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ARTICLE

Antioxidative defence mechanisms contributes to desiccation tolerance in *Haberlea rhodopensis* population naturally exposed to high irradiation

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ABSTRACT Drought induced stress is one of the most important among the environmental challenges. *Haberlea rhodopensis*, a chlorophyll-retaining resurrection plant, can survive desiccation to air-dry stage in its usual low irradiance habitat ("shade" plants). Nevertheless, in the past years, some populations living under high irradiance ("sun" plants) have been also discovered with the same ability to survive dehydration. In order to clarify the adaptation mechanisms to a high irradiation habitat, superoxide dismutase (SOD) activity determined by activity staining on polyacrylamide gels and malondialdehyde (MDA) content of sun and shade plants collected from high and low irradiance environment, respectively, were studied. Desiccation induced a significantly higher induction in SOD activity and thus a smaller increase in the MDA content in sun compared to shade plants. The MDA content and SOD activity was restored in both sun and shade plants after six-day rehydration. Nevertheless, the SOD activity remained higher in rehydrated sun leaves compared to the well-hydrated initial stage. The early enhancement of SOD activity in dehydrating sun plants contributes to the higher stress tolerance of these populations.

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KEY WORDS

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Environmental challenges deeply influence plant productivity. However, plants evolved mechanisms in order to tolerate various stresses including drought. One of the most amazing set of tolerance mechanisms is evolved by the resurrection higher plants which have a unique ability to survive dehydration to the air-dry state. *Haberlea rhodopensis* Friv. (Gesneriaceae) is a Balkan endemism that belongs to the homoiochlorophyllous (chlorophyll-retaining) resurrection type (Tuba et al. 1998). Most of the *H. rhodopensis* populations grow in deep shadow under natural conditions, and they are very sensitive to high irradiation especially during desiccation (Georgieva and Maslenkova 2006). In fact, desiccation at 350 μmol m^{-2} s^{-1} irradiance induced irreversible damages in the photosynthetic apparatus, and thus mature leaves were not able to recover during rehydration (Georgieva et al. 2008). Nevertheless, recent studies revealed a high ecological plasticity of *H. rhodopensis* in natural habitats (Daskalova et al. 2011). In spite of the majority of *H. rhodopensis* prefers deeply shaded light environment, several populations inhabit rock surfaces exposed to high light irradiance, drought, high and low temperatures. Previous results showed that the membrane integrity of sun plants is protected (Georgieva et al. 2012). They were shown to have a higher ability to dissipate absorbed excitation energy by an antennae-based non-photochemical quenching route, thus have a reduced photosystem II inactivation during desiccation in contrast to shade plants (Solti et al. 2014). It is also known that resurrection plants use antioxidative defence systems to cope with desiccation (Farrant 2000; Dinakar et al. 2012). For a better understanding of the adaptation of sun plant to the high-light habitat, the oxidative damage and antioxidative defence was compared in *H. rhodopensis* sun and shade plants.

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Materials and Methods

Plant material

Experiments were conducted on *Haberlea rhodopensis* Friv. plants growing in Rhodope Mountains, South-West Bulgaria, Orehovo region (N41°52.231; E024°36.171). Plants of various hydration stages were collected on slopes exposed to full sunlight (1500-1700 μmol m^{-2} s^{-1} average summer photosynthetically active radiation [PAR], leaf-level temperature of 30-37 °C and relative air humidity of about 15-30%; 'sun' plants). Sun plants were compared to understory plants growing in shaded habitats (25 μmol m^{-2} s^{-1} average summer PAR, leaf-level temperature of 21-25 °C and a relative humidity of 40-45%; 'shade' plants). Light intensity was measured at the surface of the collected plants by QSPAR Quantum Sensor (Hansatech Ltd., United Kingdom). Leaf temperature and

Table 1. Superoxide dismutase (SOD) activity (expressed in total pixel density of activity stained gel lanes mg^{-1} total soluble protein) in leaves of shade and sun adapted *Haberlea rhodopensis* plants under desiccation and rehydration. 90% RWC - well-hydrated control; 50% RWC – mild dehydration; 8% RWC - desiccated; R1 - rehydration for one day; R6 - rehydration for six days. Error bars represent SD values. One-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed to analyse statistical differences (P<0.05).

