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PHOTOSYNTHETIC CHARACTERISTICS OF THE RESURRECTION PLANT *HABERLEA RHODOPENSIS* FROM TWO HABITATS

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Summary: The photosynthetic characteristics of *Haberlea rhodopensis* plants from two habitats in Western Rhodope Mountain (in the regions of Trigrad and Wonderful bridges) were studied. Although the plants from both habitats were exposed to sun during part of the day, their leaves were morphologically similar to those of shade plants. The desiccation-induced damages on membrane integrity were characterized by electrolyte leakage and lipid peroxidation. Photochemical activity, energy distribution between the two photosystems and physicochemical properties of thylakoid membranes were estimated by means of chlorophyll fluorescence, 77K fluorescence and particle microelectrophoresis measurements. The mutual organization of PSI-LHCI and PSII-LHCII complexes and spillover between them were similar in thylakoids isolated from well-hydrated plants from Trigrad and Wonderful bridges. Under desiccation up to 8% RWC the F735/F685 ratio at excitation with 472 nm (chl *b*) remained almost unchanged in thylakoid membranes from Wonderful bridges while it decreased considerably in thylakoid membranes from Trigrad. The thylakoid membranes from Wonderful bridges showed a less alteration in energy distribution between both photosystems in response to dehydration. Dehydration induced stronger modifications in thylakoid membranes of plants from Trigrad, expressed by higher electrolyte leakage, lipid peroxidation and membrane surface charge density.

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Keywords: *Haberlea rhodopensis*; desiccation; photosynthesis; energy transfer; surface charge density.

Abbreviations: Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; d.c. – direct current; EPM – electrophoretic mobility; ETR – electron transport rate; LHC – light-harvesting complex; NPQ – non-photochemical quenching; PS – photosystem; RWC – relative water content; ζ – zeta potential; σ – surface charge density.

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INTRODUCTION

Resurrection plants have the unique ability to survive desiccation of their vegetative tissues to an air dry state (Gaff 1971). After rehydration they quickly recover their metabolism (Bernacchia et al., 1996). Two types of resurrection plants can be distinguished in respect to their ability to limit the light-chlorophyll interactions – poikilochlorophyllous (dismantling the photosynthetic apparatus and losing chlorophyll during dehydration) and homoiochlorophyllous (leaf folding and retaining their chlorophyll) (Tuba et al., 1998; Sherwin and Farrant, 1998). *Haberlea rhodopensis* Friv. is a perennial herbaceous rock poikilohydric plant, forming dense tufts of leaves. The plant is a preglacial relict, whose "age" is probably over two million years. It belongs to the group of homoiochlorophyllous desiccation-tolerant plants. Some *H. rhodopensis* localities show great variations in habitat characteristics including differences in temperature, water and light conditions (Daskalova et al., 2011). As usual, in most of the habitats *H. rhodopensis* plants grow on overshadowed rocks below the trees at a very low light intensity. There are only few habitats where *H. rhodopensis* plants grow on rocks directly exposed to sunlight, out of the forest coverage or exposed to sun only part of the day. That is why the investigations on desiccation tolerance of *H. rhodopensis* up to now have been conducted mainly on shade plants. In order to understand the processes underlying desiccation tolerance of *Haberlea rhodopensis* it is important to compare how plants from different habitats, growing under different environment conditions, survive and maintain their photosynthetic activity.

To survive dry environments plants must maintain their cellular integrity and limit the damage from the dehydration/rehydration process (Bewley 1995). The photosynthetic apparatus is very sensitive to oxidative damage (Halliwell 1987). On drying, the relative water content (RWC) and leaf water potential decrease, which reduces photosynthesis (Lawlor and Cornic, 2002). However, the chlorophyll molecules continue to capture light energy and direct the electrons flow to O_2. Thus, the light energy harvested by chlorophyll cannot be dissipated via photosynthesis and can cause oxidative burst in chloroplasts (de Carvalho, 2008; Smirnoff 1993). Plants have developed different antioxidant mechanisms, including enzymatic and non enzymatic scavenging systems and possess the capability to change stoichiometry of main pigment-protein complexes including photosystem I (PSI), photosystem II (PSII) and light-harvesting complexes, thus regulating the excitation energy distribution and preventing overexcitaion and production of reactive oxygen species (ROS). Energy interaction between the main pigment–protein complexes of thylakoid membranes depends on their mutual organization and distance between their light-capturing antennae complexes. The alterations of membrane organization induced by unstacking at low salt conditions, state 1 – state 2 transition and some other treatments, influence the relative intensities of the main fluorescent bands at 77 K of isolated thylakoids (Andersson 1981; Barber 1980). It is reasonable to expect that the desiccation

