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The mechanism of the ozone-induced changes in thermoluminescence glow curves of barley leaves

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Abstract

The changes in thermoluminescence (TL) signals induced by short-term ozone exposure of leaves are characterized by a down-shift of the peak-temperature of the TL B-band and an increase of a TL band at 55 °C. We investigated the relationship of these changes to photosystem 2 (PS2) photochemistry. The changes were not only detectable in the presence of ozone, but also after irradiation of dark-adapted leaves and after aging of irradiated detached leaf segments. The opposite effect on TL, an up-shift of the peak-temperature of the B-band and the decrease of the intensity of the band at 55 °C were found after infiltration of leaves with nigericin, antimycin A, and diphenyleneiodonium chloride (DPI). Propyl gallate down-shifted the peak-temperature of the B-band. 2,5-dimethyl-1,4-benzoquinone up-shifted the peak-temperature of the B-band and decreased the intensity of the 55 °C band. The intensity of the 55 °C band did not change significantly in the presence of oxygen in comparison to that in nitrogen atmosphere. It decreased with time of dark adaptation (50 % intensity was observed after 3 h of dark adaptation at room temperature), however, it was reactivated to its initial value (at 5 min of dark adaptation) after 1 single-turnover flash. The 55 °C band was not significantly changed in the presence of DCMU. Thus the ozone-induced band at 55 °C is assigned to charge recombination in PS2. Changes in the electron transport chain at the acceptor side of PS2, probably related to the cyclic electron transport around photosystem 1 and/or chlororespiration, could play an important role in the increase of the 55 °C band and the down-shift of the B-band. The changes at the acceptor side indicated by TL can be an expression of a physiological regulatory mechanism functional under stress conditions.

Additional key words: antimycin A; DCMU; 2,5-dimethyl-1,4-benzoquinone; diphenyleneiodonium chloride; *Hordeum*; nigericin; oxygen; photosystem 2; propyl gallate.

Introduction

Ozone is a phytotoxic air pollutant (for reviews see Mudd 1996, Sandermann 1996, Reichenauer and Bolhàr-Nordenkampf 1999). The chemical reactivity of O_3 in aqueous solutions with many of the plant cell-wall phenolics, olefinic compounds, and unsaturated lipids rapidly generates active oxygen species in leaf extracellular spaces. However, the ozone concentration in the intercellular air space is close to zero (Laisk *et al.* 1989), indicating a fast reaction with chemical compounds of the intercellular air space (Salter and Hewitt 1992) and the wet internal free surface area of cell walls (Castillo and Greppin 1988, Chameides 1989). Ozone triggers an oxidative burst concomitant with the enzymatic generation of reactive oxygen species (ROS) that can lead to the so-called hypersensitive response (HR) in plants (Wohlgemuth *et al.* 2002). Stomatal closure and a decrease in the rate of net assimilation of CO_2 under saturating irradiance is one of the first effects of ozone observed in green leaves. The reduction of the photosynthetic electron transport is regarded as secondary effect, caused by a consistent high ΔpH across the thylakoid membrane due to a decreased demand of ATP and NADPH in the Calvin cycle (Reichenauer and Bolhàr-Nordenkampf 1999).

Thermoluminescence (TL) can be used to study damages of the photosynthetic apparatus. Various TL bands peaking at different temperatures have been described. The majority of TL bands originate from a thermally stimulated recombination of positive charges on the donor side of photosystem 2 (PS2) with electrons on the acceptor side (for review see Vass and Inoue 1992). Changes in the intensities and positions of these TL bands yield valuable information about the functional state of electron transport in PS2. TL bands observed in the high-temperature range of the TL curve above 60 °C do not originate from charge recombination in PS2, but are associated with oxygen radicals and lipid peroxidation in the thylakoid membrane (Venedikov *et al.* 1989,

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Vavilin and Ducruet 1998). These bands give information about the stress-induced oxidative burst in the sample (Vavilin and Ducruet 1998).

In a previous report (Skotnica *et al.* 2003) it was demonstrated that ozone induces changes in TL glow curves of barley leaves measured after excitation by 1 single turnover flash. The characteristic effects of ozone on TL were a shift of the peak temperature of the B band (maximum at 22 °C in control) to lower temperatures, an increase of the band at about 55 °C together with a stimulated light emission in the temperature range from 60 to 160 °C.

Several different bands at about 50 °C had been observed in TL glow curves under various conditions. The "classical" C band appears at about 50 °C in leaves, chloroplasts, isolated PS2 membranes, and Ca^{2+}-depleted PS2 (for review see Vass and Inoue 1992). The intensity of this band was enhanced in the presence of high concentrations of DCMU (Desai *et al.* 1975). This band is suggested to arise from charge recombination between Q_A^- (quinone acceptor of PS2) and Y_D^+ (tyrosine 160 on the D_2 protein) (Demeter *et al.* 1993, Krieger *et al.* 1993, Johnson *et al.* 1994). The charge recombination corresponding to this band occurs probably in reaction centres with a modified redox potential of Q_A^- (Demeter *et al.* 1993, Krieger *et al.* 1993, Johnson *et al.* 1994) and with an inactivated water-splitting complex (Krieger *et al.* 1993). The formation of a large proton gradient across the thylakoid membrane can lead to a reversible Ca^{2+} release from the donor side of PS2 and, as a consequence, to an inactivation of the water-splitting complex and a shift in the redox potential of Q_A (Krieger and Weis 1993, Krieger *et al.* 1993). The band at about 50 °C may reflect inactive PS2 centres to be equivalent to inactive PS2 Q_B-non-reducing centres (Andrée *et al.* 1998). Another explanation for the heat-induced stimulation of a band at about 50 °C is a shift of the $Q_A \leftrightarrow Q_B^-$ equilibrium towards the left above 40 °C which is in accordance with a heat-induced rise in F_0 in this temperature range (Ducruet 1999).

Chemiluminescence bands which do not originate from charge recombination in PS2 can contribute to the TL at about 50 °C. Rózsa *et al.* (1989) observed a light-induced band in various sub-chloroplast preparations obtained by detergent treatment as well as with artificially prepared protein-pigment-detergent micelles. Skotnica *et al.* (1999) characterized a chemiluminescence band at about 50 °C in TL glow curves of barley leaves. The appearance of the band was dependent on the presence of oxygen during heating of the sample. The band was not influenced by excitation of TL and it was found only after freezing of the sample. The intensity of the band was dependent on growth conditions of the plants (*e.g.* irradiance and nutrition state). The mechanism leading to the emission of this chemiluminescence band involves thermally stimulated production of an active oxygen species.

