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BRIEF COMMUNICATION

Effects of high temperature and low pH on photosystem 2 photochemistry in spinach thylakoid membranes

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Abstract

The effects of temperature (25 - 45 °C) and pH (7.5 - 5.5) on photosystem (PS) 2 was studied in spinach (*Spinacia oleracea* L.) thylakoid membranes using chlorophyll *a* fluorescence induction kinetics. In high temperature and low pH treated thylakoid membranes a decline in the variable to maximum fluorescence ratio (F_v/F_m) and PS 2 electron transport rate were observed. More stacking in thylakoid membranes, studied by digitonin fractionation method, was observed at low pH, while the degree of unstacking increased under high temperature conditions. We conclude that the change in pH does not significantly affect the donor/acceptor side of PS 2 while high temperature does. Fluorescence emission spectra at 77 K indicated that low pH is associated with energy redistribution between the two photosystems while high temperature induced changes do not involve energy re-distribution. We suggest that both, high temperature and low pH, show an inhibitory effect on PS 2 but their mechanisms of action are different.

Additional key words: abiotic stress, electron transport chain, fluorescence, *Spinacia oleracea*.

Many environmental factors such as drought, CO_2, solar radiation, temperature and salinity affect various physiological processes including photosynthesis (Kočová *et al.* 2009, Yu *et al*. 2009, Amudha *et al.* 2010, Cai *et al.* 2010**).** The inhibition of photosystem (PS) 2 by heat stress was detected by changes in chlorophyll fluorescence (Srivastava *et al.* 1997, Wang *et al.* 2009, Mathur *et al.* 2011). Heat induced the dissociation of the Mn-stabilizing 33 kDa protein from oxygen evolving complex (OEC) followed by release of Mn atoms (Enami *et al*. 1994, Yamane *et al.* 1998, Tiwari *et al*. 2007), dissociation of light harvesting complex 2 (LHC 2), changes in grana stacking (Gounaris *et al.* 1983) and a shift of the redox equilibrium between Q_A and Q_B (Ducret and Lemoine 1985, Havaux 1989). Heat stress induced changes in PS 2 can be easily studied in isolated thylakoid membranes.

It has also been known that PS 2 activity is highly sensitive to small changes in pH. With isolated intact thylakoid membranes, inhibition occurs at acidic and alkaline pH (Chapman *et al.* 1989). During the day, the chloroplast experience a high irradiance and hence the pH of the thylakoid lumen decreases to 5.5 (Kramer *et al*. 1999). Walters *et al.* (1996) have reported proton sensitive glutamate residues located on the surface of LHC 2 proteins on the lumenal side of thylakoid membranes which act as H^+ sensors. This sensing of pH is a natural protection of PS 2 from injury. OEC is also inactivated at low pH (Krieger *et al*. 1993). Change in pH is known to alter the reorganization of thylakoid membrane. Decrease of pH in the medium from 5.5 to 4.0 leads to shrinkage of the thylakoids (Semenova 2002) and to the release of the calcium cofactor (Krieger *et al.* 1993).

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Abbreviations: DCPIP - 2,6-dichlorophenol indophenol; F_0 - minimum fluorescence; F_m - maximum fluorescence; F_v - variable fluorescence; HEPES - N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid; LHC - light harvesting complex; MES - (2-[N-morpholino]ethanesulfonic acid); M_0 - net rate of PS 2 closure [$dV/dt_0 = 4\cdot(F_{300} - F_{50})/(FM - F_{50})$]; NPQ - non-photochemical quenching; OEC - oxygen evolving complex; PEA - plant efficiency analyser; PI - performance index; PS 1 - photosystem 1; PQH_2 - plastoquinol; PS 2 - photosystem 2; Q_A - quinone type A; Q_B - quinone type B; RC - reaction center; TR - trapping; Vj - relative variable fluorescence at 2 ms or the proportion of RCs closed at 2 ms (unconnected PS 2 only) calculated as $(F_2 - F_{50})/(FM - F_{50})$.

