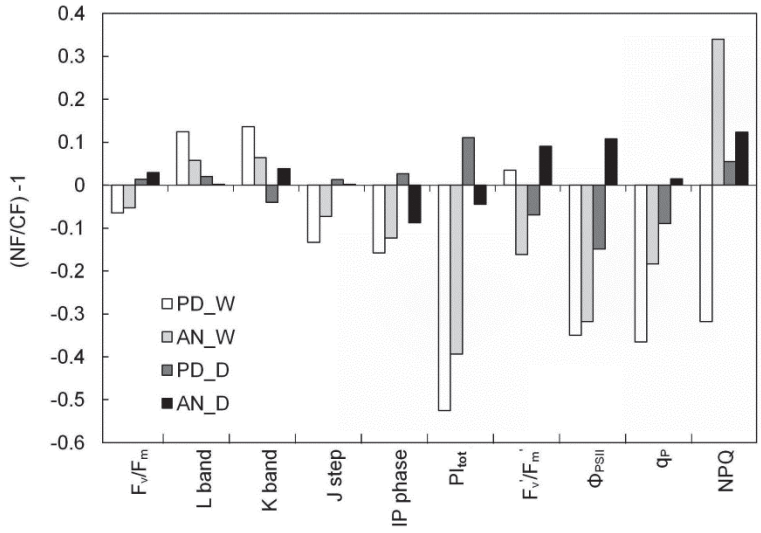

Fig. 1. Relative values of the selected parameters of direct and modulated fluorescence in the afternoon with respect to predawn (AN/PD – 1) in the different experimental conditions. Results from all dates were pooled. The significance of differences in Table 6. F_v/F_m – maximum quantum yield of primary photochemistry; L band – $V_{OK}100$; K band – $V_{OJ}300$; J phase – efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors; I phase – the efficiency of electron transport around the PSI; PI_{tot} – Performance Index total, potential performance index for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; F_v'/F_m' – PSII maximum efficiency in the light-adapted state; Φ_{PSII} – actual quantum yield of PSII, or PSII operating efficiency; q_P – photochemical quenching; NPQ – nonphotochemical quenching.

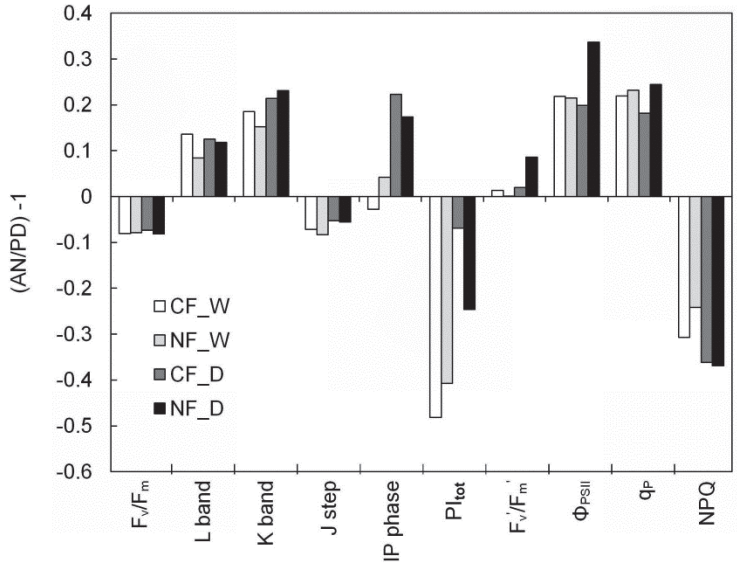

Fig. 2. Relative values of the selected parameters of direct and modulated fluorescence in the nonfiltered chambers with respect to the charcoal filtered ones (effect of ozone, NF/CF – 1). Results from all dates were pooled. Data refer to afternoon (AN) and predawn (PD) measurements, in watered (W) and dry (D) conditions. Significance of differences between NF and CF are reported in Table 7. For explication of parameters *see* Fig. 1.

and q_P increased in the afternoon, while NPQ decreased. Here, we compared the data from predawn (absence of photoinhibitory conditions) and afternoon (full exposure to sunlight). The findings shown in Fig. 1 and Table 6 highlighted significant differences between these two times of the day for many of the investigated parameters, in CF and NF chambers, both in W and D plants.