Stages	Shade plants	Sun plants
	pixel density mg^{-1} protein	
90% RWC	190.9±*45.8* [a]	197.4±*32.9* [a]
50% RWC	220.9±*11.8* [a]	382.4±*40.1* [bc]
8% RWC	266.5±*50.6* [ab]	423.9±*6.4* [c]
R1	240.6±*4.5* [a]	307.1±*37.9* [bc]
R6	211.7±*36.3* [a]	301.5±*8.2* [b]

Table 2. Malondialdehyde (MDA) content of shade and sun adapted *Haberlea rhodopensis* leaves under desiccation and rehydration. 90% RWC - well-hydrated control; 50% RWC – mild dehydration; 8% RWC - desiccated; R1 - rehydration for one day; R6 - rehydration for six days. Error bars represent SD values. One-way ANOVAs with Tukey-Kramer *post-hoc* tests were performed to analyse statistical differences (P<0.05).

Stages	Shade plants	Sun plants
	mmol MDA g^{-1} D.W.	
90% RWC	0.110±*0.020* [a]	0.155±*0.027* [ab]
50% RWC	0.128±*0.047* [ab]	0.151±*0.051* [ab]
8% RWC	0.287±*0.030* [c]	0.200±*0.030* [b]
R1	0.167±*0.070* [ab]	0.135±*0.025* [ab]
R6	0.108±*0.017* [a]	0.105±*0.032* [a]

relative humidity values were detected by a Pocket Profi-Termohygrometer (TFA, Germany). Leaves of plants with approximately 90%, 50% and 8% relative water content (RWC) were collected. Rehydration experiments were performed under laboratory conditions and samples were taken after one day (50-60% RWC – R1) and six days rehydration (90-95% RWC – R6). The RWC of *H. rhodopensis* leaves was determined gravimetrically by weighing them before and after oven drying at 80°C to a constant mass and expressed as the percentage of water content in dehydrated tissue compared to water-saturated tissues, using the equation: RWC (%) = (fresh weight – dry weight) 100/(saturated weight – dry weight). Saturated weight was measured on leaf discs maintained 16 h at 4°C in the dark floating on water.

Superoxide dismutase enzyme assay

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured according Giannopolitis and Ries (1977) with some modifications. 100 mg leaf samples were homogenized on ice in 1 ml isolating buffer (50 mM Na-K-phosphate buffer [pH 7.0], 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) Triton X-100, 2 mM polyvinylpyrrolidone and 50 mM Na-ascorbate) and centrifuged at 15000 × g for 15 min, and the supernatants were collected as crude extract. Samples were solubilised mildly (5 mM Tris-HCl [pH 6.8], 0.01% SDS, 10% glycerol and 0.001% bromophenol blue). Soluble proteins were separated by native PAGE using 10-18% gradient acrylamide gels by Laemmli (1970) containing only 0.01% SDS. Gels were stained for SOD activity in 50 mM Na-K-phosphate buffer [pH 7.8], 0.1 mM EDTA, 13 mM methionine, 60 μM riboflavin, 2.25 mM Nitro Blue Tetrazolium. Activity stained gels were evaluated by densitometry using the Phoretix software (Phoretix International, Newcastle upon Tyne, UK).

To determine the protein content of samples, solubilised proteins were run on 10-18% acrylamide gradient gels containing 0.1% SDS (Laemmli 1970). Protein content was calculated by comparing the total density of the Coomassie-stained protein lanes to that of the lane of the protein standard of known protein content.

Determination of MDA content

Malondialdehyde content was determined as described by Esterbauer and Cheeseman (1990). 250 mg leaf material was homogenized at 4 °C in 3 ml of 0.1% TCA, and centrifuged at 10000 × g for 15 min. To the supernatant, 0.5 ml of 0.1 M Tris-HCl (pH 7.6) and 1 ml of 15% (m/v) trichloracetic acid; 0.375% (m/v) thiobarbituric acid; 0.25 M HCl reaction mixture were added. This solution was boiled in water bath for 15 min, centrifuged at 2000 × g for 5 min, and the absorbance was read at 532 nm (ε = 155 mM^{-1} cm^{-1}) for the determination of MDA.