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In tropical countries, the temperature rises above 45 °C during summer and, due to high irradiance at mid-day, the pH of the thylakoid membrane drops near to 5.5. Thus, plants experience dual stress *i.e*. high temperature and a low lumenal pH. Studies done so far focused on the effect of thermal denaturation of thylakoid membranes, but a comparison of thermal effects with pH induced effects has never been made although both, high temperature and low pH, lead to inhibition of PS 2 photochemistry. In the present work, we have investigated the cumulative effects of high temperature and low pH stress on PS 2 photochemistry using Chl *a* fluorescence induction kinetics, fluorescence emission at 77 K and degree of stacking of the thylakoid membranes.

Deveined market spinach (*Spinacea oleracea* L.) was used for the isolation of thylakoid membranes using the method of Kuwabara and Murata (1982). These were stored in 50 % glycerol in liquid nitrogen until further use. The chlorophyll content was estimated by the method of Porra *et al.* (1990). The stored thylakoid membranes were thawed on ice and washed with shock medium (50 mM HEPES-NaOH, 1 mM $MgCl_2$ and 10 mM NaCl) to remove glycerol and to give an osmotic shock. These membranes were incubated for 3 min in dark and centrifuged at 8 200 *g* for 15 min. The resultant pellet was resuspended in the following medium: 0.33 M sucrose, 50 mM HEPES-NaOH (pH 7.5) or MES-NaOH (pH 6.5, 5.5), 1 mM $MgCl_2$ and 10 mM NaCl. The stock medium contained 50 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$ and 10 mM NaCl.

The thylakoid membranes were incubated at 35, 40 or 45 °C in a water bath (*Julabo F10-UC*, *Julabo*, Seelbach, Germany) in dark for 5 min with frequent shaking, and immediately after the temperature treatment, the thylakoid membranes were kept on ice until the experiment. For pH treatment, the thylakoid membranes were incubated in the buffers of the respective pH (5.5, 6.0, 6.5, 7.0, and 7.5) for 15 min.

PS 2 mediated electron transport rate was determined by measuring photoreduction 2,6-dichlorophenol indophenol (DCPIP) at 605 nm using *Visiscan 167 Systronics* spectrophotometer. The irradiance used was 150 W m^{-2}. The reaction mixture composed of the above mentioned medium with 35 μM DCPIP and thylakoid suspension equivalent to 10 μg(Chl) cm^{-3}.

Chl *a* fluorescence transients were measured with the plant efficiency analyzer (*Handy PEA*, *Hansatech*, King's Lynn, Norfolk, UK). The reaction mixture contained above mentioned medium and thylakoid suspension equivalent to 10 μg(Chl) cm^{-3}. Absorbtion in reactive centers (ABS/RC) has been calculated by using the following equation: ABS/RC = $(TR_o/RC)/(TR_o/ABS)$, where TR_o/RC = maximum trapping rate of PS2 (M_0/VJ) and TR_0/ABS = trapping probability. ABS/RC is a measure of the average absorption per (active) RC and concomitantly of the average amount of absorbing chlorophylls per (active) RC, *i.e.* of the apparent antenna size. It is influenced by the ratio of active/inactive RCs (Force *et al.* 2003).

The fluorescence emission spectra at low temperature (77 K) were measured on a spectrofluorometer *Jasco FP-6300* (Tokyo, Japan). Reaction mixture used was the above mentioned medium, thylakoid suspension equivalent to 10 μg(Chl) cm^{-3} and 50 % glycerol. The excitation wavelength was 435 nm with 2.5 nm slit width of the excitation emission monochromator. Fluorescence excitation spectra at 77 K were monitored at 730 nm while scanning the excitation radiation from 635 to 715 nm to estimate the changes in the absorption cross section of PS 1. The reaction mixture and thylakoid suspension were the same as for the emission spectra. Wavelength calibration of the equipment was done using fluorescein isothiocyanate (FITC, isomer I, *Sigma,* St. Louis, USA) and wavelengths of the spectra have been corrected.

Degree of stacking of thylakoid membranes under different pH (7.5 and 5.5) was measured by digitonin fractionation method as described in Chow *et al*. (1980). Thylakoid membranes were allowed to stand in the medium with 0.5 % digitonin of respective pH for 15 min at 0° C. The mixture was then diluted 6 - 7 folds with an ice-cold solution of 200 mM sucrose and 1.5 mM K_2HPO_4 (pH 7.0), centrifuged at 10 000 *g* for 30 min. The fraction of chlorophyll in the pellet was determined by the method of Porra (1990).