The B-band (peak temperature between 15 and 40 °C) is the best characterized TL band. It originates from the charge recombination of positive charges on the S_2 and S_3 states of the water splitting apparatus with electrons on Q_B^- (quinone acceptor of PS2). The down-shift of the B-band under stress conditions reflects changes of the donor side and/or the acceptor side of PS2. A decrease of pH in the lumen of thylakoids and/or an increase of the reduction of the plastoquinone (PQ) pool was suggested as reasons for the down-shift of the B-band (Farineau and Laval-Martin 1992, Miranda and Ducruet 1995). A similar effect due to an increased reduction of the PQ pool could be induced by a decreased ability in PQ binding or a changed protein conformation at the acceptor side.

This report is focused on the changes in TL glow curves in ozone-exposed leaves to understand in more detail the physiological effects of ozone exposure on PS2.

Materials and methods

Plants: Barley (*Hordeum vulgare* L. cv. Bomi) was grown in a growth chamber at a temperature of 23±2 °C and at a relative air humidity of 60±5 % on soil. The irradiance (fluorescent light tubes *Osram* and *Philips*) was adjusted to photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1}. An irradiation regime of 8/16 h (dark/light) was applied. Primary barley leaves from 10-d-old plants were used for measurements.

Fumigation treatment: Plants were incubated in an ozone chamber (rectangular plexiglass box) at a concentration of 0.9 mg m^{-3} of ozone for 6 h at a temperature of 22±1 °C and a relative humidity of 80±5 %. PPFD on top of the plants was adjusted to 200 μmol m^{-2} s^{-1}. The ozone chamber was continuously ventilated with a regulated flow of ozone-fumigated air passed over a charcoal filter. The ozone was produced by a mercury vapour lamp. The concentration of ozone in the chamber was analysed by an ozone detector *ML 9810* (*Monitor-Labs*, USA).

Irradiation and aging: In the case of transfer of plants from the dark into the light, the plants were dark-adapted for 16 h in a dark box and were then transferred to PPFD of 200 μmol m^{-2} s^{-1} for 10 min. In the case of aging, the detached barley leaf segments of 3 cm length were kept for 3 d on water under 16/8 h light/dark, 150 μmol m^{-2} s^{-1}.

Infiltration of leaves with different chemical compounds was performed by the application of a vacuum. Leaf segments (3 cm of length, cut off 3.5 cm from the tip) were infiltrated in a test-tube with 10 cm^3 of an infiltration medium. The vacuum in the test-tube was reached using a vacuum pump. Normal air pressure was changed with the vacuum all 10 s for a total time of 5 min. The

infiltration solutions were: 20 µM nigericin (in 1 % methanol, v/v), 100 µM antimycin A (in 1 % DMSO, v/v), 500 µM diphenyleneiodonium chloride (DPI) (in 1 % DMSO), 600 µM 2,5-dimethyl-1,4-benzoquinone (in 1 % DMSO), and 2 mM propyl gallate (in 1 % DMSO, v/v). 1 % (v/v) DMSO or 1 % (v/v) methanol (in the case of nigericin) was used as control solution. The nigericin was chosen as an uncoupler of the pH gradient, antimycin A as an inhibitor of cyclic electron transport, DPI as an inhibitor of PQ reduction, 2,5-dimethyl-1,4-benzoquinone as an acceptor of electrons from the acceptor side of PS2, and propyl gallate as an inhibitor of chlororespiratory oxidase.

Fluorescence and thermoluminescence measurements: A leaf segment of 2 cm length was cut 4 cm below the tip of the barley leaf. In the case of infiltration of leaves or the aging, a 2 cm long central part of the 3 cm segment was used as the sample for measurements. TL was measured using a set-up described in Skotnica *et al*. (2003) or similar to Ducruet *et al*. (1998). The sample was placed on the sample holder, a Peltier plate 4×4 cm (*HT6-12-40*, *Melcor*, USA) covered by a thin copper film. For better thermal contact the sample was pressed against the sample holder by several thin copper wires fixed on an open teflon ring. To increase the reproducibility of measurements, pre-excitation of the sample was done by 20 single turnover flashes (*XST 103* xenon flash lamp, *Walz*, Germany). The pre-excitation randomizes the S-states of the donor side and the negative charges of the acceptor side (Q_B/Q_B^-) yielding a well defined ratio of S_0/S_1 = 25/75 and Q_B/Q_B^- = 50/50 after the subsequent dark adaptation (Vass and Inoue 1992). After pre-excitation the leaf segment was dark-adapted for 5 min at 20 °C and then rapidly cooled to 2 °C. After a stabilization period of 60 s at 2 °C, TL was excited by 1 (or 0 if indicated in the text) single turnover flash and kept for 30 s at 2 °C in darkness. Then, the sample was linearly heated with a rate of 20 °C per min up to 80 °C with simultaneous recording of TL. The sample was kept either under nitrogen (100 %, v/v), oxygen (100 %, v/v), or normal atmosphere during the whole TL procedure.

Variable fluorescence parameters of the dark adapted state (5 min, as for TL measurements) were derived as described by Schreiber *et al*. (1995).

All data were obtained by averaging 4–8 independent measurements. Standard deviations were calculated from this number of repetitions.

Results

The effects of ozone exposure, aging of irradiated leaves, and transfer of plants from dark to light on TL glow curves: Following ozone exposure, the peak-temperature of the band being at 22 °C in control (based on its properties denoted as TL B-band, see Skotnica *et al*. 2003) was shifted to lower temperature and a TL band appeared at 55 °C (Fig. 1*A*). To analyse if these effects are specific for ozone, other conditions have been tested, too. The down-shift of the B-band and the increase of the intensity of the band at about 55 °C was also found when dark-adapted leaves (16 h) were irradiated for a short time (10 min) (Fig. 1*C*). Similar changes in TL were found when detached leaf segments were kept on water for 3 d (Fig. 1*B*) under irradiation. Hence the mechanism of changes in TL observed after O_3 exposure (increase of the band at about 55 °C and the down-shift of the B-band) might be similar compared to other conditions.

