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UV-B induced alteration of oxygen evolving reactions in pea thylakoid membranes as affected by scavengers of reactive oxygen species

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Abstract

The effect of UV-B irradiation at temperatures of 22 and 4 °C on flash induced oxygen yields, photochemical activity, and energy transfer in pea thylakoid membranes in the absence and presence of scavengers of reactive oxygen species (ROS) was studied. Three different scavengers were used: dimethyl sulfoxide (DMSO), histidine (His), and *n*-propyl gallate (nPG). As result of the UV-B treatment of isolated membranes, the flash oxygen yields were considerably affected – the amplitudes decreased and the oscillation pattern was lost. The analysis of the flash oxygen yields and initial oxygen burst showed alterations of a number of oxygen evolving centers in the S_0 state as well as changes of decay kinetics of the oxygen burst under continuous irradiation. ROS scavengers exhibited more or less expressed protective effects, nPG being the most effective against UV-B induced damages of the flash oxygen yields. At both the temperatures, photosystem II (PS II) mediated electron transport was more sensitive to the UV-B treatment in comparison with photosystem I (PS I). The analysis of 77 K fluorescence spectra showed that the fluorescence ratio F735/F685 increased by the UV-B treatment probably due to a redistribution of excitation energy between both photosystems most likely caused by partial unstacking and due to a decrease of PS II fluorescence resulting from reaction center-type quenching. The nPG was the most powerful scavenger which protected the oxygen evolution capacity of PS II in the absence and presence of an exogenous electron acceptor to the highest extent.

Additional key words: 77 K fluorescence, DMSO, flash oxygen yields, histidine, initial oxygen burst, *n*-propyl gallate, oxygen evolution, *Pisum sativum*, ROS.

Introduction

UV-B radiation (280 - 320 nm) is known to be very harmful to all biological organisms and can induce damages of their structure and function. Photosynthesizing organisms and especially higher plants are particularly sensitive to increased UV-B. Plant response to increased UV-B involves an activation of a defense system by stimulation ROS scavenging enzyme activities and accumulation of UV-B absorbing compounds (Fedina *et al*. 2007, 2010). The UV-B induces structural alterations in chloroplast grana and stroma structures and in membrane lipids (Hollosy 2002). One of the most susceptible parts is the photosynthetic apparatus and specifically the pigment-protein complexes of photosystem (PS) II. UV-B induces the impairment of PS II mediated electron transport and degradation of D1 protein (Friso *et al*. 1994, Strid *et al*. 1994, Hideg and Vass 1996). UV-B exposure of *Spirulina platensis* results in alterations of fluorescence emission of pigment-protein complexes of thylakoids and a considerable decrease of PS II activity, whereas almost no effect on PS I (Rajagopal *et al*. 2000). *In vitro* studies have shown that the most sensitive component of the PS II electron transport is the water-oxidizing complex and particularly the manganese cluster (Renger *et al*. 1989, Hideg *et al*. 1993). Besides the manganese cluster, Renger *et al*. (1989) also suggested Q_A-Q_B-apoproteins as target, while later on, Szilard *et al*. (2007) showed a correlation between UV-B induced damages and the oxidation state of the water-splitting complex. Barbato *et al*. (1995) reported that degradation of D1 protein under UV-B irradiation occurs in the presence of the functional manganese cluster on the donor side. It is believed that

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Abbreviations: BQ - 1,4 benzoquinone; Chl - chlorophyll; DCMU - 3-(3,4dichlorophenyl)-1,1-dimethyl urea; DCPIP - 2,6-dichlorophenol-indophenol; DMSO - dimethyl sulfoxide; His - histidine; MDA - malondialdehyde; MES - 2-[N-morpholino] ethanesulfonic acid; nPG - *n*-propyl gallate; PS - photosystem; ROS - reactive oxygen species; S_i - redox states of water oxidizing complex; Tricine - *N*-[tris(hydroxymethyl) methyl] glycine; Tris - 2-amino-2-hydroxymethyl-propane-1,3-diol; UVB_{BE} - biological effectiveness of UV-B radiation.

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redox components of PS II, quinone acceptors and tyrosine donors, could be affected directly by UV-B, but the possibility for impairment of these components due to damage of protein environment could not be excluded (Vass *et al*. 2005 and refs. therein). The mechanism of the UV-B damaging effect on photosynthetic apparatus (the primary target and consecutive steps) are widely discussed in literature, however, it is far from being completely understood. There is a general agreement that ROS are generated as result of UV-B irradiation and involved in signaling and inactivation processes (Jordan 1996, Mackerness *et al*. 1999, Šnyrychová *et al*. 2007). Using fluorescence sensors, Barta *et al*. (2004) have shown that UV radiation generates ROS (especially superoxide and singlet oxygen) in spinach leaves depending on the wavelengths, and concomitantly reduces photosynthetic activity measured by variable fluorescence. He and Häder (2002) have shown the generation of ROS under UV radiation in cyanobacterium *Anabaena sp*. However, there is a discrepancy about the identification of ROS involved in UV-B induced damages of photosynthetic apparatus. Whereas some authors suppose involvements of singlet oxygen, hydroxyl radical, superoxide anions, and hydrogen peroxide (Mackerness *et al*. 1999), Hideg and Vass (1996) suggested that UV-B irradiation of isolated thylakoid membranes at room temperature does not result in the production of singlet oxygen although other oxygen radicals are induced.

