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Effect of high temperature on dehydration-induced alterations in photosynthetic characteristics of the resurrection plant *Haberlea rhodopensis*

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Abstract

The effect of high temperature (HT) and dehydration on the activity of photosynthetic apparatus and its ability to restore membrane properties, oxygen evolution, and energy distribution upon rehydration were investigated in a resurrection plant, *Haberlea rhodopensis*. Plants growing under low irradiance in their natural habitat were desiccated to air-dry state at a similar light intensity [about 30 μmol(photon) m^{-2} s^{-1}] under optimal day/night (23/20°C) or high (38/30°C) temperature. Our results showed that HT alone reduced the photosynthetic activity and desiccation of plants at 38°C and it had more detrimental effect compared with desiccation at 23°C. The study on isolated thylakoids demonstrated increased distribution of excitation energy to PSI as a result of the HT treatment, which was enhanced upon the desiccation. It could be related to partial destacking of thylakoid membranes, which was confirmed by electron microscopy data. In addition, the surface charge density of thylakoid membranes isolated from plants desiccated at 38°C was higher in comparison with those at 23°C, which was in agreement with the decreased membrane stacking. Dehydration led to a decrease of amplitudes of oxygen yields and to a loss of the oscillation pattern. Following rehydration, the recovery of CO_2 assimilation and fluorescence properties were better when desiccation was performed at optimal temperature compared to high temperature. Rehydration resulted in partial recovery of the amplitudes of flash oxygen yields as well as of population of S_0 state in plants desiccated at 23°C. However, it was not observed in plants dehydrated at 38°C.

Additional key words: desiccation; photosynthesis; thylakoid membranes.

Introduction

Desiccation-tolerant or resurrection plants have the unique ability to survive desiccation to air-dry state and they quickly restore their normal physiological activity upon rehydration. In this context, resurrection plants are interesting model systems to analyze resistance to the severe desiccation (Bartels *et al*. 1996). In order to survive in dry environments, plants must limit damage from desiccation and/or rehydration to a minimum, maintain cellular integrity in the dehydrated state, and activate repair mechanisms upon rehydration (Bewley 1995). The protection against a desiccation damage in angiosperms includes the production of nonreducing di- and oligosaccharides, various compatible solutes, and specific proteins, such as the late embryogenesis abundant proteins and heat shock proteins, some changes in lipid composition, and production of antioxidants

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Abbreviations: BSA – bovine serum albumin; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; *E* – transpiration; EPM – electrophoretic mobility; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT – high temperature; g_s – stomatal conductance; LHCI – light-harvesting complex of photosystem I; LHCII – light-harvesting complex of photosystem II; MES – 2-[N-morpholino] ethanesulfonic acid; OE – oxygen-evolving; P_N – net photosynthetic rate; PS – photosystem; RWC – relative water content; σ – surface charge density; ζ – zeta potential.
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to minimize a free radical-caused damage (Farrant *et al*. 1999, Hoekstra *et al*. 2001, Quartacci *et al*. 2002). Based on the different behaviour of photosynthetic apparatus during desiccation, two groups of desiccation-tolerant plants can be distinguished: homoiochlorophyllous and poikilochlorophyllous. The homoiochlorophyllous, desiccation-tolerant plants retain their chlorophyll (Chl) during desiccation, whereas Chl is lost as a result of desiccation in poikilochlorophyllous and it must be resynthesized following rehydration (Tuba *et al*. 1998). One advantage, which homoiochlorophyllous plants possess, is the ability to resume photosynthesis promptly upon rehydration (Drazic *et al*. 1999). However, a high amount of Chl molecules retained during desiccation could be a source for potentially harmful singlet oxygen production. Thus, the photosynthetic apparatus must be maintained in a recoverable condition throughout the dehydration process in this type of resurrection plants.

Temperature is one of the main factors controlling the formation and functional activity of the photosynthetic apparatus. Among all cell functions, the photosynthetic activity of chloroplasts has long been recognized as one of the most heat-sensitive processes in plants (Berry and Björkman 1980, Sharkey and Schrader 2008). There are two principal modes of stress-induced impairment of photosynthesis: the first is a direct damage induced by the stress factor and the second is an inhibition of *de novo* protein synthesis by reactive oxygen species (Allakhverdiev *et al*. 2008). There are several target sites for elevated temperature-induced damage, such as the CO_2 fixation system, photophosphorylation, the electron transport chain, and the oxygen-evolving (OE) complex (Nash *et al*. 1985, Feller *et al*. 1998, Bukhov and Mohanty 1999, Carpentier 1999, Sharkey 2005).

Haberlea rhodopensis Friv. (Gesneriaceae) is a rare resurrection plant of the northern hemisphere, originating from the Balkan Peninsula as an endemic and relict species of the Tertiary period. From an ecological point of view, *H. rhodopensis* is a perennial, herbaceous, shade-adapted species belonging to the group of homoiochlorophyllous, poikilohydric plants. Dehydration of *H. rhodopensis* frequently occurs under high temperatures in its natural habitats.

The aim of the present study was to examine the changes occurring in photosynthetic apparatus of *H. rhodopensis* upon dehydration under HT. The rate of CO_2 fixation of plants desiccated to different extent under the optimal and HT was compared. The alterations in distribution of excitation energy between photosystem (PS) II and PSI complexes and the OE capacity of thylakoid membranes isolated from control and desiccated plants were studied. In addition, the changes in chloroplast ultrastructure and the electrokinetic properties of thylakoid membranes were investigated.

Materials and methods

Plant material, desiccation, and rehydration: Well-hydrated *Haberlea rhodopensis* plants were collected from their natural habitat, where they grow on rocks below trees under a very low irradiance. Adult rosettes from the same locality and of similar size and appearance were selected for the experiments. The tufts with naturally occurring thin soil layers were planted in pots in peat-soil. Plants were subjected to drought stress by withholding irrigation either at 23/20°C or 38/30°C day/night temperature and the irradiance of 30 μmol(photon) m^{-2} s^{-1}, 12 h photoperiod, and relative humidity of 60%. After desiccation to air-dry state, the plants were rehydrated by watering them in a modified exsicator, where the desiccant at the bottom was replaced by water. The water was pumped up, thus ensuring a permanently high humidity level. Control plants, kept at 23/20°C or 38/30°C, were regularly watered during the experiment. The measurements were conducted at moderate [relative water content (RWC) 50–70%], strong (RWC 15–20%), and severe (RWC 4–8%) dehydration as well as after 1 d and 7 d of rehydration.