The effect of infiltration by nigericin, antimycin A, and DPI: To investigate if the ozone-induced changes in TL may be influenced by the cyclic electron transport, chlororespiration, or a pH gradient across the thylakoid membrane, the samples were infiltrated by different inhibitors. Nigericin is a well known uncoupler of the trans-thylakoidal pH gradient (Shavit *et al.* 1968). Alternative electron transport processes operating under specific physiological conditions beside the non-cyclic electron transport chain can be inhibited by antimycin A and DPI. Antimycin A is an inhibitor of photosystem 1 (PS1) cyclic electron flow (Hosler and Yocum 1987) as well as of the NADPH dehydrogenase (Endo *et al.* 1998). In parallel to its effect in chloroplasts, antimycin A is a potent inhibitor of mitochondrial respiration (Singer 1979). DPI as an inhibitor of flavoproteins can influence the dark reduction of the PQ pool (Yamane *et al.* 2000). As shown in Fig. 2*B*, nigericin induces an up-shift in the peak-temperature of the B-band and a simultaneous increase of the TL intensity at 55 °C. Antimycin A (Fig. 2*D*) and DPI (Fig. 2*F*) have similar effects on the TL signals as nigericin. The uncoupler and inhibitors reverse the effect of ozone on leaves. Similar effects of infiltrations were found also in control leaves without ozone exposure (Fig. 2*A*,*C*,*E*). However, a decrease in the 55 °C band was only significant in the case of DPI (Fig. 2*E*).

The effect of infiltration by propyl gallate (PG) and 2,5-dimethyl-1,4-benzoquinone: PG, an inhibitor of the terminal PQ oxidase (PTOX, Cournac *et al.* 2000), induced a down-shift in the peak-temperature of the B-band in control leaves (Fig. 3*A*) which could be neglected in ozone treated leaves where the ozone treatment already downshifted the peak temperature of the B band (Fig. 3*B*). The intensity of the 55 °C band was not changed by PG (Fig. 3*A*,*B*). The DMBQ up-shifted the peak-temperature of the B-band and decreased the intensity of the 55 °C band (Fig. 3*C*,*D*).

The effect of dark-adaptation and various atmospheric compositions on the band at about 55 °C: TL glow curves were measured without preceding flash

excitation of the samples at 2 °C. The intensity of the band at 55 °C decreased with time and reached 50 % of the 5 min-dark adapted state approximately after 3 h of dark adaptation (Fig. 4*A*). This time is considerably higher as obtained for thylakoids (5 min, for review see Vass and Inoue 1992). One single turnover flash applied at the end of 4 h dark-adaptation increased the intensity of the band (Fig. 4*B*, the sample was dark-adapted for 5 min after one flash). The band at about 55 °C displayed an intensity that was similar to the sample dark-adapted for 5 min. The ability of reactivation by the very short flashes indicates that the band could be related to PS2 photochemistry (see Discussion).

TL was analysed also under different atmospheric compositions (Fig. 4*C*). If the sample was heated in nitrogen atmosphere, the intensity was not significantly different from that under heating in the presence of oxygen. This behaviour is opposite to that observed for the CL band previously described in barley leaves (Skotnica *et al*. 1999). It indicates that the 55 °C band is not an oxygen-dependent CL band, but is related to PS2 photochemistry.

The effect of ozone on variable fluorescence: The fluorescence parameters of dark adapted leaves, F_v, F_m, and F_v/F_m, were not changed significantly (data not shown) indicating that a non-photochemical quenching and a concomitant considerable ΔpH after 5 min dark adaptation can be ruled out.

Discussion

The band at 55 °C: Several different bands were described at about 50 °C in thermoluminescence glow curve (Desai *et al.* 1975, Rózsa *et al.* 1989, Skotnica *et al.* 1999). In leaves, the main candidates for the explanation of an ozone-induced band at about 55 °C could be the classical C-band or a contribution of an oxygen-dependent chemiluminescence band (Skotnica *et al.* 1999). The experiments with dark-adaptation and different atmospheres after the treatment with ozone support the relation of the band to PS2 photochemistry. The band was found under heating of leaves in nitrogen atmosphere and its intensity was not lower in nitrogen than in air and oxygen. In contrast to these results, the earlier reported chemiluminescence band did not appear under nitrogen atmosphere (Skotnica 1999). The ozone-induced band was re-activated after 1 single-turnover flash. This flash was strong enough to induce charge separation in the majority of PS2, but was probably too short to considerably change conditions of oxygen-dependent chemiluminescence reactions. Since a chemoluminescence band was observed only after previous freezing of the sample (Skotnica *et al*. 1999) the ozone-induced band at about 55 °C measured under non-freezing conditions is probably related to PS2 corresponding to the C-band or another type of charge recombination in PS2. The relation of the band to charge recombination in PS2 is further supported by the inhibitory effects on the intensity of the band by various chemicals influencing PS2 photochemistry (nigericin, antimycin A, DPI, 2,5-dimethyl-1,4-benzoquinone). However, the long lifetime of this band cannot be simply explained by a classical recombination between stabilized charge pairs after the single turn-over flash. The half-time of the classical C band is about 10 min (Vass and Inoue 1996) and represents the longest lifetime of stabilized radical pairs in this temperature range. Hence, the recombining radical pair responsible for the TL emission at 55 °C might not yet be fully present at the start of the TL measurements but is formed during the heating process. Ducruet (1999) suggested that between 40–55 °C electrons could be transferred in a heat stimulated process onto Q_A by reverse electron transfer from Q_B^- forming together with a long-lived positive charge on Y_D^+ at the donor side a TL active radical pair. Recombination of this radical pair will induce light emission in this temperature range. Stress might stimulate this process, probably by a shift of the redox potential of Q_A at the acceptor side to more positive values (Bukhov *et al*. 1990) or by an increased NADPH/NADP ratio when the Calvin cycle is inhibited. The latter can stimulate redox components of cyclic electron flow which could lead to reduction of the PQ pool triggering the above described mechanism. Endo *et al.* (1998) could show a dark-induced reduction of the PQ pool by addition of NADPH to osmotically ruptured chloroplasts. Furthermore, the stress-induced increase of the 55 °C band may also be attributed to a change at the donor side of PS2 that results in a higher number of reaction centres with the normally redox inactive tyrosine Y_D now being oxidized. Different stress factors inactivate the water splitting apparatus of PS2 and increase the yield of Y_D^+ (Hideg *et al*. 1993, Krieger *et al*. 1993). However, beside PS2 radical pair recombination a small contribution of a chemiluminescence component independent of oxygen can not be fully excluded.