Despite the numerous publications devoted to UV-B action on photosynthetic organisms, to the best of our knowledge, there are no data concerning the effect of exogenous scavengers of ROS on UV-B induced damages of photosynthetic apparatus and on their possible protective action on primary photosynthetic processes. In addition, no comparative study of a UV-B effect at room and low temperatures is reported although in nature, the higher doses of UV-B irradiation at high altitudes are accompanied by low temperature. Recently, it has been shown that temperature is a main factor, influencing photosynthetic performance during senescence of green leaves (Xue *et al*. 2012) and its effect on UV-B induced alteration of PS II photochemistry could be supposed. The aim of the present work was to study the effect of UV-B radiation on the oxygen evolving process and energy distribution in isolated thylakoid membranes in the presence of three scavengers of ROS, and to compare their effect at room (22 °C) and low (4 °C) temperatures. By using different scavengers – dimethyl sulfoxide (DMSO), histidine (His), and *n*-propyl gallate (nPG) – we evaluated which ROS dominated to the inactivation during the UV-B treatments of isolated thylakoid membranes at both studied temperatures.

Materials and methods

Thylakoid membranes from 14-d-old pea (*Pisum sativum* L., cv. Ran 1) leaves were isolated following the procedure described previously (Velitchkova and Popova 2005). The final pellet was resuspended in a medium containing 0.33 M sucrose, 10 mM Tricine (pH 7.5), 5 mM $MgCl_2$, and 10 mM NaCl. Chlorophyll (Chl) content was determined in acetone extracts according to Lichtenthaler (1987) by measuring absorbances at 663 and 646.8 nm using a spectrophotometer *Specord 210 Plus* (*Analityk-Jena AG*, Jena, Germany). DMSO, His and nPG were used as scavengers of ROS based on data about their use and protective effect in studies of photoinhibition of isolated thylakoid membranes and submembrane particles (Mishra *et al*. 1994, Jacob and Heber 1996, Sonoike 1996). The scavengers were applied at appropriate concentrations suggested by Jacob and Heber (1996) for DMSO, Sonoike (1996) for nPG, and Mishra *et al*. (1994) for His.

The thylakoid membranes in Petri dishes were irradiated with UV-B at 22 or 4 °C for 30 min (and where indicated for 60 min) in the absence or presence of 0.7 M DMSO, 25 mM His, or 1 mM nPG. The content of Chl *a*+*b* during the UV-B treatment was 150 μg per cm^3 of thylakoid membranes. The UV-B radiation was supplied by narrow-band UV-B (312 nm) fluorescent tubes (*TL 20W/01 R*, *Philips*, Hamburg, Germany), giving biologically effective UV-B (UVB_{BE}) of aprox. 0.3 W m^{-2}. As the emission of UV lamps decreases sharply at shorter wavelengths and is almost negligible at 280 nm, and as the results from our preliminary experiments with a cellulose diacetate filter to cut off UV-C showed almost no differences between observed effects (less than 1 % with and without the filter), further experiments were carried out without the cellulose filter. Respective control samples, without or with scavengers, were kept under the same conditions, except for the UV-B irradiation.

Photochemical activity was measured polarographically by a Clark-type electrode (model *DW1*, *Hansatech Instruments*, King's Lynn, Norfolk, UK) in a temperature controlled vessel and at saturating white radiation. Activity of PS II (steady state oxygen evolution) was determined by the rate of oxygen evolution with an exogenous electron acceptor, 0.4 mM 1,4-benzoquinone (BQ) in a reaction medium containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and a 20 mM MES buffer (pH 6.5). PS I mediated electron transport was determined by the degree of oxygen uptake in a medium containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM Tricine (pH 7.5), 0.4 μM DCMU, 0.5 mM NH_4Cl, 5 mM NaN_3, and artificial electron donors and acceptor: 0.1 mM 2,6-dichloro-phenol-indophenol (DCPIP), 4 mM Na ascorbate, and 0.1 mM methyl viologen. Both the photochemical activities were measured at 22 °C and at a Chl *a*+*b* content of 25 μg cm^{-3}. The effect of scavengers was evaluated as percentage of the activity of the respective control.