RWC of leaves was determined gravimetrically by weighing them before and after oven-drying at 80°C to a constant mass and it was expressed as the percentage of water content in dehydrated tissue compared to water-saturated tissues, using the equation:

$$RWC\ [\%] = (m_{fresh} - m_{dry}) \times 100/(m_{saturated} - m_{dry})$$

Gas-exchange measurements: Response of net photosynthetic rate (P_N) to irradiance was measured using a portable photosynthesis system *LCpro+* (*ADC BioScientific Ltd*., Hertfordshire, UK). CO_2 assimilation (P_N) in [μmol(CO_2) m^{-2} s^{-1}], transpiration (E), and stomatal conductance (g_s) in [mmol(H_2O) m^{-2} s^{-1}] were calculated according to von Caemmerer and Farquhar (1981).

Isolation of thylakoid membranes: 20 g of leaves were ground in liquid nitrogen to yield a fine powder. This powder was resuspended in buffer A (50 mM HEPES, pH 7.5, 400 mM NaCl, 10 mM $MgCl_2$, 0.2% BSA, 10 mM sodium dithionite, 0.4% ascorbate) and centrifuged for 5 min at 8,600 × *g* and 4°C. The supernatant was carefully removed and the pellet resuspended in buffer A. This washing was repeated to remove phenoloxidase activity and afterwards the sample was filtered through two layers of muslin and cotton wool. The filtrate was centrifuged for 10 min at 8,600 × *g* and the pellet resuspended in less than 50 ml of buffer B (50 mM MES,

pH 6.0, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% BSA, 0.05% ascorbate). After a further centrifugation step at 8,600 × *g* and 4°C for 10 min, the pellet was resuspended in 200 µl of buffer C (50 mM MES, pH 6.0, 5 mM $MgCl_2$, 15 mM NaCl).

Chl determination: Chl *a* and Chl *b* contents of isolated thylakoids were determined spectrophotometrically in 80% acetone according to Porra *et al.* (1989).

Low-temperature (77 K) fluorescence measurements: Samples from isolated thylakoid membranes were transferred into the tube for fluorescence measurement and immediately frozen in liquid nitrogen. Low-temperature fluorescence emission and excitation spectra were registered by a *Jobin Yvon JY3* spectrofluorometer (*Division d'Instruments S.A*., Longjumeau, France) equipped with a red sensitive photomultiplier (*Hamamatsu R928, Hamamatsu Photonics,* Japan) and a low temperature device. The width of the slits was 4 nm. Chl concentration was 10 µg(Chl) ml^{-1}. Data were digitised by an in-built A/D converter and transferred to an online IBM-compatible computer for further retrieval and analysis. The spectra were analyzed by *Origin 6.0* (*Microcal Software*). The emission spectra were recorded under excitation with 436 nm (Chl *a*) and 472 nm (Chl *b*). Excitation spectra were recorded for emission at 685 nm (PSII) and 735 nm (PSI) in a red region (710–600 nm) and Soret region (400–510 nm).

Measurement of oxygen flash yields and initial oxygen burst: Determination of oxygen flash yields and initial oxygen burst was performed using a home-constructed equipment, described in details in Zeinalov (2002). Its main device is a fast oxygen rate electrode equipped with a system for flash, modulated, and continuous illumination permitting an estimation of oxygen production. Each sample (100 µl) was preilluminated with 25 flashes followed by a 5 min dark adaptation. For measurements of flash oxygen yields, thylakoid membranes were illuminated with short (10 µs), saturating (4 J) flashes with a dark period of 0.466 s between flashes. For continuous illumination measurements, a cold light supplier (*LED LXHL-LW3C, Luxeon, Philips Lumileds Lighting Company*, San Jose, USA) providing irradiation of 420 µmol m^{-2} s^{-1} on the surface of sample was used. Data were digitized by a built-in A/D converter and transferred to an online IBM-compatible computer for further analysis. The S_i state population, misses (α) and double hits (β), were calculated by software based on least square deviations fitting of the theoretically calculated oxygen burst yields according to the noncooperative Kok's model of oxygen evolution (Kok *et al*. 1970) to the experimentally obtained values. The induction curves after continuous illumination showed biphasic exponential decay. For determination of kinetic parameters of initial oxygen burst, a deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components:

$A_1e^{-(t/\tau 1)} + A_2e^{-(t/\tau 2)}$, where A_1 and A_2 are amplitudes and τ_1 and τ_2 are time constants of the fast and slow components, respectively. For all measurements, 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 concentration of 150 µg ml^{-1}, without addition of an artificial electron acceptor.

Microelectrophoretic measurements: The electrophoretic mobility (EPM) measurements were performed using the particle electrophoresis technique with the *OPTON* cytopherometer (*Feintechnik Ges., m.b.H*., Wien, Austria). Electrophoretic migration was measured in a rectangular chamber at a constant electric field of 7 mA and 25°C. The movement of 15–30 thylakoids over a known distance (32 µm) was timed using microprocessor equipment for both forward and backward (reversed field) runs. The observation light [with intensity of 13 µmol(quantum) m^{-2} s^{-1}] was filtered through a green (545 nm) interference filter. The thylakoids were observed under a light microscope connected to a *Sony* video camera (*Video Camera Head CH-1400 CE, Sony Corporation*, Japan) providing 800-fold magnification. Images were recorded on a *Sony* video recorder *RDR-GX700/S (Sony,* Hungary). The electrical conductance and viscosity of different media, including the thylakoids, were measured using a *Thermo Fisher Scientific PC 510* (*Oakton Instruments/Eutech Instruments Pte Ltd*., USA/Singapore) pH/conductivity meter and a *Rheo* (*VEB MLW Prüfgeräte-WERK,MEDINGEN/SITZ FREITAL/ GDR, Typ 202*, Germany) viscometer, respectively.