A direct influence of a pH gradient on the shape of TL glow curves can be neglected, since significant quenching of the variable fluorescence F_v was not observed after the 5 min dark adaptation. Hence, the most important changes in TL curves induced by stress and/or inhibitor infiltration might be due to changes in the ratio of reduced to oxidized PQ and the associated changes in the reducing and energy equivalents. However, the presence or absence of a pH gradient before the application of 20 single turn-over flashes at room temperature, can indirectly influence the ratio of reduced to oxidized PQ at the start of the TL measurements. The ratio of cyclic to non-cyclic electron transport, depending significantly on the physiological or stress status of the photosynthetic apparatus, will also influence the reduction state of the PQ pool. Furthermore, different mechanisms, such as

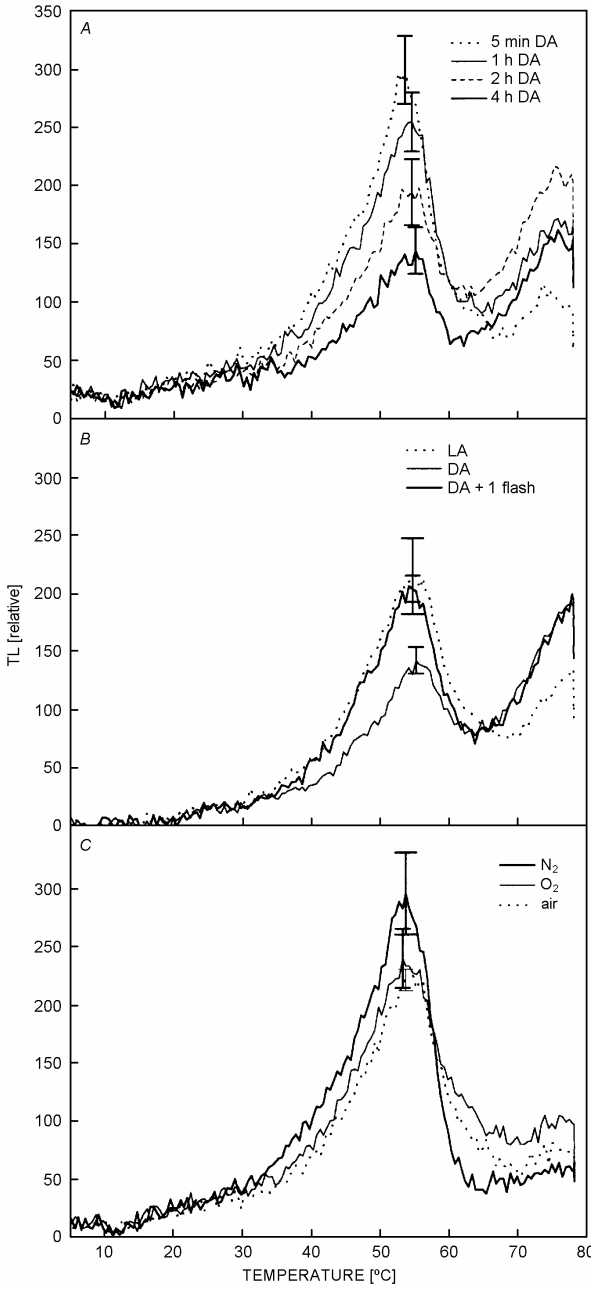

Fig. 1. The effects of ozone exposure (*A*), aging of leaves under irradiation (*B*), and transfer of plants from dark to light (*C*). *A*: The leaves were exposed by ozone (0.9 mg m^{-3}, 6 h, 200 µmol m^{-2} s^{-1} PPFD) and then TL was measured as described in Materials and methods (5 min of dark-adaptation before cooling, 1 flash). *B*: The detached barley leaf segments of 3 cm length were kept for 3 d on water under regime 16/8 h light/dark, PPFD of 150 µmol m^{-2} s^{-1}, and then TL was measured as described in Materials and methods (5 min of dark-adaptation before cooling, 1 flash). *C*: The plants were dark-adapted for 16 h in a dark box and then were transferred for 10 min to PPFD of 200 µmol m^{-2} s^{-1}. Then TL was measured as described in Materials and methods (5 min of dark-adaptation before cooling, 1 flash). LA = light-adapted (8 h, 200 µmol m^{-2} s^{-1}; DA = 16 h dark-adapted and 10 min light-adapted at 200 µmol m^{-2} s^{-1}.

chlororespiration, the photoaccumulation of reducing equivalents (Asada *et al*. 1993, Mano *et al*. 1995) or the metabolite and energy exchange between chloroplasts and mitochondria can lead to a reduction of the PQ pool in the dark, *i.e*. within the 5 min period of dark adaptation. The experiments with inhibitors, uncouplers, and artificial electron acceptors provide strong evidence for the significant influence of the redox state of the PQ pool on the shape and the emission intensity of TL curves.

The suppression of the stress-induced 55 °C band by antimycin can be explained by its function as an inhibitor of cyclic electron transport around PS1 which results in a more oxidized PQ pool after 20 single turn-over flashes due to the exclusively operating linear electron transport chain. A more oxidized PQ pool will diminish the probability for a thermally induced back-transfer of electrons onto oxidized Q_A *via* Q_B generating the TL active radical pair state $Y_D^+Q_A^-$ after ozone or other stresses. Furthermore, antimycin can inhibit the NADPH dehydrogenase (NDH) that plays an important role in the dark reduction of the PQ pool and the concomitant post-irradiation increase in fluorescence (Endo *et al*. 1998). Alternatively, beside a direct effect on chloroplast functions an indirect effect of antimycin A by the inhibition of mitochondrial respiration can not be excluded. According to Gans and Rebeille (1990) the accumulation of reducing power is provoked by inhibition of mitochondrial respiration. Due to shuttling of reducing equivalents between both organelles, significant reduction of the PQ pool was reported by these authors in response to inhibition of mitochondrial activity.

Nigericin, as an H^+/K^+ exchanger, will uncouple the electron transport chain by quenching the trans-thylakoid pH gradient. The consequence is a higher electron transport rate in the light and a more oxidized PQ pool that will probably remain also more oxidized until the start of TL measurements. However, also cyclic electron flow around PS1 and NDH dependent electron flow is inhibited by nigericin that will result in a more oxidized PQ pool too (Endo *et al*. 1998). The result will again be a reduction of the 55 °C band.