Rate of PS 2 mediated electron transport was maximum at pH 7.5 and decreased as we lowered the pH to 5.5 (Fig. 1*A*). At still lower pH (pH 4.5 and 4.0), total loss of PS 2 activity was observed (data not shown), probably because of the total disorganization of the pigment-protein complexes of PS 2. The rates of PS 2 mediated electron transport decreased as the temperature was increased from 25 to 45 ºC (Fig. 1*B*). Almost total loss of PS 2 activity was observed at 45 ºC.

Effects of combined stress were analyzed in experiments with temperature stress given in the isolated thylakoid membranes at different pH values (5.5, 6.5, 7.5). The representative Chl *a* fluorescence induction curves (designated OJIP curves) in spinach thylakoid membranes after treatment with high temperature and different pH are shown on Fig. 1*C*,*D*. The O-J step is related to the PS 2 primary electron acceptor (Q_A) reduction. The J-I and I-P phases attribute to the reduction of the acceptor side of PS 2, or more specifically to the reduction of two distinct plastoquinone pools (Boisvert *et al.* 2006). The lack of a clear I step in isolated thylakoid is related to a change in reduction and oxidation kinetics of PQ in this material as compared to intact leaves (Joly *et al.* 2010). We observed that both, low pH and high temperature, lead to a decrease in PS 2 photochemistry.

The OJIP test represents a translation of the original data to biophysical parameters that quantify the energy flow through PS 2. The shape of the O-J-I-P transient has been found to be very sensitive to stress caused by

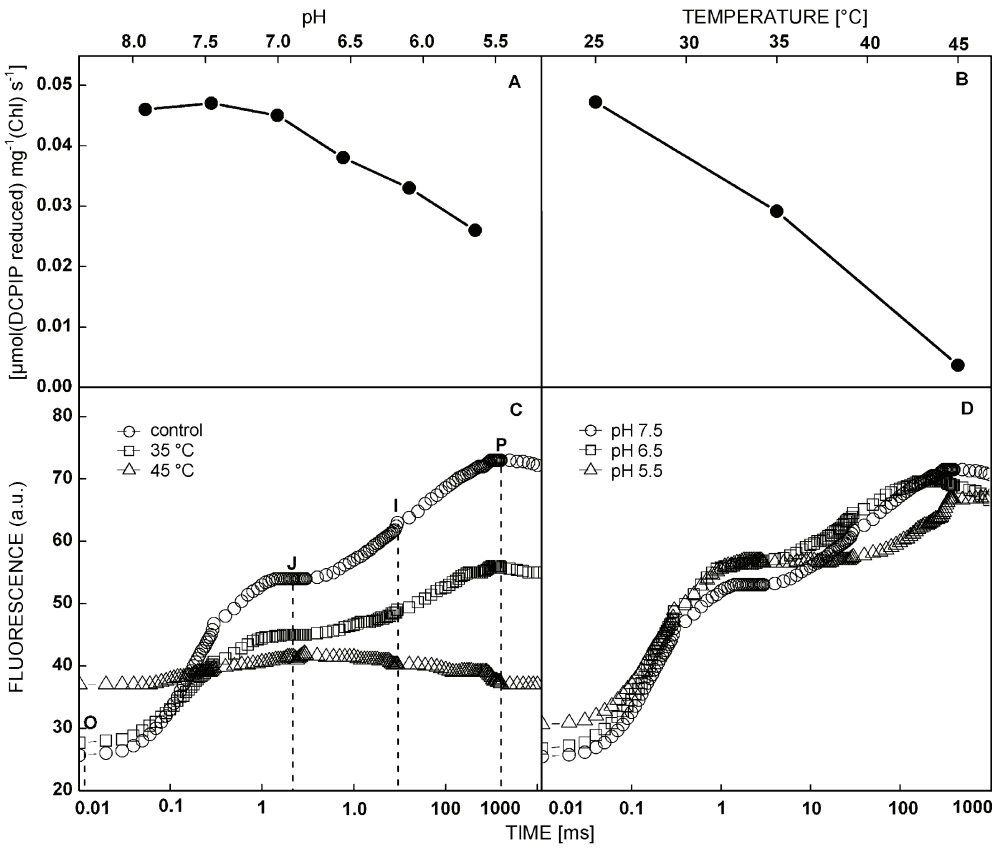

Fig. 1. *A* - Effect of various pH (8.0 - 5.5) on Hill reaction activity at 25 °C. *B* - Effect of various temperature (25, 35 and 45 °C) on Hill reaction activity at pH 7.5. *C* - Effects of various temperatures on Chl *a* fluorescence induction kinetics. J represents the momentary maximum of Q_A, I reflects the concentration of Q_A and Q_B , P reflects the peak concentration of Q_A, Q_B and PQH_2. *D* - Effects of different pH on Chl *a* fluorescence induction kinetics, a.u stands for arbitrary units.