The zeta potential (ζ) was calculated from the electrophoretic mobility, *u*, using Helmholtz-Smoluchowski equation:

$$\zeta = \frac{\eta \times u}{\varepsilon_r \times \varepsilon_0}$$

where ε_r is the dielectric constant of the aqueous phase (ε_r = 78.5 at 25°C), ε_0 is the permittivity of free space ($\varepsilon_0 = 8.8542 \times 10^{-12}$ F m^{-1}), η is the viscosity of the aqueous phase (η = 0.0010 Pa s, 50 mM MES, 5 mM $MgCl_2$, 15 mM NaCl, pH 6.0). Thylakoids [final concentration of 6 µg(Chl) ml^{-1}] were diluted in 25 ml of appropriate buffer.

We used the following equation for a negatively charged surface bathing in a mixed solution containing monovalent: monovalent and divalent: monovalent salts:

$$\sigma = -(4\,R\,T\,\varepsilon_r\,\varepsilon_0)^{1/2}\left[C'\left(\mathrm{Cosh}\,y_0 - 1\right) + 2C''\left(\mathrm{Cosh}^2 y_0 - 1\right)\right]$$

where the surface charge density (σ) is in [C m^{-2}], $y = F\,\psi/(R\,T)$, ψ is the electric potential at a distance *x*

from the charged surface, and ($\psi \approx \zeta$) is in [mV], F the Faraday constant (96,485.3365 C mol^{-1}), R is the gas constant (8.3143 J K^{-1} mol^{-1}), T the absolute temperature (298 K), ε_r the dielectric constant (78.5), ε_0 the absolute permittivity of free space (8.85415×10^{-12} C V^{-1} m^{-1}) (Chow *et al*. 1991).

Chloroplast structure: The material used was taken from the middle portion of fully hydrated and dehydrated leaves. Leaf segments (1 mm^2) were fixed in 5% (w/v) glutaraldehyde in Na-cacodylate buffer (pH 7.2) at 4°C for 3 h, washed and post-fixed in 1.3% (w/v) OsO_4 in the same buffer. The samples were buffer-washed and dehydrated through a graded ethanol series (25, 50, 75, 96, and 100%); ethanolpropylene oxide (1:1, v/v); propylene oxide and propylene oxide/*Durcupan ACM* (1:1, v/v), and embedded in *Durcupan ACM* (*Fluka AG*, Buchs, Switzerland).

Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a *JEM 100B* transmission electron microscope (*JEOL*, Japan).

Statistical analysis: Control and water-stress treatments were statistically compared. Comparison of means from three separate experiments, each in three replications, was done by the *Student*'s *t*-test.

Results

Haberlea rhodopensis plants, growing under a low irradiance in their natural habitat, were desiccated to air-dry state at a similar light intensity (about 30 μmol m^{-2} s^{-1}) under optimal (23/20°C, day/night) or high (38/30°C) temperature. Dehydration of plants at HT increased the rate of water loss 3-fold. RWC of leaves reached 8% after 24 d or 7 d of drought at 23°C and 38°C, respectively. After rewatering, severely dehydrated plants at 23°C and 38°C recovered quickly: RWC of leaves was 80% and 60%, respectively, on 1st day of rehydration, and it reached 95% after 7 d of rehydration.

RWC of plants, which were regularly watered but exposed to HT (control plants for the HT treatment), was 85% at a time when dehydrated plants had only 70% RWC, and it did not change further till the end of the experiment. Moreover, the values of the investigated parameters were very similar in the course of the high temperature treatment. For that reason, the data about HT controls were given as means of four measurements, each made at the time of the respective degree of dehydration (70, 50, 20, and 8% RWC). Throughout the experiments, the leaves of fully hydrated, control plants kept at 23°C showed no significant differences in RWC.

The rate of photosynthesis turned out to be very sensitive to desiccation (Fig. 1*A*). P_N decreased by 50% in moderately desiccated plants (50% RWC) and it was strongly inhibited when the RWC dropped to 20%. The HT treatment decreased P_N by 36% and it was more significantly reduced when plants were desiccated at 38°C compared with those left at 23°C. After 7 d of rehydration (R), the rate of CO_2 assimilation increased both in plants desiccated under optimal temperature and HT, but it was lower than the respective controls. g_s and the water-use efficiency (WUE) declined with the decreasing water loss and they were more reduced when desiccation was carried out at 38°C (Fig. 1*B*,*C*).

The alterations of 77 K fluorescence bands attributed to PSII and PSI complexes of thylakoid membranes, isolated from control and desiccated plants, were compared in order to evaluate the effect of desiccation