DPI inhibits formation of Q_A^- at high temperature by inhibition of a putative flavoenzyme (Yamane *et al.* 2000). Chloroplast NDH complex mediates the dark-reduction of the PQ pool, especially in response to heat stress in leaves (Sazanov *et al.* 1998). Since NDH might also have a role in cyclic electron transport around PS1 (Endo *et al*. 1998), DPI could also inhibit cyclic electron flow in the light as well as the dark reduction of the PQ pool by the chlororespiratory electron pathway in the dark. Both will lead to a more oxidized PQ pool and a suppression of the 55 °C band.

The effect of DMBQ can be explained in a similar way. As an artificial electron acceptor of PS2 it will also induce a more oxidized PQ pool and hence a reduction of

the 55 °C band.

Opposite is the observation in case of PG, an inhibitor of the PQ oxidase in the chlororespiratory electron transport pathway. PG did not change the 55 °C band in ozone stressed leaves, since in ozone stressed leaves the PQ pool seems already to be distinctly reduced compared to leaves without ozone fumigation. Hence, the inhibition of PQH_2 oxidation by PG could not increase the reduction state of the PQ pool.

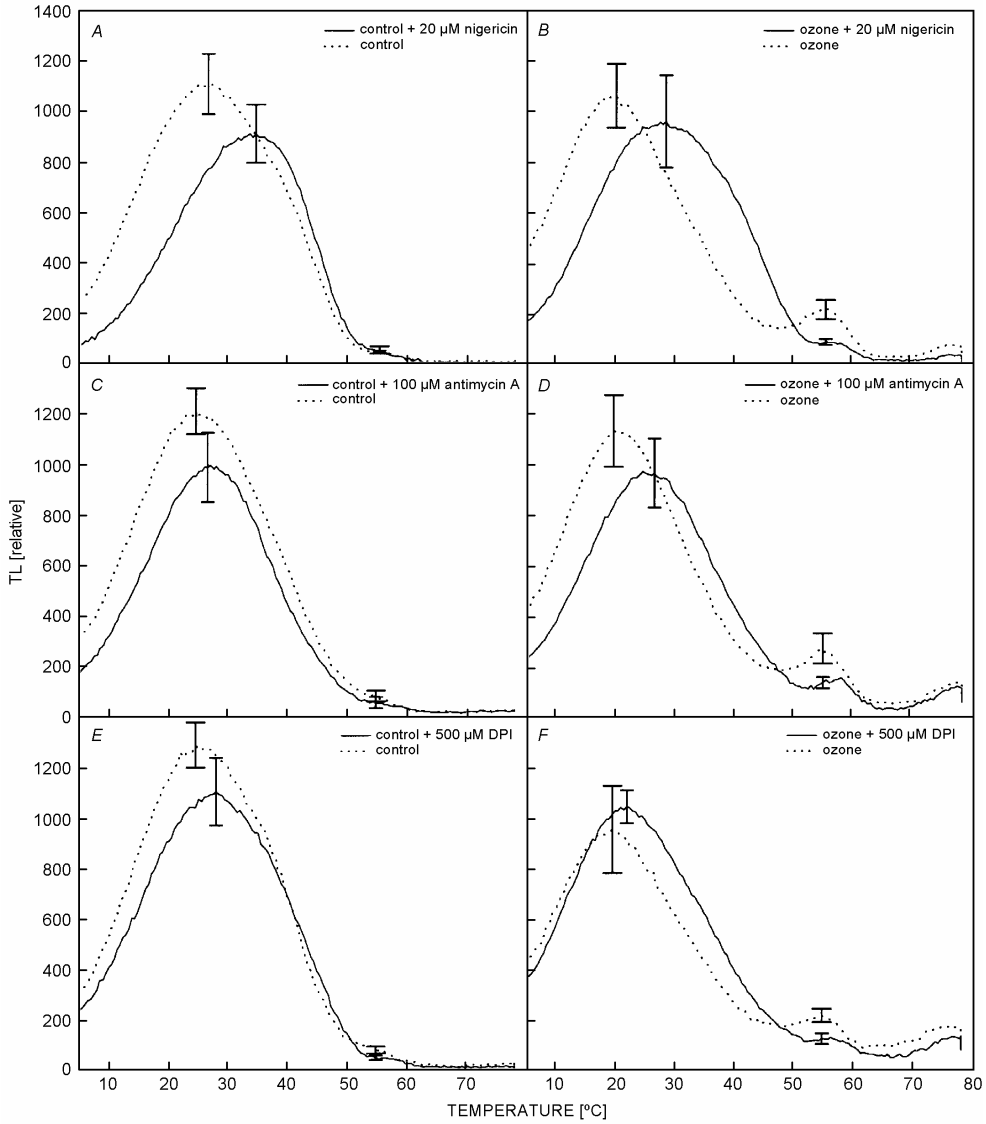

Fig. 2. The effects of infiltration of control and ozone-exposed leaves by 20 µM nigericin (*A, B*), 100 µM antimycin A (*C, D*), and 500 µM DPI (*E, F*). The leaf segments detached from light-adapted leaves were infiltrated for 5 min in dark with the chemicals and then TL was measured as described in Materials and methods (5 min of dark-adaptation before cooling, 1 flash).

The B-band: The main band at about 20 °C corresponds to the TL B band previously observed in various plant material. The band was suppressed by DCMU and its intensity oscillated with a period of four (data not shown). The ozone exposure of leaves induced a down-shift of the peak temperature of the B band. Generally, it can be caused by a modification of donor as well as acceptor side of PS2 (see Vass and Inoue 1992). In literature, the down-shift of the B-band was mainly explained by the increase of the trans-thylakoid pH gradient or by the increased reduction state of the plastoquinone pool (for example see Miranda and Ducruet 1995). The site of damage in case of ozone stress is not absolutely clear from the data: it can affect the dark reactions of the Calvin cycle, the acceptor or the donor side of PS2. However, according to the above discussion of the 55 °C band the acceptor side, *i.e*. the redox state of the PQ pool might play also the major role in the peak shift of the B band. All chemical infiltrations that induce a more oxidized PQ pool, such as antimycin, nigericin,