As for the ozone effect, (NF *vs*. CF chambers; Table 7, Fig. 2), F_v/F_m was significantly lower at predawn in NF-W (*vs*. CF-W) plants from date 3 (8 July 2008). At the same date 3, IP phase was significantly lower in the afternoon in NF-D (*vs*. CF-D) plants; whereas at sampling date 4 (22 July 2008), it was also significant at predawn (NF-W *vs*. CF-W plants). PI_{tot} was significantly lower in the afternoon, at sampling date 3, in NF (both on W and D plants), with respect to CF chambers. As far as modulated fluorescence parameters are concerned, Φ_{PSII} (date 4) and q_P (dates 4 and 5, 22 July and 4 August 2008) were depressed in both afternoon and predawn measurements in NF-W (*vs*. CF-W) plants, although differences were firstly significant in the afternoon measurements.

A more detailed description of findings is given for date 4, 22 July 2008 (Figs. 3–5). Fluorescence transient (Fig. 3) showed there was an evident behaviour difference between W plants (Fig. 3*A*) and D ones (Fig. 3*B*). In the W plants, the transients of NF plants developed a lower fluorescence intensity than CF ones. Yet, the difference in fluorescence intensity (ΔF) between CF and NF plants was always greater at predawn than in the afternoon. Conversely, in the D plants there was a difference between predawn and afternoon, but there was no clear distinction between CF and NF at the two times of day. The K and L bands in relation to sunlight and ozone are evidenced by means of the curves, where $\Delta V = (V_{treated} - V_{control})$ (Figs. 4,5). V indicates the relative variable fluorescence at any time (t) between F_0 and F_J $[V_{0J} = (F_T - F_0)/(F_J - F_0)]$ to evidence the K band (Fig. 4),

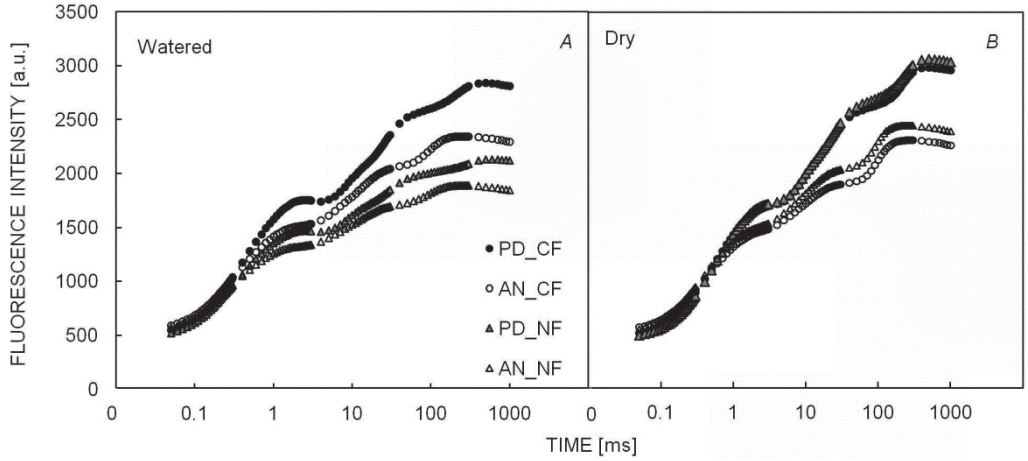

Fig. 3. Mean fluorescence transients at the date 4 (22 July), in the afternoon (AN) and predawn (PD) in charcoal-filtered (CF) and nonfiltered (NF) chambers. Transients are subdivided in watered (*A*) and dry (*B*) conditions.