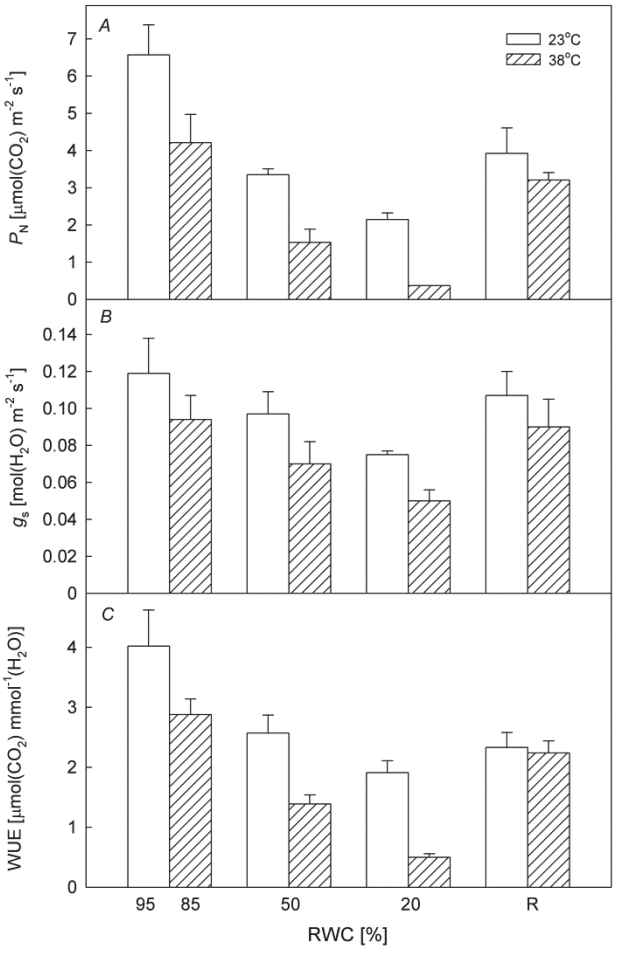

Fig. 1. Changes in CO_2 assimilation, P_N (*A*), stomatal conductance, g_s (*B*), and water-use efficiency, WUE (*C*) during dehydration of *Haberlea rhodopensis* at optimal (23°C) and high temperature (38°C) and low light intensity (30 μmol m^{-2} s^{-1}), as well as after 7 days of rehydration (R). Mean from 3 separate experiments, each in 3 replications.

degree on energy distribution between both photosystems and to compare the effect of stress treatments on energy

interactions within the photosynthetic apparatus. 77 K fluorescence emission spectra under excitation with 436 nm are presented in Fig. 2. With the increasing extent of desiccation, the overall intensity of fluorescence emission decreased and the relative intensities of main bands at 735 nm and 685 nm also changed. After rehydration, a restoration of fluorescence intensity was observed, but it was more pronounced in thylakoid membranes, isolated from plants desiccated at 23°C (Fig. 2*A*) compared with those desiccated at 38°C (Fig. 2*B*). The ratios of F_{735}/F_{685} under excitation with 436 nm (preferential excitation of Chl *a*) and 472 nm (Chl *b*) are presented in Table 1. It must be noted that the value of F_{735}/F_{685} for nondesiccated plants kept at 38°C was higher than that at 23°C. It is well known that at higher temperature a partial destacking destacking of grana thylakoids occurs followed by randomization of PSI and PSII complexes. It was supposed that a dissociation of LHCII from PSII complex occurred and some dissociated complex could migrate to PSI enriched regions of thylakoid membranes. It is possible that this LHCII can serve as additional antenna of PSI and thus more excitation energy is delivered to PSI. This was confirmed also by the data from excitation spectra – the ratio of E_{680}/E_{650} of fluorescence at 735 nm was lower in control plants kept at 38°C indicating relative increase of Chl *b* participation in energy supply of PSI. In addition, it was possible that bigger portion of energy would be transferred from PSII to PSI (so called spillover) due to heat-induced inactivation of PSII.

During desiccation at 23°C, the ratio F_{685}/F_{695} did not change, which meant that no significant changes within PSII-inner antennae complex occurred (Table 1). However, this ratio increased as a result of desiccation at 38°C by about 10% ($p<0.05$) indicating an alteration within organization of PSII core complex and its proximal antenna.

Desiccation up to 8% resulted in an increase of the F_{735}/F_{685} ratio (Table 1). It could be due to an increase of energy delivered to PSI and more pronounced decrease of fluorescence emission from PSII complex in comparison to PSI emission, as a result of rearrangement and/or randomization of pigment-protein complexes of PSI and

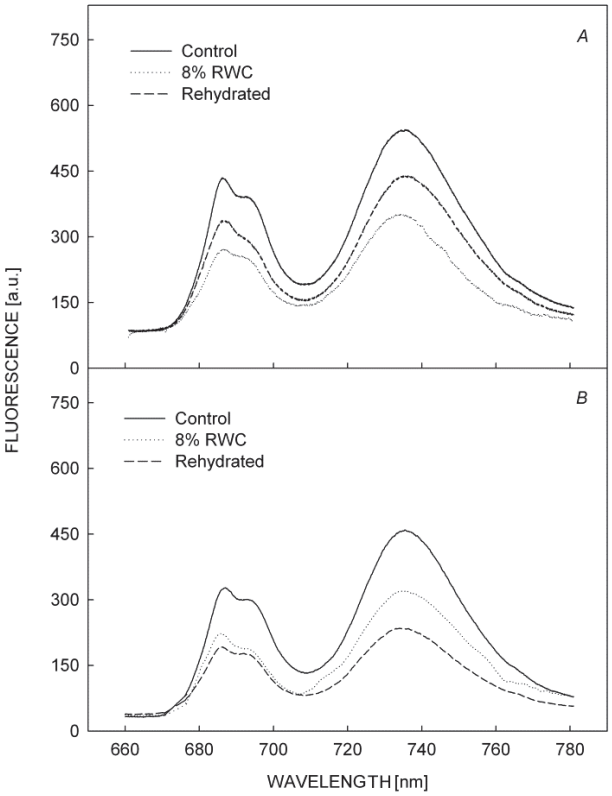

Fig. 2. Fluorescence emission spectra of thylakoid membranes isolated from *Haberlea rhodopensis* plants dehydrated at 23°C (*A*) and 38°C (*B*). *Solid line* – control plants, *dotted line* – 8% RWC, *dashed line* – rehydrated plants. Excitation wavelengths – 436 nm. Resuspending medium and chlorophyll concentration are as in Materials and methods.

Table 1. Ratios F_{735}/F_{685} and F_{685}/F_{695} estimated from emission spectra and E_{680}/E_{650} and E_{470}/E_{436} estimated from fluorescence excitation spectra of thylakoid membrane isolated from *Haberlea rhodopensis* dehydrated at 23°C and 38°C. Mean values ± SE are determined from 3 independent experiments. R – rehydrated.