Determination of oxygen flash yields and an initial oxygen burst without the addition of the exogenous electron acceptor was performed using a home-constructed equipment described in details by Zeinalov (2002). Its main device is a fast oxygen rate electrode equipped with a system for flashes, and modulated and continuous irradiation, permitting the estimation of oxygen production reactions. The rate electrode used in our work is a modified Joliot-type rate electrode. The sample compartment is 0.1 cm^3 and this bigger sample volume increases reproducibility of flash oxygen yields, so we obtained stable and repeatable signals at multiple experiments. Each sample (0.1 cm^3) was pre-treated with 25 flashes followed by a 5 min dark adaptation. For measuring flash oxygen yields, the thylakoid membranes were irradiated with white short (10 μs) saturating (4 J) flashes with a dark period of 0.466 s between the flashes. For continuous irradiation (10 s) measurements, a cold radiation supplier (*LED LXHL-LW3C*, *Luxeon*, *Philips Lumileds Lighting Company*, San Jose, USA) was used, providing white irradiation of 420 μmol m^{-2} s^{-1}on the surface of the sample. Data were digitized by a built-in A/D converter and transferred to a computer for further analysis. The flash oxygen yields were calculated by a software based on fitting the experimentally obtained values to theoretically calculated oxygen burst yields according to the non-cooperative Kok's model of oxygen evolution (Kok *et al*. 1970). For determination of kinetic parameters of an initial oxygen burst, a deconvolution of the oxygen burst decay was performed by fitting the function with two exponential components with different rate constants and different amplitudes (A_1 for the "fast" component and A_2 for the "slow" one). For all measurements, the thylakoid membranes were resuspended in a buffer containing 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and 20 mM MES (pH 6.5) at a Chl *a+b* content of 150 μg cm^{-3}, without the added artificial electron acceptor.

For 77 K fluorescence measurements, samples were taken from the control and UV-B irradiated suspensions and diluted to the Chl *a+b* content of 10 μg cm^{-3}, transferred into a tube for fluorescence measurement and immediately frozen in liquid nitrogen. Low temperature (77 K) fluorescence emission spectra were registered by a *Jobin Yvon JY3* (*Division d'Instruments S.A.*, Longjumeau, France) spectrofluorometer equipped with a red sensitive photomultiplier (*Hamamatsu 928, Hamatsu Photonics,* Japan) in a low temperature device in the presence of 2 μM fluorescein isothiocyanate as internal standard. The width of excitation and emission slits were 4 nm. Data were digitized by an in-built A/D converter and transferred to a computer for further retrieval and analysis. Fluorescence ratios F735/F685 and F685/F695 were calculated after subtraction of the baseline.

The degree of lipid peroxidation in the thylakoid membranes was evaluated as amount of malondialdehyde (MDA). MDA was determined by the modified method of Esterbauer and Cheeseman (1990). Briefly, 0.4 cm^3 of 0.1 % (m/v) thrichloroacetic acid, 0.5 cm^3 of 0.1 M Tris (pH 7.6), and 1 cm^3 of 0.5 % (m/v) thiobarbituric acid (in 20 % thrichloroacetic acid) were added to 0.1 cm^3 of the thylakoids with the Chl *a+b* content of 150 μg cm^{-3}. This solution was boiled for 30 min in a water bath, centrifuged at 3 200 *g* for 15 min, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. Absorbance at 600 nm for non-specific turbidity was subtracted. The MDA content was determined using the coefficient of absorbance of 154 000 M^{-1} cm^{-1}.

Results

The UV-B treatment was performed at room (22 °C) and low (4° C) temperatures in the absence and presence of ROS scavengers. Irradiation with UV-B at the room temperature for 30 min resulted in the inactivation of PS II to different extent. The well-known oscillation pattern of flash induced oxygen yield was observed in non-irradiated thylakoid membranes. The maximum yield was observed after the third flash (the amplitude Y3 is maximal). The amplitudes and oscillations of the oxygen flash yields were inhibited as result of UV-B irradiation at room temperature for 30 min in the absence of scavengers (Fig. 1*A*) as well as in the presence of either DMSO or His (Fig. 1*B*,*C*). A protective effect was observed in the presence of nPG (Fig. 1*D*). Pronounced UV-B induced changes of decay kinetics of the initial oxygen burst were registered and expressed as slow decline (Fig. 2). The changes of amplitude A were related to the changes of amplitudes of "fast" and "slow" components (A1 and A2, respectively), determined on the basis of deconvolution of experimental oxygen decay traces by two exponential components. Similar traces of flash oxygen yields and initial burst were observed after irradiation of the isolated membranes at 4 °C.

Using the software based on the Kok's non-cooperative mechanism, the population of oxygen evolving centers in S_0 state (the lowest oxidation state) as well as the probabilities for misses (α) (centers do not convert to higher state-zero step advance) and double hits (β) (double step advance) were calculated (Table 1). It is worth noting that in the presence of nPG, the number of centers in S_0 state was significantly lower in the non-irradiated thylakoids at the room and low temperatures. At both the temperatures, the UV-B treatment resulted in an increase of centers in S_0 state, more for the membranes in the absence of any scavengers. At the room temperature, the increase of the S_0 population was detected in thylakoids treated with DMSO and nPG but it was not statistically significant. For all samples, a UV-B-induced increase of centers in S_0 state was more pronounced at the low temperature. The changes of calculated misses and double hits were not significant at the room temperature. At the low temperature, the misses