Table 1. Effect of pH and temperature on various parameters of Chl *a* fluorescence induction curves in the thylakoid membranes: F_v/F_m - variable to maximum fluorescence ratio; ABS/RC - absorption per reaction center; F_v/F_0 - variable to initial fluorescence ratio. Means from 6 independent samples are shown. Standard deviation in all cases was less than 0.01.

Treatments	25 °C			35 °C			45°C		
	F_v/F_m	ABS/RC	F_v/F_0	F_v/F_m	ABS/RC	F_v/F_0	F_v/F_m	ABS/RC	F_v/F_0
pH 7.5	0.685	2.680	2.174	0.536	3.490	1.240	0.143	18.500	0.167
pH 6.5	0.653	3.003	1.880	0.530	3.660	1.154	0.140	20.800	0.162
pH 5.5	0.588	3.140	1.729	0.514	4.010	1.056	0.132	22.200	0.152

changes in different environmental conditions (Srivastava *et al.* 1997). Minimum fluorescence, F_0, is defined as the fluorescence when all PS 2 RCs are open, *i.e*. when the first electron acceptor of PS 2, Q_A, is oxidized. Maximum fluorescence, F_m, is defined as the fluorescence when all the PS 2 RCs are closed, *i.e*. when all Q_A is reduced. An increase in F_0 value can be interpreted as a reduction of the rate constant of energy trapping by PS 2 centers (Havaux 1993, Lazar 1999), which could be the result of a dissociation of LHC from PS 2 core as has been observed in several plant species after heat damage. The ratio of F_v/F_m has been used as an indicator for stress tolerance or sensitivity (Murkowski 2001, Thach *et al.* 2007). A significant decrease in F_v/F_m ratio with increasing temperature (Table 1) indicated that the photosynthetic quantum yield has been affected due to damage at the donor side. However, a change in pH affected F_v/F_m ratio (~12 % decrease) less than increasing temperature (~79 % decrease). This suggests decreased efficiency of energy transferred to the PS 2 reaction centers. The F_v/F_0 ratio decreased by ~13 % in low pH and by ~90 % in case of high temperature stress, indicating that the photochemical efficiency of the sample decreased and structural alterations occurred on the donor

side of PS 2. An increase in ABS/RC indicates a decrease in the size of chlorophyll antenna serving each RC. If this ratio increases, it is assumed that number of closed reaction centers in PS 2 has increased. The ratio of ABS/RC was found to increase drastically in temperature stressed thylakoid membranes and by ~20 % in pH treated thylakoid membranes. Based on these results, we suggest that a damage of the PS 2 RC occurs under temperature stress but not by change in low pH.

Irrespective of pH, effects caused by high temperature remain the same, *i.e.* inhibition in F_v/F_m by ~78 % at 45 °C was observed at all pH values (pH 7.5, 6.5, 5.5) and *vice versa*, irrespective of temperature, effects caused by changing the pH remained the same, *i.e.* ~12 % inhibition of PS 2 caused by lowering the pH to 5.5 was observed at all temperatures (25, 35 and 45 °C). This observation led us to propose that the effects of low pH and high temperature on PS 2 photochemistry are independent of each other and are based on different mechanisms.