RWC [%]	Excitation [nm]				Emission [nm]		
	436		472		735		685
	F_{735}/F_{685}	F_{685}/F_{695}	F_{735}/F_{685}	F_{685}/F_{695}	E_{680}/E_{650}	E_{470}/E_{436}	E_{470}/E_{436}
23°C							
95	1.32 ± 0.02	1.14 ± 0.01	1.15 ± 0.01	1.14 ± 0.01	1.75 ± 0.03	2.31 ± 0.02	3.27 ± 0.03
8	1.41 ± 0.03	1.13 ± 0.03	1.30 ± 0.03	1.14 ± 0.02	2.13 ± 0.04	2.07 ± 0.04	3.80 ± 0.05
R	1.41 ± 0.03	1.2 ± 0.02	1.16 ± 0.02	1.24 ± 0.02	2.05 ± 0.03	2.27 ± 0.03	3.33 ± 0.06
38°C							
85	1.44 ± 0.01	1.11 ± 0.01	1.21 ± 0.02	1.10 ± 0.01	1.63 ± 0.02	2.49 ± 0.04	3.57 ± 0.06
8	1.52 ± 0.02	1.23 ± 0.04	1.05 ± 0.03	1.21 ± 0.03	1.72 ± 0.06	2.26 ± 0.06	3.82 ± 0.08
R	1.28 ± 0.01	1.11 ± 0.03	1.12 ± 0.02	1.10 ± 0.01	1.56 ± 0.05	2.57 ± 0.03	3.62 ± 0.07

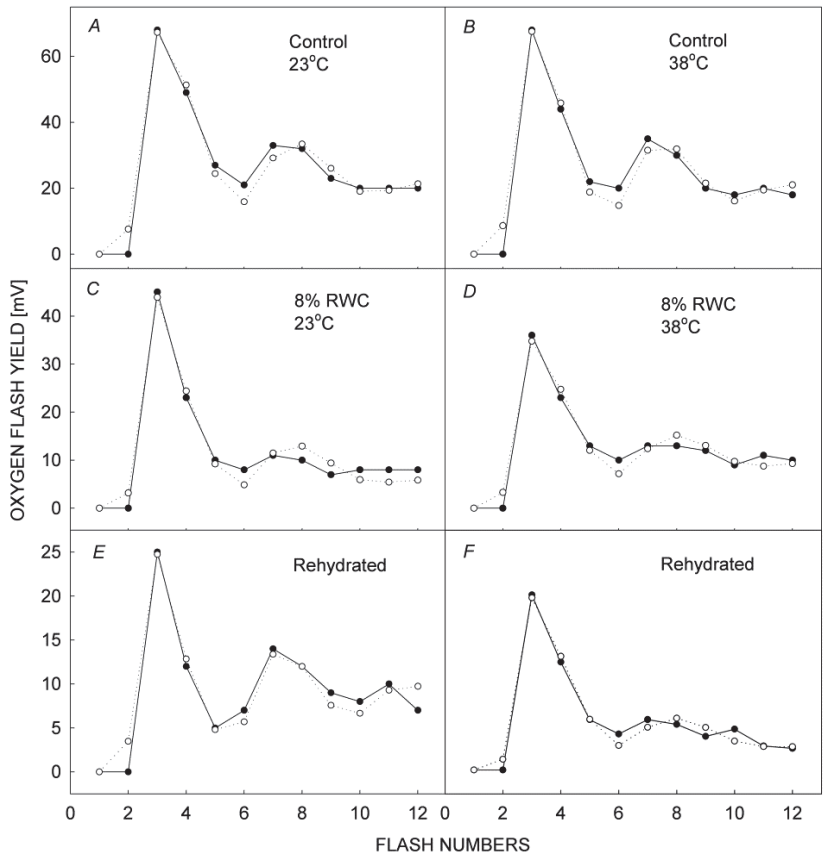

Fig. 3. Effect of dehydration on the flash-induced oxygen evolution in thylakoid membranes isolated from *Haberlea rhodopensis* dehydrated at 23°C (*A*,*B*,*C*) and 38°C (*D*,*E*,*F*) (*squares* – experimental traces; *circles* – calculated on Kok's model). RWC is as follow: *A,D* – controls – 95% and 85%; *B,E* – 8%; *C,F* – rehydrated.

Table 2. Parameters of flash oxygen yields and initial oxygen burst for thylakoid membranes isolated from *Haberlea rhodopensis* plants dehydrated to different extent of RWC at 23°C and 38°C. Numbers of oxygen-evolving centres in S_0 and S_1 states are presented as % of all centres. α and β are probabilities for misses and double hits, respectively. Mathematical fit of the photosynthetic oxygen evolution amplitudes was performed using the computer-simulating program according Kok's model. Mean values ± SE are determined from 3 independent experiments. R – rehydrated.

RWC [%]	S_0 [%]	S_1 [%]	α	β
23°C				
95	27 ± 2.1	73 ± 4.2	0.188 ± 0.017	0.035 ± 0.003
8	1 ± 0.1	99 ± 4.4	0.222 ± 0.019	0.018 ± 0.007
R	14 ± 1.1	86 ± 3.8	0.139 ± 0.015	0.029 ± 0.008
38°C				
85	28 ± 2.2	72 ± 5.6	0.150 ± 0.011	0.043 ± 0.005
8	6 ± 0.3	94 ± 7.7	0.245 ± 0.019	0.025 ± 0.007
R	7 ± 0.7	93 ± 6.8	0.245 ± 0.014	0.015 ± 0.006

PSII in the plane of thylakoid membranes due to desiccation. The involvement of Chl *a* and Chl *b* in energy supply of both photosystems was estimated by the ratios of E_{680}/E_{650} and E_{470}/E_{436} calculated from the excitation spectra of fluorescence at 735 nm and 685 nm (Table 1). The ratio of E_{680}/E_{650} for fluorescence emission at 735 nm remained lower in thylakoids from plants desiccated at 38°C in comparison with those desiccated at 23°C. It is worth nothing that the ratios of E_{680}/E_{650} and E_{470}/E_{436} from desiccated plant at both temperatures indicated an apparent decrease of Chl *b* participation in energy supply of PSI at very low RWC. Upon this very severe dehydration, even partial grana destacking might occur and pigment-protein complexes might be reorganized, the dissociated part of LHCII could not interact with PSI-LHCI complex and energy transfer to PSI was not efficient.