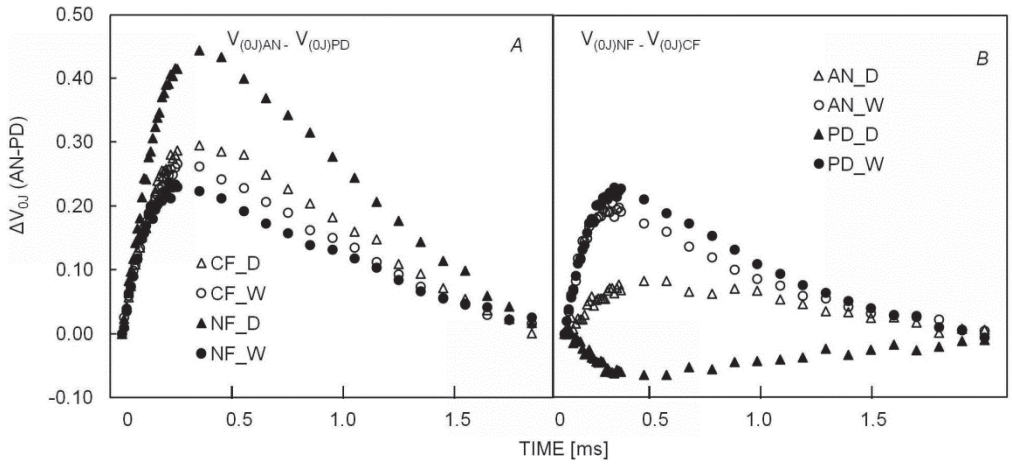

Fig. 4. ΔV_{0J} curves, obtained by subtracting the relative variable fluorescence of the transients normalized between F_0 and F_J (V_{0J}). *A*: Effect of the hour of the day [$V_{(0J)MD} - V_{(0J)PD}$]. *B*: Effect of ozone [$V_{(0J)NF} - V_{(0J)CF}$]. Gain: 4. The K band, occurring at ~300 μs, is evidenced.

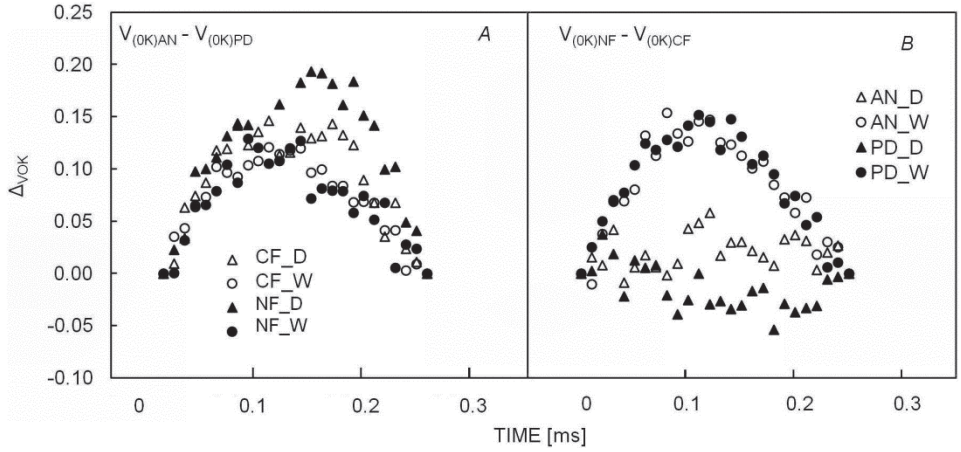

Fig. 5. ΔV_{0K} curves, obtained by subtracting the relative variable fluorescence of the transients normalized between F_0 and F_K (V_{0K}). *A*: Effect of the hour of the day [$V_{(0K)MD} - V_{(0K)PD}$]. *B*: Effect of ozone [$V_{(0K)NF} - V_{(0K)CF}$]. Gain: 4. The L band, occurring at ~150 μs, is evidenced.

and between F_0 and F_K [$V_{0K} = (F_T - F_0)/(F_K - F_0)$] to evidence the L band (Fig. 5). These curves highlighted the differences of the relative fluorescence signals at each time t. The onset of K and L bands in the afternoon ($V_{AN} - V_{PD}$) was evident for all treatments (CF-D, NF-D, CF-W, NF-W) (Figs. 4*A*, 5*A*). As far as the effect of ozone is concerned ($V_{NF} - V_{CF}$), the K and L bands appeared only in the W treatments (both at predawn and afternoon) (Figs. 4*B*, 5*B*).

Fig. 6 provides analysis of the daily trend of some fluorescence parameters in NF-W and CF-W plants. Regarding F_v/F_m, these findings showed that the difference between the two treatments, which was significant at predawn ($p<0.05$), was again significant ($p<0.05$) at the end of the day (in the evening measurements, when PAR decreases to values around 300 μmol m^{-2} s^{-1}). The same behaviour was observed for L and K bands, whereas for PI_{tot} significant differences were found only at predawn.