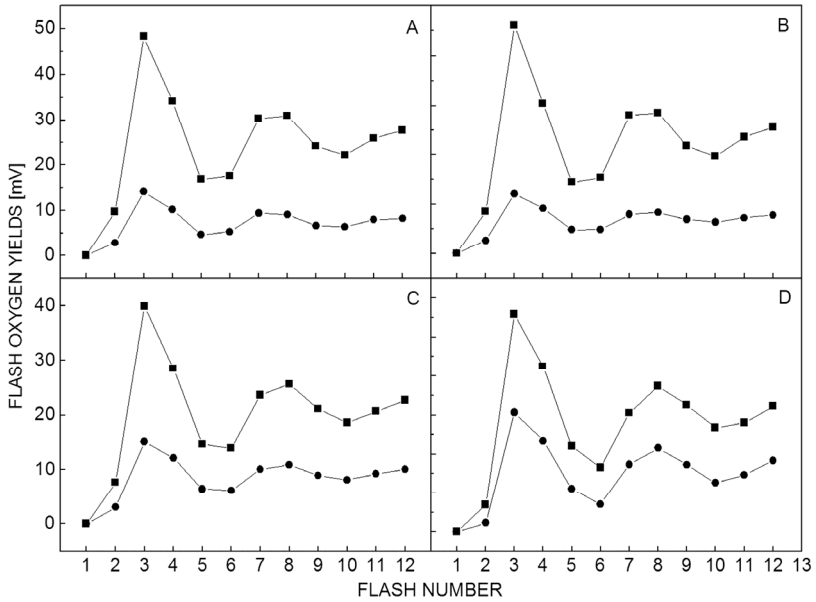

Fig. 1. The effect of UV-B irradiation on flash oxygen yields of pea thylakoid membranes in the absence and presence of three ROS scavengers. The UV-B treatment was performed at room temperature for 30 min. Non irradiated thylakoids (■) and UV-B-treated ones (●). The flash oxygen yields were calculated by the software based on fitting the experimentally obtained values to theoretically calculated oxygen burst yields according to the non-cooperative Kok's model. Control thylakoid membranes without scavengers (*A*), in the presence of 0.7 M DMSO (*B*), 25 mM His (*C*), and 1 mM nPG (*D*).

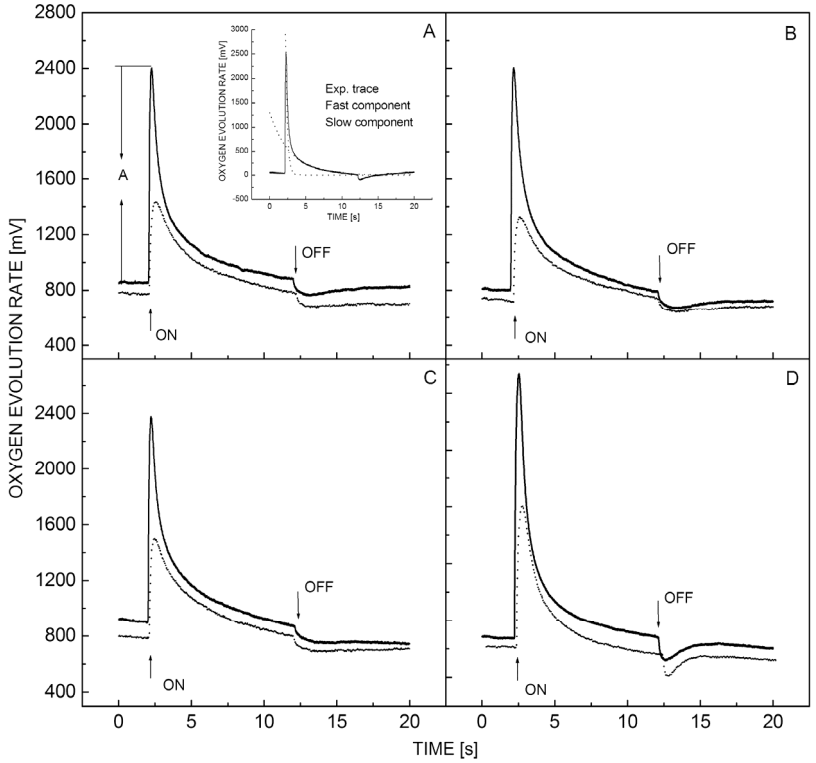

Fig. 2. The effect of UV-B irradiation on initial oxygen burst kinetics under continuous irradiation. Control thylakoid membranes without scavengers (*A*), in the presence of 0.7 M DMSO (*B*), 25 mM His (*C*), and 1 mM nPG (*D*). Non-irradiated thylakoids - *solid line*, UV-B treated - *dotted line*. The UV-B treatment was carried out at 22 °C for 30 min. The inset graph in *A* shows the example deconvolution of experimental trace made by two exponential decay fit. *Solid line* - experimental trace; *dotted lines* - "fast" and "slow" components. ON - turn on the continuous white light, OFF turn off the continuous white light, A - the amplitude of the initial oxygen burst.

Table 1. The number of oxygen-evolving centers in S_0 state [percentage of all centres] and probabilities for misses (α) and double hits (β) for thylakoid membranes irradiated with UV-B at 22 and 4 °C for 30 min. The sum of successful hits (γ), misses (α), and double hits (β) is 1. The mathematical fit of the photosynthetic oxygen evolution amplitudes was performed using the computer-simulating program according to the Kok's model. Mean values ± SE were calculated from 3 (room temperature) and 4 (low temperature) independent experiments. Statistically significant differences of UV-treated samples to its respective controls are calculated (Student *t*-test) and marked by *asterisks* (* - $P < 0.05$; ** - $P < 0.01$) and of non-irradiated membranes with scavengers to control membranes by *crosses* ($^{+}$ - $P < 0.05$; $^{++}$ - $P > 0.01$).