The OE capacity of thylakoids, isolated from plants desiccated to different extent at optimal and HT, was studied also by means of flash oxygen yields. Typical traces of experimentally obtained and calculated flash oxygen yields are presented in Fig. 3. The well-known oscillation pattern was observed for control plants at both temperatures with maximum amplitude at 3rd, 7th, *etc.* flashes. Dehydration up to 8% RWC resulted in a considerable decline of oxygen yield and a well expressed loss of oscillations (Fig. 3*C*,*D*). It must be noted that plants dehydrated at 23°C restored to a great extent not only the amplitude of oxygen yields but also oscillations, whilst oscillations were not restored in plants dehydrated at 38°C (Fig. 3*E*,*F*).

On the basis of oxygen yield traces and fitting program based on Kok's model, population of S_i states, misses (α) and double hits (β) were calculated. The effect

of dehydration resulted in reduction of the number of OE centres in S_0 state (Table 2). Usually, the percentage of centres in S_0 state in control plants is about 25% (Bacon 2001) and the values obtained for control plants kept at 23°C and 38°C were close to this value. Dehydration of plants both at optimal and HT led to a strong reduction of the population of centres in S_0 state. Misses increased for dehydrated plants at both temperatures. Rehydration of plants desiccated at 23°C restored up to 50% of population of S_0 centres, while there was not any restoration after rehydration of plants desiccated at 38°C.

The decay kinetics of the initial oxygen burst of *H. rhodopensis* thylakoid membranes was biphasic (two exponentials components), which reflected the functioning of fast and slow operating PSII centres with different time constants. The existence of two components of decay was attributed to two different mechanisms of the oxygen production – noncooperative and cooperative (Zeinalov and Maslenkova 1996, Zeinalov 2009, Zeinalov 2010). According to Kok's model (Kok *et al*. 1970), the noncooperative mechanism is related to the fast decay component, whereas the cooperative mechanism involves recombination between various OE centres (*i.e.* diffusion of precursors in membranes) and corresponds to the slow component. The two components with two different time constants are related with functioning of "fast" and "slow" operating centres from grana and stroma lamella regions and related to PSIIα and PSIIβ, respectively. Desiccation up to 8% RWC at 23°C and 38°C resulted in an increase of time constant of fast component from 0.56 s up to 0.86 s at 23°C and from 0.45 s to 0.79 s at 38°C. Concerning the slow components, the changes were more pronounced – from 7.72 s to 25.5 s and from 9.53 s to 23.7 s as a result of desiccation at 23°C and 38°C, respectively. After rehydration the values of time constant of the slow components restored near to the initial values. Involvement of "fast" and "slow" centres in oxygen evolution in the absence of an exogenous electron donor was estimated on the basis of amplitude of the fast and slow components of the second order decay kinetic of the initial oxygen burst. The portion of "fast" centres (grana situated) was lower in plants treated at 38°C. The amplitudes of both components also changed during desiccation – the ratio of amplitude of fast to slow component increased at 8% RWC and it was restored near to the control level after rehydration, being better at 23°C.

The effect of desiccation on the electrokinetic properties of thylakoid membranes was estimated by measuring the electrophoretic mobility (EPM), zeta potential (ζ) as well as surface charge density (σ) (Fig. 4). The results showed that the EPM of thylakoids, isolated from moderately desiccated plants (50% RWC) at 23°C, was not affected, but it increased in thylakoids from severely desiccated plants (8% RWC). Following rehydration, EPM was similar to the control values (Fig. 4*A*).

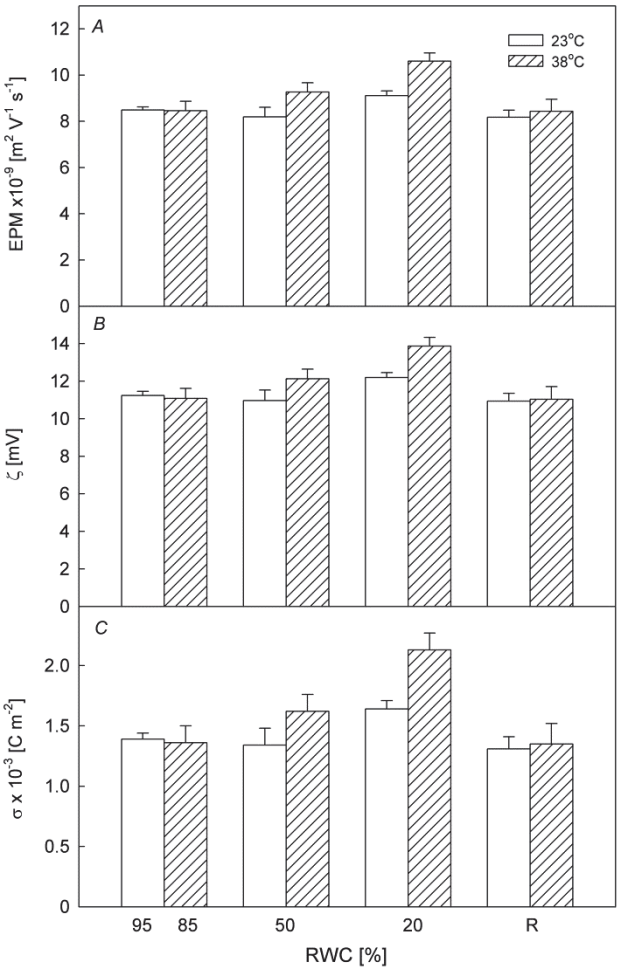

Fig. 4. Changes in the electrophoretic mobility (EPM) (*A*), zeta potential (ζ) (*B*), and surface charge density (σ) (*C*) of thylakoid membranes isolated from *Haberlea rhodopensis* plants dehydrated at 23°C and 38°C after 7 d of rehydration (R).