DPI, and DMBQ resulted in a peak shift of the B-band to higher temperatures as well in control as in ozone treated leaves. This indicates that also under physiologically unstressed conditions different light and dark induced electron transfer processes lead to a partially reduced PQ pool of barley leaves at the start of TL measurements. Beside the peak shifting also a significant decrease in the amplitude of the B band could be observed, however, only in case of DMBQ treatment. Since DMBQ can accept electrons from Q_B^-, this artificial electron acceptor will reduce the number of TL active radical pair states $S_2Q_B^-$ and therefore TL emission. That the redox state of the PQ pool obviously plays a major role for the peak temperature of the B band is further confirmed by the effect of PG. In control leaves, infiltration with PG results in a strong downshift of the peak temperature of the B band. Since PG inhibits the PQ oxidase, the PQ pool will be more strongly reduced by the NDH or flavoenzyme activity shifting the peak of the B band to 20 °C compared to 25 °C in controls without PG. Also ozone treatment shifts the B band to 20 °C. However, after that treatment the PQ pool seems to be distinctly reduced and the inhibition of the PQ oxidase by PG does not further increase its reduction and hence no change in the peak position of the B band occurs. Addition of chemicals to control leaves resulting in a more oxidized PQ pool shifts the B band to 28–35 °C, indicating that the PQ pool of barley leaves is also under physiologically non-stressed conditions in a partially reduced state after 5 min of dark adaptation.

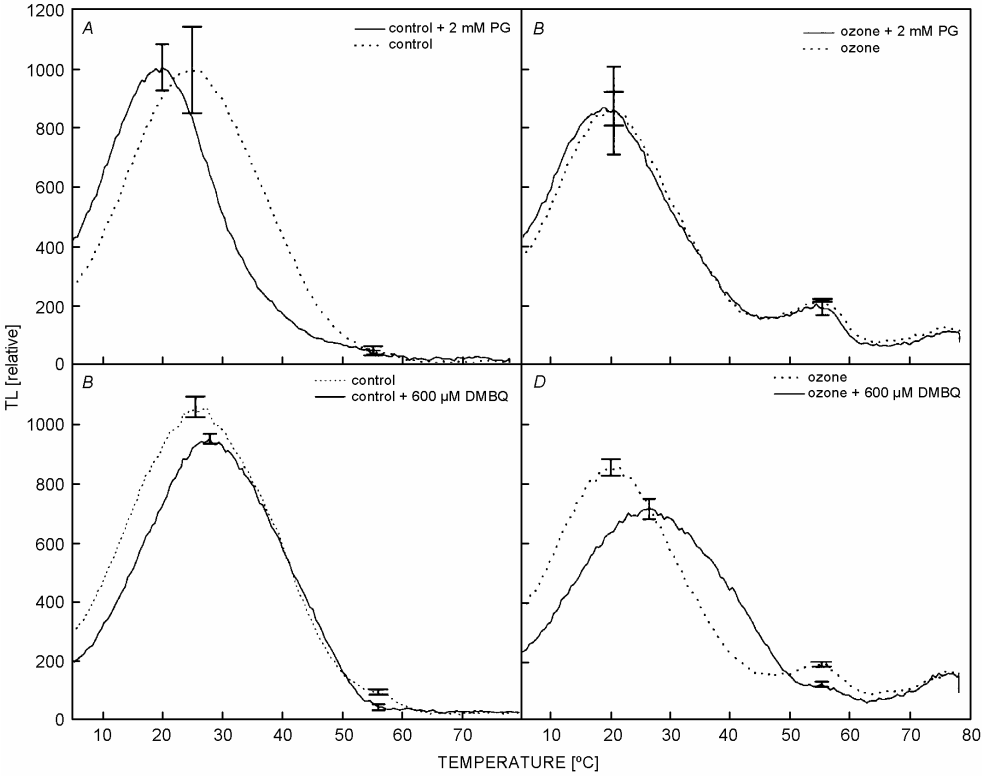

Fig. 3. The effects of infiltration of control and ozone-exposed leaves by 2 mM propyl gallate (*A*, *B*) and 600 µM 2,5-dimethyl-1,4-benzoquinone (*C*, *D*). The leaf segments were infiltrated for 5 min in dark with the chemicals and then TL was measured as described in Materials and methods (5 min of dark-adaptation before cooling, 1 flash).

The effects of different inhibitors in TL measurements are indicating altogether a more reduced PQ pool after ozone fumigation. However, we could not detect an increase in the initial fluorescence level F_0, which has been observed by several authors under *in vivo* and *in vitro* conditions after, *e.g.*, dark adaptation (Endo *et al.* 1998, Feild *et al.* 1998, Chemeris *et al.* 2004). The increase of F_0 might be a consequence of PQ reduction, which is also expected to result in an equilibrium distribution of electrons among the PS2 quinone acceptors, leading to a partial reduction of Q_A (Velthuys and Amesz 1974). The differences between our results and these of other authors might be due to specific variations in experimental conditions. Those who measured under *in vivo* conditions in the dark did it at rather high temperatures above 30 °C (Feild *et al.* 1998, Chemeris *et al.* 2004). This will also considerably influence the equilibrium constant between the PS2 acceptors. Since we measured the fluorescence parameters at about 23 °C, the partial reduction of Q_A was perhaps still neglectable. On the other hand, the partial reduction of Q_A due to a more reduced PQ pool has been