Treatment	S_0		α		β	
	22 °C	4 °C	22 °C	4 °C	22 °C	4 °C
Control - UV-B	22 ± 2	27 ± 1	0.132 ± 0.011	0.101 ± 0.007	0.076 ± 0.004	0.056 ± 0.003
Control + UV-B	27 ± 3	34 ± 4	0.121 ± 0.010	0.125 ± 0.014	0.072 ± 0.009	0.060 ± 0.008
DMSO - UV-B	24 ± 1	24 ± 1^{+}	0.127 ± 0.013	0.107 ± 0.007	0.078 ± 0.006	0.052 ± 0.004
DMSO + UV-B	25 ± 2	30 ± 1**	0.176 ± 0.022	0.142 ± 0.022	0.080 ± 0.009	0.068 ± 0.007*
His - UV-B	25 ± 3	28 ± 1	0.147 ± 0.020	0.113 ± 0.009	0.062 ± 0.010	0.049 ± 0.004
His + UV-B	30 ± 4	35 ± 2*	0.156 ± 0.014	0.112 ± 0.005	0.073 ± 0.012	0.064 ± 0.008
nPG - UV-B	16 ± 0^{+}	17 ± 1^{+++}	0.166 ± 0.016	0.127 ± 0.002	0.051 ± 0.004	0.045 ± 0.003
nPG + UV-B	19 ± 2	23 ± 1**	0.157 ± 0.008	0.145 ± 0.022^{++}	0.042 ± 0.006^{++}	0.048 ± 0.007^{+}

Table 2. The effect of UV-B irradiation at 22 and 4 °C for 30 min on the PS II and PSI photochemical activities (determined by the electron transport rate $H_2O \rightarrow$BQ nad DPIP.H2→MV, respectively), amplitudes of initial oxygen burst (A) and amplitudes of third flash (Y3), both obtained from the original traces. Data are presented as percentages of the respective non-irradiated controls. Mean values ± SE were calculated from 3 independent experiments. Significant differences of UV-B induced inhibition between samples without and in presence of scavengers are marked by *asterisks* (* - $P < 0.05$; ** - $P < 0.01$).

Treatment	PS II		PS I		A		Y3	
	22 °C	4 °C	22 °C	4 °C	22 °C	4 °C	22 °C	4 °C
Control - UV-B	100	100	100	100	100	100	100	100
Control + UV-B	73 ± 4	78 ± 3	104 ± 3	102 ± 3	48 ± 3	41 ± 5	29 ± 4	32 ± 5
DMSO - UV-B	100	100	100	100	100	100	100	100
DMSO + UV-B	83 ± 7	94 ± 3**	105 ± 2	109 ± 7	47 ± 5	43 ± 7	32 ± 7	26 ± 5
His - UV-B	100	100	100	100	100	100	100	100
His + UV-B	82 ± 1*	78 ± 1	98 ± 2	107 ± 4	43 ± 6	49 ± 4	32 ± 5	34 ± 4
nPG - UV-B	100	100	100	100	100	100	100	100
nPG + UV-B	92 ± 6*	88 ± 4*	101 ± 2	110 ± 7	60 ± 3*	56 ± 5*	59 ± 3**	44 ± 3*

increased after the UV-B treatment of the thylakoid membranes without an added scavenger as well as after DMSO addition.

A UV-B induced alteration in the photochemical activity of PS II with and without the added scavengers was measured by an oxygen Clark electrode in the presence of BQ, an artificial electron acceptor of PS II. The presence of the scavengers in the mentioned concentrations did not influence the PS II photochemical activity. The UV-B treatment decreased the photochemical activity of PS II by about 30 % in the absence of any scavenger (Table 2). DMSO and His protected the PS II activity against UV-B damage in a comparable extent (18 - 20 %), but for DMSO this protection was not statistically significant although it was detected as tendency in all experiments. The most effective protection was observed in the presence of nPG where the PS II activity was inhibited by only 8 % (Table 2). These data were compared with the UV-B induced inhibition of oxygen burst under the continuous irradiance evaluated by changes of burst amplitude A (Table 2). The oxygen burst was measured in the absence of exogenous electron acceptor and any damage at the acceptor side of PS II could not be compensated. The observed inhibition of the amplitudes of oxygen burst was more pronounced in comparison with the oxygen evolution rate in the presence of BQ. The reduction of A was accompanied by the retardation of decay kinetics (Fig. 2). The best-fit deconvolution of decay resulted in two exponential components with different rate constants (see Fig. 2*A*, the insert). It is worth mentioning that UV-B affected more considerably the amplitude of the "fast" component (A1) than that of the "slow" one (A2). For thylakoid membranes without any scavenger, A1 decreased by 64 % and A2 by 24 %. The addition of nPG resulted in some protection of A1 and it was reduced after the UV-B treatment by about 46 % (data not shown). Due to the UV-B irradiation, the amplitude of third oxygen flash (Y3) in the control thylakoid membranes decreased up to 29 % in comparison with the non-irradiated control. No

significant protection was observed in the presence of DMSO and His, whereas the most protective effect was realized in the presence of nPG (Y3 was reduced by about 40 %; Table 2). At the room temperature, nPG is the most effective scavenger against UV-B-induced damage of oxygen-evolving activity of thylakoids (Table 2).