process will affect the distribution of excitation energy between PSII and PSI and this effect can be followed by the changes of shape and intensities of 77K fluorescence spectra.

The aim of the present study was to compare the photosynthetic characteristics of *Haberlea rhodopensis* plants from two habitats (Trigrad and Wonderful bridges), where plants were exposed to sun during part of the day. The experiments were carried out with well-hydrated, moderately dehydrated, completely desiccated and rehydrated plants. We analysed the activity of PSII by measuring the chlorophyll fluorescence emission of leaves and the changes of 77K fluorescence spectra of isolated thylakoid membranes. In addition, the effect of desiccation on the electrokinetic properties of thylakoid membranes was studied. To our knowledge, investigations on the physiological activity of *Haberlea rhodopensis* plants from these habitats have not been carried out.

MATERIALS AND METHODS

Haberlea rhodopensis plants from two different habitats (in the regions of Trigrad, at 1200 m a.s.l. and Wonderful bridges at 1400 m a.s.l.) were used in this study. Plants from both habitats grow on rocks exposed to sun during part of the day. The light intensity measured in the midday in July was 500 μmol m^{-2} s^{-1} PPFD, but the leaves were morphologically similar to those of shade plants studied earlier (Georgieva et al., 2005; Georgieva et al., 2012). Plants were desiccated to different extent in laboratory conditions. Rehydration of plants desiccated to an air-dry state was carried out in a modified desiccator, where the desiccant at the bottom was replaced by water. All measurements were conducted on fully expanded mature leaves from control (90% RWC), moderately dehydrated (50% RWC) and dried leaves (8% RWC) as well as after 7 days of rehydration of the dry plants (R7).

The RWC was determined gravimetrically by weighing leaves before and after oven drying at 80°C to a constant weight and expressed as a percentage of water content in dehydrated tissue (m_{dry}) compared to water-saturated tissues ($m_{saturated}$), using the equation:

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

Electrolyte leakage from leaf tissues was measured with a conductivity meter after 24 h incubation of leaf disks (total weight 0.25 g) in 25 ml double-distilled water. Following each measurement the maximum leakage of the tissue was determined by boiling the leaves 15 min at 100°C. The results are expressed as percentage of the maximum leakage.

Light curves of chlorophyll (Chl) fluorescence were measured with a portable fluorometer PAM-2500 (Heinz Walz GmbH, Effeltrich, Germany). Electron transport rate (ETR) and non-photochemical quenching (NPQ) were calculated by the formulas:

$$ETR = PAR \times ETR\text{-}Factor \times P_{PS2}/P_{PPS} \times Y(II)$$

where PAR – photosynthetic active radiation;

ETR-Factor – absorbance of photons by photosynthetic pigments = 0.84;

P_{PS2}/P_{PPS} – photons absorbed by PSII

relative to photons absorbed by photosynthetic pigments = 0.5;

Y(II) – effective photochemical quantum yield of PSII.

$$NPQ = Fm/Fm' - 1$$

where Fm and Fm' – maximum fluorescence levels measured by saturating light pulses in dark- and light-adapted state, respectively.

Thylakoid membranes were isolated as described by Georgieva et al. (2009).