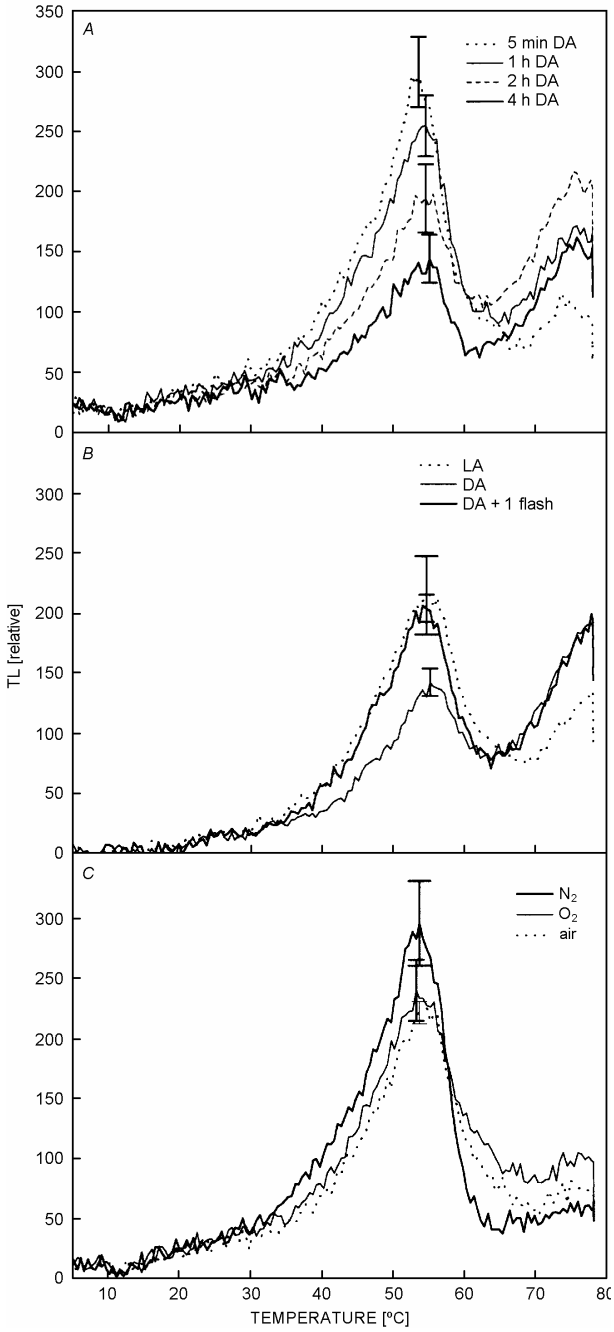

Fig. 4. The effects of dark-adaptation and detection of TL in various gaseous atmospheres on the band at about 55 °C. *A*: The sample prepared from ozone-exposed leaves was dark-adapted for various times (5 min, 1 h, 2 h, 4 h) at room temperature before TL cooling. The sample was not excited by flashes during TL procedure. *B*: The sample prepared from ozone-exposed leaves was used directly for TL measurements (indicated by LA) dark-adapted for 4 h and than measured (indicated by DA) or dark-adapted for 3 h and excited by 1 single turnover flash before TL measurement. During the TL procedure, the sample was cooled after 5 min of dark-adaptation at room temperature and 0 flashes were applied. *C*: The sample prepared from ozone-exposed leaves was heated in various gaseous atmospheres during TL measurement (5 min dark-adaptation, 1 flash).

questioned recently by Berry *et al*. (2002). They found that an 80 % reduction of the PQ pool in *Synechocystis* had no influence on F_0 indicating that a higher equilibrium constant between Q_A and PQ, which also strongly depends on the ΔpH, might prevent the partial reduction of Q_A, even at a rather high reduction state of the PQ pool. Hence, our findings that F_0 does not increase due to ozone fumigation need not to be a contradictory result to the assumed more reduced state of the PQ pool derived from the TL measurements and inhibitor studies.

Since the ozone effect on the peak temperature of the B band can be largely restored by the addition of chemicals directly or indirectly oxidizing the PQ pool, the effect of ozone on PS2 might indeed be an indirect one, leading to regulative changes in PS2 concerning the donor and acceptor side. The same is also true for the ozone induced 55 °C band that is largely suppressed by the chemical treatments. When ozone really damages mainly the ribulose-1,5-bishosphate carboxylase/oxygenase or other enzymes of the Calvin cycle this will result in a photoaccumulation of reductants and energy equivalents concomitant with a more reduced PQ pool triggering the observed changes in PS2 photochemistry.

Conclusions: TL measurements provide an interesting tool in the investigation of stress physiological ozone responses in higher plants. TL indicates that ozone might decrease the ratio of electron delivery to electron consumption and hence changes PS2 photochemistry in a way that TL can detect these changes with high sensitivity. The very sensitive behaviour of the peak temperature of the B band depending on the redox state of the PQ pool could represent an interesting characteristic to monitor this regulative switch in-between both photosystems.

References

Andrée, S., Weis, E., Krieger, A.: Heterogeneity and photoinhibition of photosystem II studied with thermoluminescence. – Plant Physiol. **116**: 1053-1061, 1998.

Asada, K., Heber, U., Schreiber, U.: Electron flow to the intersystem chain from stromal components and cyclic electron flow in maize chloroplasts, as detected in intact leaves by monitoring redox change of P700 and chlorophyll fluorescence. – Plant Cell Physiol. **34**: 39-50, 1993.

Berry, S., Schneider, D., Vermaas, W., Rögner, M.: Electron transport routes in whole cells of *Synechocystis* sp strain 6803: the role of the cytochrome bd-type oxidase. – Biochemistry **41**: 3422-3429, 2002.

Bukhov, N.G., Sabat, S.C., Mohanty, P.: Analysis of chlorophyll *a* fluorescence changes in weak light in heat treated *Amaranthus* chloroplasts. – Photosynth. Res. **23**: 81-87, 1990.

Castillo, F.J., Greppin, H.: Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. – Environ. exp. Bot. **28**: 231-238, 1988.

Chameides, W.L.: The chemistry of ozone deposition to plant leaves: Role of ascorbic acid. – Environ. Sci. Technol. **23**: 595-600, 1989.

Chemeris, Y., Shenderova, L.V., Venediktov, P.S., Rubin, A.B.: Activation of chlororespiration increases chlorophyll fluorescence yield in *Chlorella* adapted to darkness at high temperature. – Biol. Bull. **31**: 143-150, 2004.

Cournac, L., Redding, K., Ravenel, J., Rumeau, D., Josse, E. M., Kuntz, M., Peltier, G.: Electron flow between photosystem II and oxygen in chloroplasts of photosystem I-deficient algae is mediated by a quinol oxidase involved in chlororespiration. – J. biol. Chem. **275**: 17256-17262, 2000.

Demeter, S., Goussias, C., Bernát, G., Kovács, L., Petrouleas, V.: Participation of the g = 1.9 and g = 1.82 EPR forms of the semiquinone-iron complex, $Q_A^-.Fe^{2+}$ of photosystem II in the generation of the Q and C thermoluminescence bands, respectively. – FEBS Lett. **336**: 352-356, 1993.

Desai, T.S., Sane, P.V., Tatake, V.G.: Thermoluminescence studies on spinach leaves and *Euglena*. – Photochem. Photobiol. **21**: 345-350, 1975.

Ducruet, J.M.: Relation between the heat-induced increase of F_0 fluorescence and a shift in the electronic equilibrium at the acceptor side of photosystem 2. – Photosynthetica **37**: 335-338, 1999.

Ducruet, J.M., Toulouse, A., Roman, M.: Thermoluminescence of plant leaves. Instrumental and experimental aspects. – In: Garab, G. (ed.): Photosynthesis: Mechanisms and Effects. Vol. V. Pp. 4353-4356. Kluwer Academic Publ., Dordrecht – Boston – London 1998.