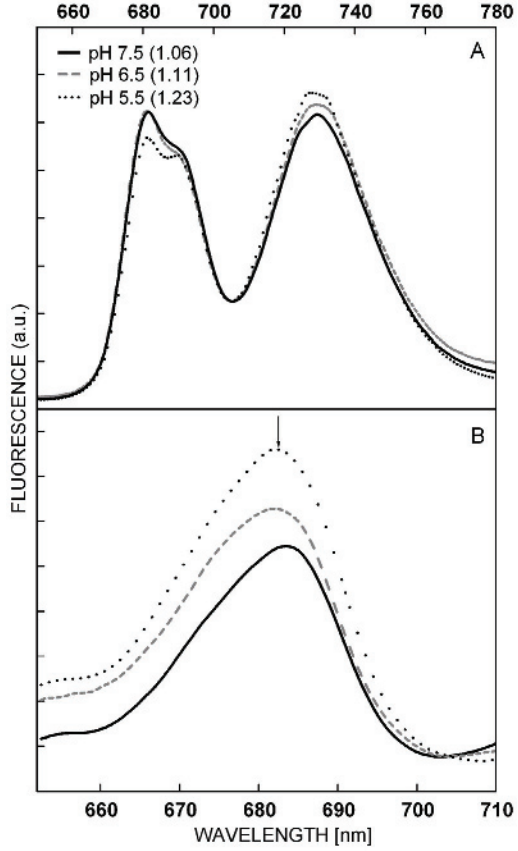

Fig. 2. *A* - Chl *a* fluorescence emission spectra measured at 77 K, recorded at excitation wavelength of 435 nm. The values given in brackets in inset depict F730/F685 ratio. Spectra have been normalized at 705 nm. *B* - Fluorescence excitation spectra at 77 K monitored at 735 nm while scanning the excitation radiation from 635 to 715 nm to estimate the changes in the absorption cross section of PS 1. Spectra have been normalized at 705 nm.

We also studied changes in the structural organization (stacking/unstacking) of the thylakoid membranes by digitonin fractionation. Percent Chl in the pellet gives an estimate of the relative amount of grana (stacked or appressed region) and stroma (unstacked or non-appressed region).The degree of stacking increased from ~64 % (at pH 7.5) to ~84 % (at pH 5.5), while with increasing temperature from 30 to 45 °C, the membranes became less stacked (from ~72 to ~42 %, respectively). Heating is known to cause destacking of thylakoid membranes and this destacking is expected to abolish differences between PS 2 centers located in granal and stromal thylakoids (Pospíšil and Tyystjarvi 1999). This indicated that, although the rate of electron transport mediated by PS 2 decreased in low pH and high temperature, their effects on the conformation of thylakoid membrane were different, and thus their mechanism of action may be totally different**.**

Low temperature (77 K) Chl *a* fluorescence emission spectra of spinach thylakoid membranes incubated at different pH values in dark were recorded. F_{685} and F_{696} peaks represented the fluorescence from PS 2 while F_{730} peak originated from PS 1 (Govindjee 1995). As evident from Fig. 2*A*, there was a consistent decrease in F_{685} and F_{690} nm and a concomitant increase in F_{730} when pH was changed from 7.5 to 5.5. The ratio F_{730}/F_{685} was 1.06 at pH 7.5, 1.11 at pH 6.5 and 1.23 at pH 5.5. An increase in this ratio indicated that more energy is received by PS 1 at low pH. The fluorescence excitation spectra (77 K) at 730 nm at each pH treatment, normalized to the content of red pigment at 705 nm, showed that the thylakoid membranes treated at pH 6.5 and 5.5 received more excitation energy from chlorophylls absorbing around 683 - 684 and 654 - 655 nm (Fig. 2*B*). This indicates that the absorption cross-section of PS 1 increased due to the association of LHC 2. Thus, low pH caused distribution of absorption excitation energy more in favour of PS 1.

Our earlier results (Tiwari *et al.* 2008) have shown that F_{685} decreased with increasing temperature while F_{730} is largely unaffected. Thus, although the ratio (F_{730}/F_{685}) increased, no energy transfer at the expense of PS 2 took place at high temperature. In our present work, a decrease in PS 2 fluorescence and a simultaneous increase in PS 1 fluorescence (and hence an increased F_{730}/F_{685} ratio) was observed at low pH, which indicates that some energy was transferred from PS 2 to PS 1.

In conclusion, both low pH and high temperature inhibit PS 2 photochemistry but the mechanisms of action are different. Change in pH (7.5 to 5.5) does not affect donor/acceptor side of PS 2 significantly while high temperature does. Low pH causes stacking of the thylakoid membranes while high temperature cause unstacking. Low pH is associated with energy redistribution between the two photosystems while high temperature induced changes do not involve energy re-distribution.

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