PS I mediated electron transport rate was almost not affected by 30 min UV-B irradiation at the room temperature (Table 2). Even at prolonged UV-B irradiation up to 60 min, no significant changes of the rate of PS I mediated electron transport were detected.

As the generation and scavenging of ROS are temperature-dependent processes, we carried out UV-B treatment of the same samples at 4 °C. In the absence of scavengers, PS II activity was less inhibited than that at the room temperature although the difference was not significant. In the presence of exogenous electron acceptor PS II photochemical activity was protected to the highest extent by the presence of DMSO when compared with its protective effect at the room temperature. At the low temperature, nPG was a less effective protector for Y3 in comparison with its action at the room temperature (Table 2). The UV-B treatment at the low temperature also changed the effect of the scavengers on A and its components A1 and A2. In the absence of the scavengers, A2 was more reduced (by 45 %) after the treatment with UV-B at 4 °C. At 4 °C, DMSO exhibited a more protective effect on A2 than at the room temperature (the reduction was by 15 % compared with 30 % at the room temperature) which correlates with the more pronounced protection of total O_2 evolution. During the UV-B treatment at the low temperature, the protective efficiencies of nPG for Y3 and A2 were lower. The PS I activity was almost not affected by the UV-B irradiation at the low temperature (Table 2).

Table 3. The MDA content in thylakoid membranes after their irradiation with UV-B at 22 and 4 °C for 30 min. Data are presented as percentage of the content of MDA in the UV-B unexposed samples without or with added scavengers. Mean values ± SE were calculated from 4 independent experiments. Significance of differences are marked by *asterisks* (* - $P < 0.05$; ** - $P < 0.01$)

Sample	22 °C	4 °C
No scavengers	118 ± 7	103 ± 3
+DMSO	105 ± 2	107 ± 10
+His	98 ± 5*	95 ± 5
+nPG	92 ± 3**	102 ± 9

In order to check the effect of the UV-B treatment on thylakoid membrane integrity, and the possible protective effect of the studied scavengers, the lipid peroxidation in thylakoid membranes was tested according to MDA content. At the room temperature, the presence of the scavengers decreased MDA content and nPG exhibited the highest protection. At the low temperature, the changes of MDA content were not significant. Significant differences in MDA content were observed in the samples without the scavengers after the UV-B treatment at the room temperature.

UV-B induced changes of energy distribution between PS I and PS II were followed by analyzing 77 K fluorescence emission spectra of thylakoid membranes in the absence and presence of the scavengers. The typical 77 K fluorescence emission spectrum after an excitation at 436 nm exhibits three major bands at 685, 695 and 735 nm. The UV-B irradiation at the room temperature changed the spectral shape and the intensity of different bands. At prolonged irradiation, a decrease of the overall intensity of fluorescence was observed, but the ratio between fluorescence at 735 and 685 nm was preserved. (Fig. 3*A*, Table 4). The addition of the scavengers did not alter the ratio F_{735}/F_{685} of the non-irradiated samples indicating that using the scavengers did not affect energy interaction in the non-irradiated thylakoids. The UV-B radiation influenced considerably the ratio F_{735}/F_{685} in all samples indicating an increase of energy delivery to PS I and/or a quencher formation in PS II reaction centers.

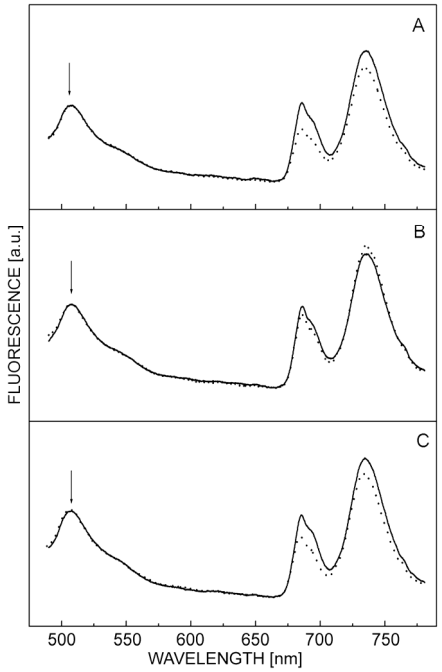

Fig. 3. Fluorescence emission spectra of isolated thylakoid membranes at 77 K. *A* - thylakoid membranes without the scavenges – non-irradiated (*solid line*) and treated with UV-B at room temperature for 60 min (*dotted line*); *B* - thylakoid membranes without the scavengers – non-irradiated (*solid line*) and treated with UV-B for 30 min at room temperature (*dotted line*); *C* - thylakoid membranes in the presence of 1 mM nPG – non-irradiated (*solid line*) and treated with UV-B at room temperature for 30 min (*dotted line*). The *arrows* indicate the emission maximum of an internal standard, fluorescein isothiocyanate. Excitation and emission slits were 4 nm. Excitation wavelength was 436 nm. Chl (*a*+*b*) content was 10 μg cm^{-3}.