Low temperature (77 K) fluorescence emission and excitation spectra of isolated thylakoid membranes were registered using a Jobin Yvon JY3 spectrofluorometer equipped with a red sensitive photomultiplier (Hamamatsu 928) and a low temperature device. Chlorophyll concentration was 10 μg/ml. The emission spectra were recorded under excitation with 436 nm (Chl *a*) and 472 nm (Chl *b*). The excitation spectra were recorded for emission at 685 nm (PSII) and 735 nm (PSI) in the red region (600–710 nm) and Soret region (400-510nm).

The electrophoretic mobility (EPM) measurements were performed using the particle electrophoresis technique with the OPTON Cytopherometer as described by Velitchkova et al. (2013).

The lipid peroxidation of thylakoid membranes was determined by production of TBARS and expressed in μM using an extinction coefficient of 154 mM^{-1} cm^{-1} (Halliwell and Gutteridge, 1989).

The experiments were performed in triplicate. The significant differences between means were determined by ANOVA. One-way analysis of variance was performed with Dunn's test for all pairwise comparisons against a control group following rank-based ANOVA.

RESULTS AND DISCUSSION

Membrane integrity was assessed by measuring the extent of electrolyte leakage. The electrolyte leakage from well-hydrated (control) plants from Wonderful bridges was two times higher compared to that from Trigrad (Fig. 1). Upon desiccation the electrolyte leakage gradually increased in plants from both habitats and it was 7-fold and 3-fold higher in dry plants (8% RWC) from Trigrad and Wonderful bridges, respectively, indicating some changes in membrane configuration, probably damage. The recovery after 7 days of rehydration showed that these damages were reversible. The 7-fold increase of electrolyte leakage during desiccation of plants from Trigrad showed that the membrane integrity of these plants was more affected by oxidative stress.

The activity of PSII was studied by measuring the chlorophyll fluorescence emission. The light-response curves of electron transport rate (ETR) and non-photochemical quenching (NPQ) of *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges are shown in Fig. 2. The ETR in control plants from Trigrad was slightly higher compared to that of Wonderful bridges. Upon moderate water loss (50% RWC) it was not significantly affected in plants from Trigrad but decreased in plants from Wonderful bridges (Fig. 2). The decline of ETR at this stage of dehydration of plants from Wonderful bridges was accompanied by enhancement of NPQ. ETR and NPQ were fully inhibited in dry leaves (8% RWC) from both habitats. Thus, the thermal energy dissipation via NPQ had a major role in preventing photoinhibition

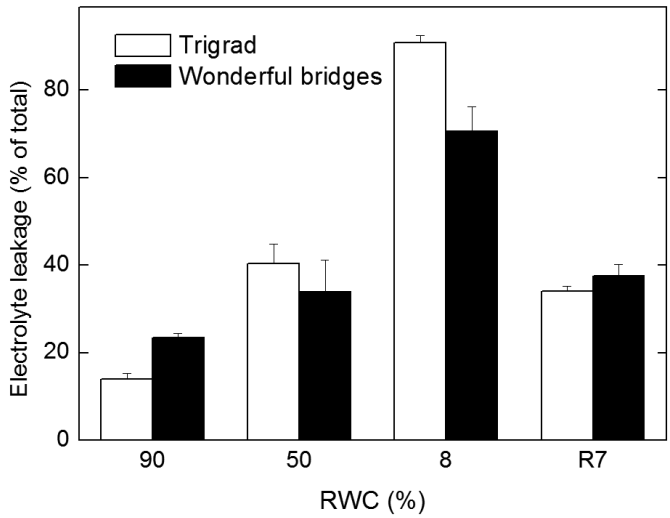

Figure 1. Changes in electrolyte leakage from leaf tissue during dehydration as well as after 7 days of rehydration (R7) of *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges. Data are presented as means of three separate experiments, each in three replications with standard error.