Endo, T., Shikanai, T., Sato, F., Asada, K.: NAD(P)H dehydrogenase-dependent, antimycin A-sensitive electron donation to plastoquinone in tobacco chloroplasts. – Plant Cell Physiol. **39**: 1226-1231, 1998.

Farineau, J., Laval-Martin, D.: Oxygen-evolving system and secondary quinonic acceptors are highly reduced in dark-adapted *Euglena* cells: A thermoluminescence study. – Photosynth. Res. **32**: 167-180, 1992.

Feild, T.S., Nedbal, L., Ort, D.R.: Nonphotochemical reduction of the plastoquinone pool in sunflower leaves originates from chlororespiration. – Plant Physiol. **116**: 1209-1218, 1998.

Gans, P., Rebeille, F.: Control in the dark of the plastoquinone redox state by mitochondrial activity in *Chlamydomonas reinhardtii*. – Biochim. biophys. Acta **1015**: 150-155, 1990.

Graan, T., Ort, D.R.: Detection of oxygen-evolving photosystem II centers inactive in plastoquinone reduction. – Biochim. biophys. Acta **852**: 320-330, 1986.

Hideg, É., Sass, L., Barbato, R., Vass, I.: Inactivation of photosynthetic oxygen evolution by UV-B irradiation: A thermoluminescence study. – Photosynth. Res. **38**: 455-462, 1993.

Hosler, J.P., Yocum, C.F.: Regulation of cyclic photophosphorylation during ferredoxin-mediated electron transport. Effect of DCMU and the NADPH/$NADP^+$ ratio. – Plant Physiol. **83**: 965-969, 1987.

Johnson, G.N., Boussac, A., Rutherford, A.W.: The origin of 40-50°C thermoluminescence bands in Photosystem II. – Biochim. biophys. Acta **1188**: 85-92, 1994.

Krieger, A., Weis, E.: Energy dependent quenching of chlorophyll-*a*-fluorescence: The involvement of proton-calcium exchange at photosystem 2. – Photosynthetica **27**: 89-98, 1992.

Krieger, A., Weis, E., Demeter, S.: Low-pH-induced Ca^{2+} ion release in the water-splitting system is accompanied by a shift in the midpoint redox potential of the primary quinone acceptor Q_A. – Biochim. biophys. Acta **1144**: 411-418, 1993.

Laisk, A., Kull, O., Moldau, H.: Ozone concentration in leaf intercellular spaces is close to zero. – Plant Physiol. **90**: 1163-1167, 1989.

Mano, J., Miyake, C., Schreiber, U., Asada, K.: Photoactivation of the electron flow from NADPH to plastoquinone in spinach chloroplasts. – Plant Cell Physiol. **36**: 1589-1598, 1995.

Miranda, T., Ducruet, J.M.: Effects of dark- and light-induced proton gradients in thylakoids on Q and B thermoluminescence bands. – Photosynth. Res. **43**: 251-262, 1995.

Mudd, J.B.: Biochemical basis for the toxicity of ozone. – In: Yunus, M., Iqbal, M. (ed.): Plant Response to Air Pollution. Pp. 267-283. John Wiley and Sons, Chichester 1996.

Reichenauer, T.G., Bolhàr-Nordenkampf, H.R.: Mechanisms of impairment of the photosynthetic apparatus in intact leaves by ozone. – Z. Naturforsch. **54c**: 824-829, 1999.

Rózsa, Z., Droppa, M., Horváth, G.: On the origin of the thermoluminescence band at around +50 °C in isolated subchloroplast particles. – Biochim. biophys. Acta **973**: 350-353, 1989.

Salter, L., Hewitt, C.N.: Ozone-hydrocarbon interactions in plants. – Phytochemistry **31**: 4045-4050, 1992.

Sandermann, H., Jr.: Ozone and plant health. – Annu. Rev. Phytopathol. **34**: 347-366, 1996.

Sazanov, A.L., Burrows, P.A., Nixon, P.J.: The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. – FEBS Lett. **429**: 115-118, 1998.

Schreiber, U., Hormann, H., Neubauer, C., Klughammer, C.: Assessment of photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis. – Aust. J. Plant Physiol. **22**: 209-220, 1995.

Shavit, N., Dilley, R.A., San Pietro, A.: Ion translocation in isolated chloroplasts. Uncoupling of photophosphorylation and translocation of K^+ and H^+ ions induced by nigericin. – Biochemistry **7**: 2356-2363, 1968.

Singer, T.P.: Mitochondrial electron-transport inhibitors. – Methods Enzymol. **55**: 454-462, 1979.

Skotnica, J., Fiala, J., Ilik, P., Dvorak, L.: Thermally-induced chemiluminescence of barley leaves. – Photochem. Photobiol. **69**: 211-217, 1999.

Skotnica, J., Gilbert, M., Weingart, I., Wilhelm, C.: Thermo-

luminescence as a tool for monitoring ozone-stressed plants. – Environ. Pollut. **123**: 15-20, 2003.

Vass, I., Inoue, Y.: Thermoluminescence in the study of photosystem II. – In: Barber, J. (ed.): The Photosystems: Structure, Function and Molecular Biology. Pp. 259-294. Elsevier, Amsterdam – London – New York – Tokyo 1992.

Vavilin, D.V., Ducruet, J.M.: The origin of 115-130 °C thermoluminescence bands in chlorophyll-containing material. – Photochem. Photobiol. **68**: 191-198, 1998.

Velthuys, B.R., Amesz, J.: Charge accumulation at the reducing side of system 2 of photosynthesis. – Biochim. biophys. Acta **333**: 85-94, 1974.

Venedikov, P.S., Matorin, D.N., Kafarov R.S.: [Chemiluminescence of chlorophyll upon lipid photoperoxidation in thylakoid membranes.] – Biofizika (Moskva) **34**: 241-245, 1989. [In Russ.]

Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., Weigel, H.J., Overmyer, K., Kangasjärvi, J., Sandermann, H., Langebartels, C.: Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. – Plant Cell Environ. **25**: 717-726, 2002.

Yamane, Y., Shikanai, T., Kashino, Y., Koike, H., Satoh, K.: Reduction of Q_A in the dark: Another cause of fluorescence F_o increases by high temperatures in higher plants. – Photosynth. Res. **63**: 23-34, 2000.