HT treatment of well-watered (control) plants did not influence EPM. However, desiccation of *H. rhodopensis* at 38°C increased EPM by 10% in thylakoids, isolated from moderately desiccated plants (50% RWC), and by 25% from severely desiccated plants (8% RWC). ζ is genetically determined and is used as a marker for the stability of the colloidal system. The results showed that there was also a significant change in ζ of thylakoids isolated from plants desiccated to air-dry state (RWC 8%) at 23°C and 38°C (Fig. 4*B*). Similarly to EPM, ζ was higher when plants were desiccated under high temperature. Our electrokinetic measurements showed that ζ of the thylakoids from control plants kept at 23°C and 38°C was –11.2 mV and –11.1 mV, respectively, but its values increased up to –12.2 mV and –13.9 mV at 8% RWC. As shown in Fig. 4*B*, thylakoids from rehydrated plants possessed approximately the same ζ as control. Desiccation of *H. rhodopensis* plant under both optimal and HT enhanced σ more than ζ (Fig. 4*C*). σ of thylakoid

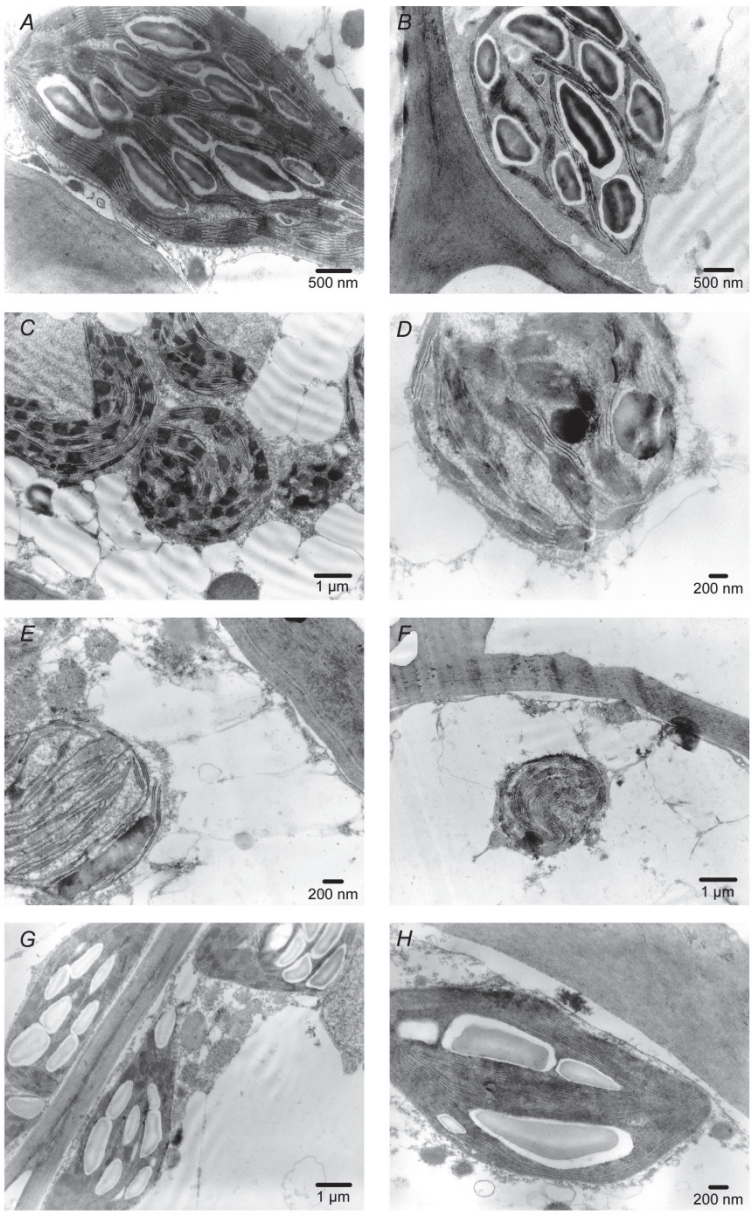

Fig. 5. Electron microscopy of chloroplast of control (*A*,*B*), dehydrated to 50% RWC (*C*,*D*), 8% RWC (*E*,*F*), and rehydrated (*G*,*H*) *Haberlea rhodopensis* plants. *A*,*C*,*E*,*G* – desiccated at 23°C; *B*,*D*,*F*,*H* – desiccated at 38°C.

membranes from plants desiccated to 8% RWC at 23°C and 38°C increased by 18% and 57%, respectively, but it was recovered to the control values after rehydration.

Alterations of physico-chemical properties of thylakoid membranes as well as energy interaction of both photosystems are highly dependent on the internal architecture of chloroplasts – appresed and nonappresed thylakoid membranes. In order to check to what extent desiccation at both temperatures induced structural changes of chloroplast internal structure, the electron microscopy study was done. Fully hydrated leaves from plants kept at 23°C and 38°C contained chloroplasts elongated in shape and located near to the cell wall (Fig. 5*A*,*B*). In the stroma of both variants, a great number of starch grains were visible. Chloroplasts showed a well-organized internal membrane system arranged into grana and a number of stromal thylakoids. The grana were much more expressed in the plants at 23°C. The progressive desiccation up to 50% RWC led to the formation of rounded chloroplasts withdrawn from the cell wall and missing starch grains in the stroma (Fig. 5*C*,*D*). Desiccation at 38°C resulted in pronounced loss of grana, while at 23°C they persisted. At 8% RWC, the integrity of the outer chloroplast membrane was disturbed and the internal thylakoid network consisted of granal thylakoids with a lower stacking degree and a higher number of stromal thylakoids (Fig. 5*E*,*F*). Upon rehydration the chloroplast structure improved and became similar to the fully hydrated leaves (Fig. 5*G*,*H*).