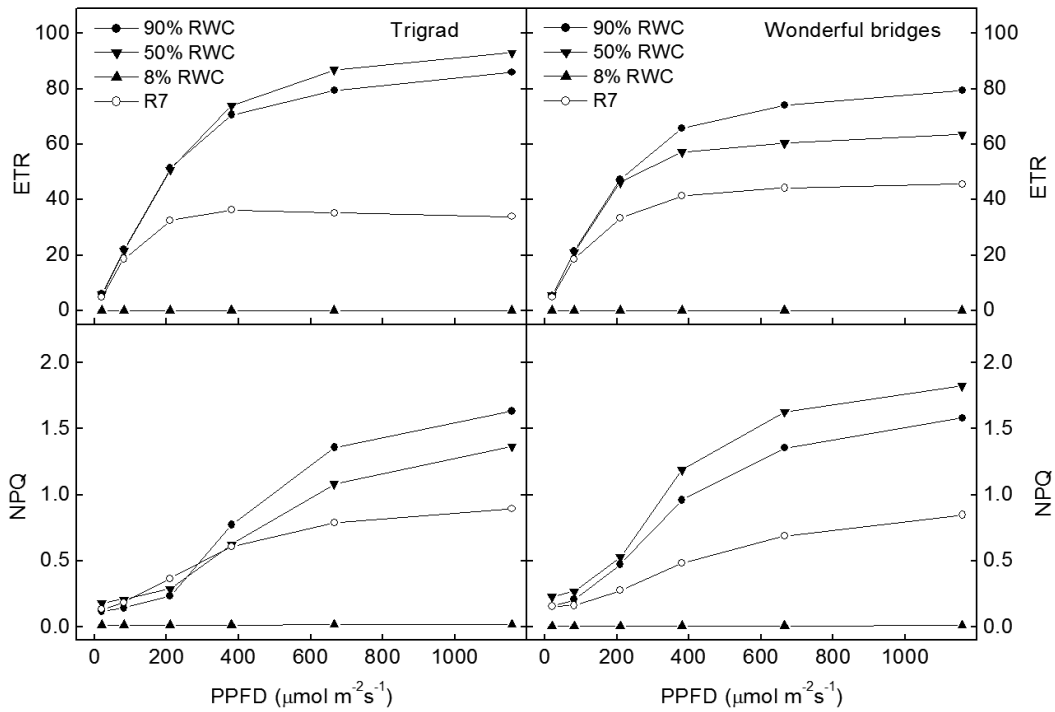

Figure 2. Light response curves of ETR and NPQ in leaves during dehydration as well after 7 days of rehydration (R7) of *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges. Data are presented as means of three separate experiments, each in three replications. The standard error does not exceed 10%.

at the early stage of dehydration, while other mechanisms might have become important at extremely low RWC to avoid photodamage. Moreover, the decline in ETR observed in *Haberlea* leaves at low RWC might represent a protective mechanism against toxic oxygen production in order to maintain membrane integrity and ensure cell survival (Di Blasi et al., 1998; Degl'Innocenti et al., 2008).

The effect of desiccation on energy distribution between PSII and PSI was studied by measuring 77K fluorescence spectra of isolated thylakoid membranes. Representative fluorescence emission spectra of thylakoid membranes from control and dehydrated plants at excitation with 436 nm (preferential excitation of Chl *a*) are presented in Fig. 3 (solid lines). The low temperature (77K) fluorescence emission spectra of thylakoid membranes exhibited two major emission bands (685 nm and 735 nm) and a shoulder at about 695 nm. The fluorescence in the region 685 nm - 695 nm and at 735 nm is emitted by PSII and PSI complexes, respectively and the intensities of bands depend on populations of both photosystems, energy delivery to them and on spillover of excited energy from PSII to PSI (Krause and Weis, 1991). The F735/F685 (F735/F695) ratios reflect these changes and they are usually used for estimation of changes of energy distribution and energy interaction between both photosystems (Briantias et al., 1986; Velitchkova and Popova, 2005). Data on fluorescence ratios for thylakoid membranes from both habitats are presented in Table 1. The fluorescence emission ratios F735/F685 and F685/F695 were calculated after extraction of base line. The ratios of band intensities at 735 nm and 685 nm (F735/F685) for thylakoid membranes from control plants did not differ too much between the two habitats, indicating that the mutual organization of PSI-LHCI and PSII-LHCII complexes and spillover