Discussion

The results of this study confirmed the daily change of F_v/F_m, which reached its lowest values during daytime hours even in the dark-adapted leaves. The dark adaptation (20 min) with leaf clips during the daytime hours ensures release of the trans-thylakoid pH gradient (Quich and Stitt 1989), a reoxidation of the plastoquinone pool (Papageorgiou and Tismilli-Michael 2007) and the reversal of the effect of high-light exposure on a large portion of deepoxidation state of the xanthophyll pigments (Thiele *et al.* 1998). F_v/F_m reduction in daytime hours happened probably because the dark adaptation time was too short to allow new synthesis of the protein D1, whereas at predawn the synthesis of D1 had occurred.

In the initial portion of fluorescence transient's trajectory, the K and L bands were sensitive to photoinhibitory conditions. The K band is known to be sensitive to high temperatures (Srivastava *et al.* 1997), but our results confirmed its sensitivity also to the diurnal light cycles (*see* also Desotgiu *et al.* in press). Moreover, in the very first part of the fluorescence transient ($F_{50\mu s} - F_{300\mu s}$), the L band at 100–150 μs indicated loss of stability in the tripartite system that controls the first stages of light harvesting (light-harvesting compounds – core antenna – reaction centre). Daytime changes in the investigated parameters were observed in CF and NF plants, both in W and D conditions.

The amplitude of the IP phase is considered to be very sensitive to sunlight, reaching maximum values in daylight (Desotgiu *et al.*, in press) and in sun leaves (Cascio *et al*. 2010). In this research, that behaviour was observed only in the nonwatered (D) conditions, both for CF and NF treatments. The decrease of IP phase in the afternoon in W plants may be considered part of a mechanism to dissipate the excess of energy in high light conditions (Lin *et al*. 2009), whereas the opposite behaviour in D plants suggests the involvement of alternative electron pathways, *i.e*. cyclic and/or pseudocyclic (Asada 1999).

The IP phase of the fluorescent transient is depressed by ozone (Bussotti *et al.* 2011) and by acute water stress (Oukarroum *et al.* 2007, 2009). Schansker *et al*. 2005 demonstrated that the IP phase depended on electron flow through PSI toward the final acceptors beyond PSI, and

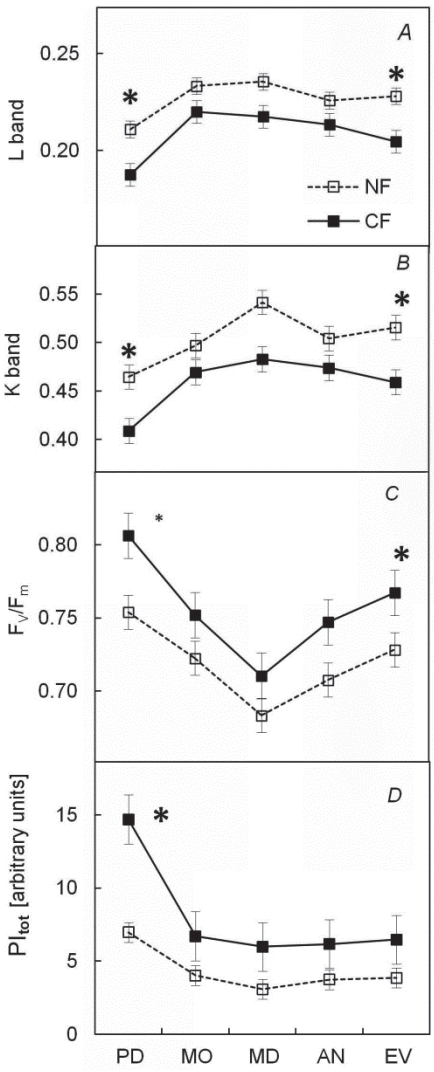

Fig. 6. Daily behaviour (PD – predawn; MO – morning; MD – midday; AN – afternoon; EV – evening) of selected parameters from fluorescence transient analysis, in nonfiltered (NF) and charcoal-filtered (CF) plots, assessed at the date 4 (22 July), only watered plants. *A*: L band; *B*: K band; *C*: F_v/F_m; *D*: PI_{tot}. Bars indicate standard error; asterisks indicated significant differences ($p<0.05$) between NF and CF chambers. $n = 6$ (mean ± SE).