Statistical analysis

Measurements were repeated in six independent replications, the similarity of the samples were analysed using one-way ANOVA with Tukey-Kramer *post-hoc* test (p<0.05) analysed by InStat v. 3.00 software (GraphPad Software, Inc.).

Results

In well-hydrated (90% RWC) leaves, a notable SOD activity was measured which showed no significant differences between the samples, regardless of the habitat of plants (Table 1). At the same time, the changes in SOD activity under desiccation and rehydration showed habitat dependence. Desiccation induced an increase in SOD activity in both the sun and shade leaves. Though the highest SOD activity was measured in fully desiccated leaves (8% RWC) in both populations, the SOD activity of sun leaves was more enhanced compared to shade leaves. Similarly, rehydration caused a decrease in the total activity in both type of leaves but the total SOD activity remained significantly higher compared to that of the initial,

well-hydrated stage in sun leaves, while relaxed to the control value in shade leaves.

The MDA content showed no differences in well-hydrated stage, whereas changed differently during desiccation and rehydration in shade and sun leaves (Table 2). Moderate dehydration had negligible effect on the MDA content of either shade or sun leaves, which only increased drastically in desiccated shade leaves. In sun leaves, no significant increase of MDA content was measured even in desiccated stage. In the rehydration phase, the MDA content decreased tendentiously in shade and also somewhat in sun leaves, thus it reached the typical value of well-hydrated plants after 6-days rehydration. Nevertheless, in sun plants, the MDA content was somewhat higher in the well-hydrated than in rehydrated stage.

Discussion

Reactive oxygen species (ROS) are common by-products in aerobic organisms. Environmental stresses such as drought increases the ROS level in cells, particularly in chloroplasts (Cruz de Carvalho 2008). The photosynthetic apparatus have a high sensitivity against oxidative damage that primarily causes inactivation of photosystem II. To protect cells against the damaging effects of ROS and maintain the redox equilibrium, antioxidative defence systems, including the SOD enzymes, have evolved (Asada 2006). In chloroplasts, SOD isoforms are responsible for the elimination of superoxide anion radicals which are among the most dangerous ROS.

Antioxidative defence systems were shown to be involved in the protection of resurrection plants during desiccation (Farrant 2000; Dinakar et al. 2012). We also confirmed their role in desiccation tolerance of *H. rhodopensis* sun and shade populations: increased SOD activity during desiccation was accompanied by only moderate elevation of MDA content in both shade and sun leaves. Desiccation increased the SOD activity, which correlated with the total antioxidative defence of the plant in many plant species (e.g. *Pisum*, Moran et al. 1994; *Glycyrrhiza*, Pan et al. 2006).

Desiccation-induced oxidative stress was significantly higher in shade than in sun leaves of *H. rhodopensis*. The lower MDA accumulation together with the higher SOD activity in sun leaves indicates that ROS eliminating mechanisms are more enhanced in sun than in shade leaves. Earlier studies on SOD activity under desiccation and rehydration of flowering and post-flowering *H. rhodopensis* plants (Yahubyan et al. 2009) found that mild desiccation decreases, whereas strong desiccation increases the SOD activity in shade *H. rhodopensis* leaves. They found a significantly higher SOD activity in well-hydrated flowering, compared to post-flowering plants. In plants of similar origin (Bachkovo region) to that studied in the paper of Yahubyan et al. (2009), Georgieva et al. (2012) found a decreased MDA content under desiccation, whereas a stable level in a population originated from Sitovo region, grown under higher irradiance. Concerning our results, the sun populations of Orehovo region also showed a significantly higher elevation in SOD activity under desiccation than the shade ones collected from a close locality. In agreement, sun plants were shown to cope with less photoinhibition during desiccation compared to shade ones (Solti et al. 2014). As one of the reasons for photoinhibition is the ROS accumulation, the enhanced SOD activity and the consequently decreased oxidative stress (MDA content) of desiccating sun *H. rhodopensis* plants contribute to the maintenance of photosynthetic activity under mild desiccation and prevent the stronger damage under total dehydration. The SOD activity in rehydrated sun leaves remained significantly higher compared to the initial well-hydrated stage, which may also contribute to the adaptation to the unfavourable conditions in their natural habitat.

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