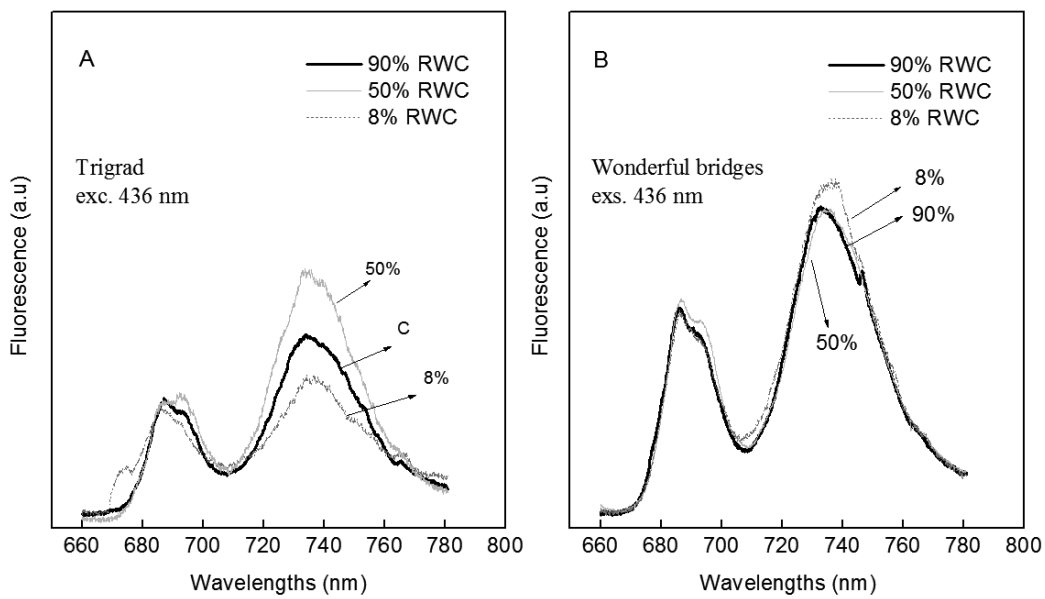

Figure 3. 77K fluorescence emission spectra of thylakoid membranes isolated from *Haberlea rhodopensis* plants from Trigrad (A) and Wonderful bridges (B) during dehydration.

Table 1. Values for the F735/F685, F735/695 and F685/F695 fluorescence ratios and the E680/E650, E480/E436 excitation ratios for thylakoid membranes isolated from *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges during dehydration.

Habitat		exc.436			exc. 472			em 735	em. 735	em. 685
	RWC	F735/F685	F735/F695	F685/F695	F735/F685	F735/F695	F685/F695	E680/E650	E480/E436	E480/E436
Trigrad	control	1.58	1.76	1.12	1.41	1.54	1.09	1.4	3.72	4.4
	50%	2.07	1.94	0.94	1.51	1.66	1.09	1.45	2.73	3.84
	8%	1.3	1.51	1.12	0.94	1.19	1.25	1.87	1.5	5.83
Wonderful bridges	control	1.5	1.72	1.14	1.32	1.46	1.1	1.6	2.7	4.15
	50%	1.42	1.58	1.11	1.15	1.29	1.12	1.74	2.5	4.15
	8%	1.63	1.89	1.16	1.35	1.58	1.15	1.73	3.23	5.07

between them were similar in plants from Trigrad and Wonderful bridges. However, the F735/F685 ratio was higher in plants from Trigrad in comparison with those from Wonderful bridges and this difference was more pronounced under preferential excitation of Chl *b* (472 nm). Preferential excitation of chl *b* (excitation wavelength 472 nm) resulted in a decrease of both F735/F685 and F735/F695 ratios, which was an expected result taking into account the pigment composition of PSII-LHCII and PSI-LHCI. Under excitation with 472 nm the F735/F685 ratio was higher in thylakoid membranes isolated from plants from Trigrad which implied that the excitation of chl *b* was more effective for PSI fluorescence thus indicating a higher antenna size of PSI and/or participation of LHCII to the energy supply of PSI. The relative involvement of chl *a* and chl *b* in the energy supply of PSI and PSII was estimated by the intensity of bands at 680 and 650 nm as well as at 480 and 436 nm of fluorescence at 735 nm (PSI) and at 685 nm (PSII). The E480/436 ratio was higher for the emission at 685 nm in comparison with that for the emission at 735 nm, reflecting greater participation of LHCII in the energy supply of PSII.