fluorescence rise in the IP phase has been shown to on PSI relative content (Oukarroum *et al.* 2009). The parallel the re-reduction of plastocyanin PC^+ and P_{700}^+ in photosystem PSI (Schansker *et al.* 2003). The increasing values of the IP phase as a consequence of high light (Pollastrini *et al.* 2011) enhance carbon reduction (Cascio *et al.* 2010) and represent an effective way of photochemical de-excitation. In this study, the increase of IP phase under high sunlight was confirmed only for non-watered plants. PSII operating efficiency (Φ_{PSII}) showed higher values in the afternoon measurements than in the predawn. This behaviour has already been described by Pollet *et al*. (2002) for CAM species of the *Phalaenopsis* genus: the authors attributed the finding to high daytime temperatures which favour the flow of electrons through PSII. In daytime, the flow of electrons supplies photochemical needs (increase of q_P), depressing NPQ.

The effect of ozone (NF and CF chambers) was influenced both by the time of day at which measurements were performed and by the water treatment. As expected, ozone exerted its harmful action primarily in well watered conditions. Dry plants, with reduced g_s, reduced their absorption of this pollutant and it thus delayed the onset of symptoms (Gerosa *et al.* 2009, Marzuoli *et al.* 2009). In well watered conditions, differences in the parameters measuring the effect of ozone (NF *vs*. CF chambers) were detectable starting from sampling 8 July 2008 (date 3). These differences (namely decreased F_v/F_m and PI_{tot} and increased L and K bands) were significant at the early sampling time (predawn), but not later in the day (afternoon). This behaviour can be explained by the different amplitude of modifications recorded in daytime in the two ozone treatments, NF and CF, thus suggesting that NF plants did not recover (or recovered partially) from photoinhibition during nighttime.

Ozone sensitivity of IP phase has been discussed by Bussotti *et al*. (2011). The slowed activity of end acceptors reduction was considered a consequence of a lower request of reductants, connected to the decrease of P_N as early response to ozone. Among of the fluorescence-modulated parameters, the ozone sensitivity of Φ_{PSII} can be also connected to the reduced demand of electrons by Calvin-Benson cycle (Baker 2008). Both these parameters showed greater sensitivity to ozone (significant differences between NF and CF chambers) in the afternoon than at predawn.

Finally, it is interesting to note that the IP phase was ozone-sensitive even in nonwatered plants, despite the fact that they have been proved to have a reduced ozone absorption through the stomata. No visible foliar symptoms appeared on plants in the dry-treatment group, further confirming that alterations in parameters connected to the IP phase were an early (presymptomatic) manifestation of ozone injury (Desotgiu *et al.* 2010).

Conclusions: Analysis of the findings yielded by this study, based on the analysis of Chl *a* fluorescence in open top chamber conditions, showed that response sensitivity to treatment was influenced by the hour of the day at which measurements were taken. The initial hypothesis (the differences between ozone-treated and control plants is emphasized in high-sunlight condition) was not confirmed for F_v/F_m and other parameters like L and K bands. These parameters in fact displayed the highest differences between ozone treated and control plants in dark conditions (at predawn). It is likely that the results reported in the literature are taken from measurements performed during daytime; this could help explain many of the findings presented in the review by Bussotti *et al*. (2011) and the alleged low sensitivity of the parameter F_v/F_m to ozone stress.

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Appendix

$PI_{tot} = (RC/ABS)\ [\varphi_{Po}/(1 - \varphi_{Po})]\ [\Psi_{Eo}/(1 - \Psi_{Eo})]\ [\delta_{Ro}/(1 - \delta_{Ro})]$, where:

$RC/ABS = \varphi_{Po}\ (V_J/M_0)$, where $M_0 = [4\ (F_K - F_0)/(F_m - F_0)]$. It represents the initial slope of the fluorescence induction curve, and is defined as the net rate of PSII closure;

$\varphi_{Po} = F_v/F_m$ = maximum quantum yield of primary photochemistry in the dark-adapted state;

$\Psi_{Eo} = 1 - V_J$ = efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors;

$\delta_{Ro} = (1 - V_I)/(1 - V_J) = (F_m - F_I)/(F_m - F_J)$. Probability that an electron is transported from reduced PQ to the electron acceptor side of PSI.