Discussion

Plant response to environmental stress factors is very complex including structural changes and alterations in efficiency of some physiological processes in a way to help a plant to cope and survive under severe conditions. Recently, we have investigated *in vivo* the effect of HT during desiccation of the resurrection plant *H. rhodopensis* (Mihailova *et al*. 2011). The results clearly showed that water deficit decreased the photochemical activity of PSII and PSI and this effect was stronger when desiccation was carried out at 38°C. PSII was more sensitive than PSI to both HT treatment and desiccation (Mihailova *et al*. 2011). *H. rhodopensis* belongs to the group of homoiochlorophyllous species, which maintain most of Chl in the dried state. However, this ability requires that the photosynthetic apparatus is maintained in a recoverable form throughout the entire period of dryness. Our previous results showed that the amounts of Chl-protein complexes remain stable during desiccation and rehydration of *Haberlea* (Georgieva *et al*. 2007). Some reduction in the amount of the main PSI and PSII proteins was observed, especially in severely desiccated *H. rhodopensis* leaves, but their content fully recovered after rehydration (Mihailova *et al.* 2011). In the present study, we focused on the effect of HT during desiccation on photosynthesis and on properties and structure of thylakoid membranes. Since photosynthesis is the most sensitive of all physiological processes to water deficit in most of plants, we measured P_N. Our results showed that HT alone reduced the photosynthetic activity and desiccation of plants under HT had more detrimental effect compared to desiccation at optimal temperature (Fig. 1).

Another process affected by desiccation at optimal and at HT is the distribution of excitation energy between both photosystems, which was studied in isolated thylakoid membranes. Our data showed that at 38°C the ratio F_{735}/F_{685} was higher; this means that PSI received more energy. This could be related to partial destacking as a result of higher temperature and/or desiccation and this was consistent with the data from electron microscopy (Fig. 5) showing a decrease in a number of grana as a result of desiccation, more pronounced at higher temperature (38°C). In addition, as it can be seen from Fig. 4*C*, σ of thylakoid membranes desiccated at 38°C was higher in comparison with those at 23°C, which was in line with the reduction of membrane stacking. Recently it was reported that desiccated plants showed a higher PSI emission relative to PSII emission compared with controls plants, thus supporting the idea that antenna movement from antenna proteins of PSII to PSI takes place during desiccation (Georgieva *et al*. 2009). On the other hand, elevated temperature led to destacking of thylakoids and to an increase of the F_{735}/F_{685} ratio in plants subjected to heat stress (Weis 1984, Ivanov and Velitchkova 1990, Bukhov and Mohanty 1999). It seems reasonable that both factors – heat and dehydration – have synergetic effect on energy distribution, most probably inducing some destacking. Other results, which need attention, were the changes of relative intensity of bands associated with PSII complex – 685 nm and 695 nm. No significant changes of the F_{685}/F_{695} ratio were observed in plants dehydrated at 23°C. However, this ratio increased during desiccation at 38°C, which was the most probably due to the decrease of fluorescence intensity at 695 nm in comparison to that at 685 nm. Thus, it indicated the detachment and a possible separation of CP47 from the reaction centre complex of PSII and it caused interruption of the effective transfer of quanta absorbed by the inner antenna to the reaction centre.

Comparing the data of E_{670}/E_{650} from fluorescence excitation spectra at 735 nm, it was evident that more Chl *b* molecules participated in energy supply of PSI at 38°C. However, under severe desiccation (8% RWC), the destacking and reorganization of pigment-protein complexes was not probably typical (due to dehydration of thylakoid membranes and changes of physico-chemical properties of lipid bilayer) and no effective energy transfer from Chl *b* to PSI proceeded. Our previous data showed that the amount of PSI, which binds also LHCII (PSI+LHCI+LHCII complex), decreased in dry leaves upon desiccation of *H. rhodopensis* (unpublished data). The rehydration of plants restored to high extent the fluorescent properties of plants when desiccation was carried out at 23°C, while the restoration was to lesser extent in those treated at 38°C.

The OE complex of thylakoid membranes is the most sensitive part of the photosynthetic apparatus towards to different stress treatments including dehydration. Our results showed that OE capacity was modified as a result of dehydration at 23°C and 38°C. Flash-induced oxygen evolution is associated with OE complexes situated in the grana regions. Dehydration led not only to the decrease of the amplitude of flash oxygen yields, but also to a loss of oscillation pattern. This means that the number of OE centres from grana regions was reduced and that the centres still evolving oxygen did not work "properly". This was confirmed also by the data concerning the state of a population after dehydration – a number of centres in S_0 state was strongly reduced. The values of misses (centres do not convert to higher state or zero-step advance) increased with decreasing RWC at both temperatures, while the number of double hits (double step advance) decreased. Rehydration resulted in partial recovery of the amplitude of flash oxygen yields as well as the population of S_0 state in plants desiccated at 23°C. However, it was not observed in plants dehydrated at 38°C.

Biphasic decay kinetics of oxygen burst was reported in several plants and cyanobacteria (Zeinalov and

Maslenkova 1996, Dobrikova *et al.* 2012). It has been also shown that in chlorine *f2* mutant with almost no grana stacks, a cooperative mechanism dominates. The loss of oscillation of flash oxygen yields indicated an inactivation of fast centres from grana regions. On the other hand, our data showed that destacking of thylakoids occurred in *H. rhodopensis* plants under desiccation at 38°C. On the basis of our data, it can be concluded that the "slow" centres from stroma regions were also affected by dehydration. Not only that some of the centres were inactivated, but the remaining centres were modified; their time constant increased considerably. Better restoration of oxygen yields after rehydration at 23°C showed that the temperature during desiccation was an important factor and desiccation at higher temperature resulted in more severe damage of photosynthetic apparatus.

In summary, our results showed that HT during desiccation increased the detrimental effect of dehydration and influenced the rate of recovery after rehydration in respect to the photosynthetic apparatus. PSII and OE centres were more affected by desiccation especially at HT. Inhibition of flash oxygen amplitudes and the loss of oscillation pattern as well as the changes of time constants showed that the desiccation-induced inactivation of OE apparatus was the limiting step, which determined the retarded recovery after rehydration at 38°C.

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