The alterations in the fluorescence ratio F_{685}/F_{695}, indicative of the energy interaction within the supercomplex of PS II, were insignificant. For the thylakoid membranes not containing the scavengers and irradiated with UV-B, the ratio F_{685}/F_{695} decreased due to the relatively higher peak at 695 nm. In the presence of DMSO and His, the decline of the ratio F_{685}/F_{695} diminished, and was completely abolished by the presence of nPG. At the 30 min UV-B treatment at 4 °C, the 77 K fluorescence ratios were also altered, the changes being very similar to those observed for the room temperature (data not shown).

Discussion

The supramolecular complex of PS II and especially the oxygen-evolving complex are known to be among the most sensitive components of chloroplasts towards stress factors. Enhanced UV-B irradiation causes serious damages of photosynthetic machinery (Renger *et al*. 1989, Hideg *et al*. 1993, Vass *et al*. 2005). The present study permitted us to characterize in more details UV-B induced damage of oxygen evolving complexes. The results show that after 30 min of the UV-B irradiation at room temperature, the flash oxygen yields were more inhibited in comparison with the oxygen evolution measured in the presence of an electron acceptor BQ. The lower extent of inhibition in the presence of BQ shows that the UV-B irradiation caused a damage in the Mn-cluster at the donor side but also at the acceptor side of PS II. The damage of acceptor side components of PS II has been earlier suggested (Renger *et al*. 1989) and experimentally demonstrated *in vitro* (spinach PS II membranes) and *in vivo* (*Synechocystis* sp. PCC 6803) (Vass *et al*. 1996). It has been shown that during UV-B irradiation of isolated spinach PS II membranes, the primary damage occurs at the water-oxidation complex and subsequently the Q_AFe^{2+} acceptor complex is modified and/or inactivated (Vass *et al*. 1996). Later, Vass *et al*. (1999) have demonstrated that a UV-B treatment of cyanobacteria cells results in a donor side inactivation accompanied by a modification of the Q_B site, thus affecting binding plastoquinone. Benzoquinones, and especially BQ, used in our study, accept electrons mainly from the Q_B site (Satoh *et al*. 1995) and therefore can compensate UV-B induced damage of the Q_B-binding pocket. The observed inhibition of the flash oxygen yields was accompanied by an increased amount of centers in S_0 state (Table 1). This could be due to structural alterations and/or changes of functionality of the oxygen evolving centers as the UV-B treatment could directly affect the Mn-cluster (Vass *et al*. 2005) or might lead to detachment of the 33 kD protein from the PS II complex (Nedunchezhian and Kulandaivelu 1991) which is important for the functional conformation of the catalytic Mn-cluster.

Table 4. The effect of UV-B irradiation at room temperature for 60 min on fluorescence ratios F735/F685 and F685/F695. Data were calculated from the 77 K fluorescence emission spectra after an excitation at 436 nm. Mean values ± SE were calculated from three independent experiments. Significance of differences for every sample to the respective control are marked by *asterisks* (* - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$).

Sample	F_{735}/F_{685}	F_{685}/F_{695}
Control -UV-B	1.54 ± 0.06	1.31 ± 0.15
Control +UV-B	2.45 ± 0.30*	1.11 ± 0.02
DMSO -UV-B	1.40 ± 0.04	1.20 ± 0.07
DMSO +UV-B	2.37 ± 0.12***	0.98 ± 0.04*
His -UV-B	1.39 ± 0.05	1.17 ± 0.15
His +UV-B	2.17 ± 0.18***	1.09 ± 0.03
nPG -UV-B	1.34 ± 0.01	1.17 ± 0.02
nPG +UV-B	1.83 ± 0.10**	1.18 ± 0.09

The UV-B irradiation did not induce a remarkable inhibition of PS I as it has been reported earlier for cyanobacteria and for chloroplasts from higher plants (Rajagopal *et al*. 2000, Vass *et al*. 2005 and reference therein). The UV-B treatment for 30 min did not lead to the inhibition of the photochemical activity of PS I, and even at prolonged irradiation (60 min), no inhibition was observed. UV-B induced damages on PS I (if any) were not significant or they were fully compensated in the presence of artificial electron donors and acceptors used for determination of the electron transport through PS I.

In our experiments, we observed a difference between the extent of inhibition of the flash oxygen yields (Y3) and the amplitude of the initial oxygen burst (A) under the continuous irradiance, both measured without the added electron acceptors. The analysis of the exponential decay shows that the decrease of the amplitude of the oxygen burst was due mainly to the reduction of contribution of "fast" component A1 (about a 60 % inhibition of A1 for the control, DMSO, and His supplied thylakoids), thus indicating a decrease of the number of active oxygen evolving centers and/or modifications of their functionality leading to slower turnover.