The fluorescence spectra of thylakoid membranes of plants from the two habitats at different degrees of desiccation under excitation with 436 nm are shown in Fig. 3. Desiccation of leaves led to alterations of the relative intensities of emission bands of PSII (685-695 nm) and PSI (735 nm). Data on all fluorescence ratios are given in Table 1. For plants from Wonderful bridges, desiccated up to 50% RWC, the values for F735/F685 were close or lower to those for controls. The decrease of the RWC of leaves up to 8% RWC resulted in an increase of the F735/F685 ratio in plants from Wonderful bridges, which was in line with an earlier observed increase of the F735/F685 ratio in fully desiccated plants (Velitchkova et al., 2013). In plants from Trigrad this ratio increased at 50% RWC, and the values at 8% RWC were lower in comparison with the respective controls. It must be noted that the spectra of plants from Wonderful bridges did not change considerably with the alterations of RWC in comparison with those from Trigrad. Under desiccation up to 8% RWC the F735/F685 ratio at excitation with 472 nm (chl *b*) remained almost unchanged for thylakoid membranes from Wonderful bridges while it decreased considerably for thylakoid membranes from Trigrad. The thylakoid membranes from Wonderful bridges were more stable and showed less alteration in energy distribution between both photosystems in response to dehydration.

The electrokinetic properties of thylakoid membranes were investigated by measuring the electrophoretic mobility (EPM) and zeta potential (ζ) and surface charge density (σ) were calculated according to Chow et al. (2001). The thylakoids from control plants from Trigrad possessed a high polarity and separation and reorientation of some aggregates during movement in electric field upon illumination. The thylakoids isolated from control plants from Wonderful bridges were characterized by a reorientation of the smaller and bigger thylakoid aggregates during movement in direct current electric field, as well as the binding of couple of aggregates and formation of supracompıexes in suspending media. However, there were no significant changes in aggregation in

thylakoids from plants from Wonderful bridges during desiccation to 50% RWC and 8% RWC.

The values of electrophoretic mobility, zeta potential and surface charge density of thylakoids from control plants from both habitats did not differ significantly (Fig. 4). Desiccation of the plants from Trigrad up to 50% RWC increased the EPM and ζ of thylakoids by 43% ($p<0.001$) and an increase in the net negative surface electric charge (σ) from –0.86 mC m^{-2} to –1.79 mC m^{-2} was observed. The values of all parameters characterizing the electrokinetic properties of the tylakoid membranes remained higher than control at 8% RWC. In contrast to Trigrad, the EPM, ζ and σ of thylakoids from moderately desiccated plants from Wonderful bridges (50% RWC) was not affected (Fig. 4). On the other hand, EPM and ζ increased by 37% ($p<0.001$) in thylakoids from severely desiccated plants (8% RWC) while σ - from –0.90 mC m^{-2} to –1.71 mC m^{-2}.

Desiccation increased ROS production in chloroplasts. In thylakoid membranes from Trigrad the secondary products of lipid peroxidation (TBARS) increased by 60% and 82% in moderately desiccated (50% RWC) and fully desiccated plants (8% RWC), respectively (Fig. 5). However, desiccation of the plants from Wonderful bridges led to a less pronounced increase

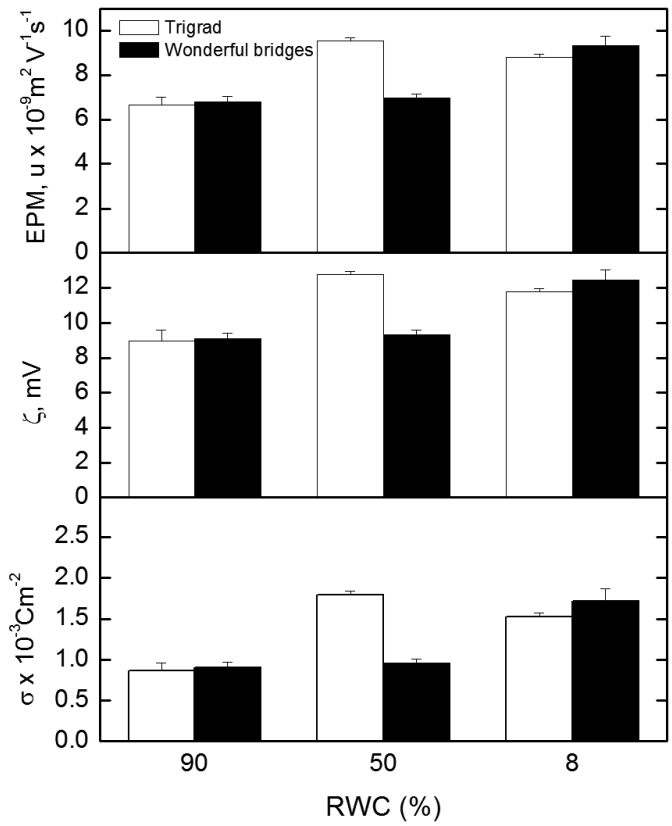

Figure 4. Changes in the electrophoretic mobility (EPM), zeta potential (ζ) and surface charge density (σ) of thylakoid membranes isolated from *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges during dehydration.

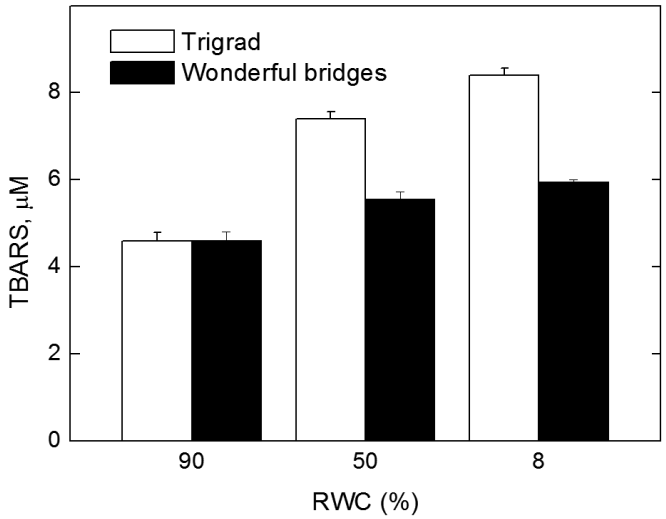

Figure 5. Changes in the secondary products of lipid peroxidation (TBARS) measured in thylakoid membranes isolated from *Haberlea rhodopensis* plants from Trigrad and Wonderful bridges during dehydration.

of TBARS - by 20% and 30% at 50% and 8% RWC, respectively.

In summary, the increased electrolyte leakage from desiccating leaves indicated changes in membrane integrity, which were stronger in plants from Trigrad. In addition, the lipid peroxidation measured in thylakoid membranes isolated from moderately dehydrated and dry *Haberlea rhodopensis* plants from Trigrad was also higher compared to that from Wonderful bridges. The thylakoid membranes of plants from Wonderful bridges showed a less alteration in energy distribution between both photosystems in response to dehydration. The higher surface charge density on thylakoid membranes from moderately dehydrated plants (50% RWC) from Trigrad correlated with an increased energy transfer from PSII to PSI as could be expected for low stacked membranes. Although the studied plants grow in a similar environment with respect to the light conditions, our data showed that thylakoid membranes isolated from plants form Trigrad and Wonderful bridges responded in a different manner to desiccation up to 8% RWC. The thylakoids of plants from Trigrad showed more flexibility, changing the mutual organization of pigment-protein complexes and balancing energy distribution.

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