Thylakoid lipids are also targets of a UB-V attack, and lipid peroxidation may directly affect membranes and further initiate free radical cascades leading to damages of proteins and other components (Vass *et al*. 2005). In the course of the 30 min UV-B irradiation, several ROS are generated and further involved in the complex process of inactivation of photosynthetic apparatus. The application of three different ROS scavengers, and the analysis of their protective effect gave the possibility to propose if ROS were involved in the UV-B induced inhibition of oxygen evolution, and if so, which ROS dominated to the inactivation process at the room and low temperatures. DMSO penetrates biomembranes and can

serve as trapping reagent for hydroxyl radicals (Jacob and Heber 1996). The effect of His as scavenger of singlet oxygen, and of nPG as scavenger of hydroxyl and alkoxyl radicals has been reported by Sonoike (1996) and by Rajagopal *et al*. (2005). It should be noted that scavengers are not strictly specific and some of them could quench more than one type of ROS. The reaction of scavengers with different ROS could occur but always with different affinity and kinetics, and the effectiveness of every scavenger will depend on the dominant ROS at different conditions. The highest protection of the flash oxygen yields, initial oxygen burst, and oxygen evolution in the presence of BQ is observed in the presence of nPG (Table 2). Although His scavenges singlet oxygen, and DMSO traps hydroxyl radicals, both scavengers protected the oxygen evolution to a comparable extent and the protective effect was not statistically significant. Hideg and Vass (1996) have reported that singlet oxygen is not produced under UV-B irradiation, but in our experiments, we observed a certain protection of the PS II activity at the room temperature. This could be due to either a non-typical occurrence of singlet oxygen or an unknown nonspecific response of His.

The protective effect of DMSO was less expressed than that observed for nPG, thus indicating a different mechanism of protection due to different affinity for trapping hydroxyl radicals or a different site of action. The proposed quenching activity of nPG as powerful antioxidant for alkoxyl radicals and for singlet oxygen as well (Miyao 1994, Mukai *et al*. 2005) could explain its higher effectiveness. Obviously, the hydroxyl radicals were involved in the inhibition process and were responsible for the observed UV-B induced damages of the oxygen evolving machinery in the pea thylakoid membranes. As hydroxyl radicals are highly reactive, act in close distance, and are short living, they are supposed to be produced in the near vicinity of oxygen evolving centers.

To check whether and to what extent the UV-B induced damages were temperature dependent, we carried out the same experiments at 22 and 4 °C. It is worth noting that the protective effect of DMSO at 4 °C was more pronounced than that at the room temperature. The PS II photochemical activity after the UV-B irradiation at 4 °C in the presence of DMSO was 94 % of the non-irradiated sample, whereas after the UV-B treatment at the room temperature, the activity of PS II in the presence of DMSO was 83 % of the control (Table 2). Probably, the UV-B irradiation at 4 °C led to an increase of the content of hydroxyl radicals, and the action of DMSO was more pronounced as the concentration of the hydroxyl radicals was higher and they dominated to the inactivation at this temperature. At 4 °C, nPG was a more powerful protector for oxygen evolution without an exogenous electron acceptor, whereas DMSO showed the highest protection for oxygen evolution in the presence of BQ. It is worth noting that the effectiveness of nPG at 4 °C was lower than at the room temperature, probably due to its lower solubility at low temperature and non-uniform distribution of the scavenger in the sample.

In order to check if UV-B irradiation leads to any structural changes of the PS II complex and/or alterations of energy distribution between pigment-protein complexes of PS I and PS II, we analyzed 77 K fluorescence emission spectra. Under optimal conditions and in the absence of any quenching, the F_{685}/F_{695} ratio is informative about the energy interaction in the complex of PS II and its proximal antenna, whereas F_{735}/F_{685} characterizes the energy distribution between both photosystems (Krause and Weis 1991, Andrizhiyevskaya *et al*. 2005). The analysis of emission spectra at 77 K indicated that no considerable changes in respect to an energy interaction within PS II complexes occurred as the changes of F_{685}/F_{695} ratio were very small (Table 4). As result of the UV-B irradiation, the F_{735}/F_{685} ratio increased in the controls and scavenger-supplied thylakoid membranes thus indicating a possible redistribution of excitation energy in favor of PS I, and quenching PS II fluorescence. It has been reported that in *in vitro* systems (thylakoids and PS II-enriched membranes), the inhibition of electron donation to PS II may allow the special pair Chl_{680}^{+} to act as quencher (Horton and Ruban 1992, Bruce *et al*. 1997). The observed inactivation at the donor side of PS II was more probably accompanied by the formation of a P_{680}^{+} quencher and led to a decrease of PS II fluorescence in the 685 nm region. The ratio F_{685}/F_{695} did not change significantly which means that the inhibition of oxygen evolution did not relate to the alteration of inter-connection between reaction centre and pigment-protein complexes CP43 and CP47.

Our experiments show that temperature during the UV-B treatment did not affect significantly the extent of inhibition of the PS II activity, initial oxygen burst, and flash oxygen yields. However, the protective effect of the used scavengers on oxygen evolution and the parameters of the flash oxygen yields depended on temperature. DMSO exhibited better protection on oxygen evolution in the presence of the exogenous electron acceptor at the low temperature, whereas nPG was more effective for the flash oxygen yields at the room temperature.

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