

# Characterization of oxidative and antioxidative events during dehydration and rehydration of resurrection plant *Ramonda nathaliae*

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**Abstract** In order to investigate changes of oxidative status in relation to the activity of the various protective mechanisms in resurrection plant *Ramonda nathaliae*, we have analysed time and relative water content (RWC) related changes in lipid peroxidation and ion leakage, hydrogen peroxide accumulation, changes of pigment content and antioxidative enzyme activity, together with expression of dehydrins. The results indicate that enhanced oxidative status during dehydration, not previously reported for resurrection plants, could play an active role in inducing the desiccation adaptive response in *R. nathaliae*. A critical phase is shown to exist during dehydration (in the range of RWC between 50 and 70%) during which a significant increase in hydrogen peroxide accumulation, lipid peroxidation and ion leakage, accompanied by a general decline in antioxidative enzyme activity, takes place. This phase is designated as a transition characterized by change in the type of stress response. The initial response, relying mainly on the enzymatic antioxidative system, is

suspended but more effective, desiccation specific protective mechanisms, such as expression of dehydrins, are then switched on. The expression of dehydrins in *R. nathaliae* could be inducible as well as constitutive. In order to cope with the oxidative stress associated with rapid rewatering, *R. nathaliae* reactivated antioxidative enzymes. We propose that controlled elevation of reactive oxygen species, such as hydrogen peroxide, could be an important mechanism enabling resurrection plants to sense dehydration and to trigger an adaptive programme at an appropriate stage during the dehydration/rehydration cycle.

**Keywords** Resurrection plants · *Ramonda nathaliae* · Dehydration/rehydration · Antioxidative systems

## Introduction

The higher plants exhibit several strategies to cope with life in dry environments. However, only a few of them, designated resurrection plants, have the ability to survive nearly complete water loss in their vegetative tissues for extended periods and to “resurrect” from this desiccated state to recover full metabolic competence upon rehydration. These plants are able to survive several dehydration/rehydration cycles during their life, tolerating various detrimental influences, such as water deficit, osmotic, mechanical and oxidative stress.

Current evidence suggests that multiple mechanisms and several signalling pathways are involved in the dehydration/rehydration response (Bartels 2005). The general adaptive strategy for surviving dehydration/rehydration stress is achieved by the ability to limit cellular damage and maintain membrane integrity, as well as to mobilize repair mechanisms rapidly and effectively upon rehydration

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and to minimize free radical production (reactive oxygen species—ROS) (Bewley and Krochko 1982; Sherwin and Farrant 1996; Oliver et al. 2000, 2005). Oxidative stress is known as one of the most deleterious consequences of water deficit in plants (Mundree et al. 2000). Plants have developed mechanisms to protect them from ROS that are both non-enzymatic (e.g. glutathione, ascorbate, tocopherols) and enzymatic. The enzymatic defence mechanism includes enzymes regenerating the reduced forms of antioxidants, such as ascorbate peroxidase (APX) and glutathione reductase (GR), and ROS-interacting enzymes (ROS scavengers), such as superoxide dismutase (SOD), catalase (CAT), non-specific peroxidase (POD) and ascorbate peroxidase (APX). Investigation of antioxidative enzyme activities in various resurrection plants has confirmed the hypothesis that there is no universal scheme of antioxidative defence, but that each plant has its own specific mechanism (Farrant 2000). In addition, it is apparent that ROS, especially hydrogen peroxide, play an important signalling role in the response to biotic and abiotic environmental stimuli, and that the level of ROS in plant cells has to be tightly regulated (Mundree 2002). However, the dynamics and extent of ROS production during dehydration and rehydration of resurrection plants have not been analysed in detail.

Desiccation and recovery also provoke expression of other mechanisms that protect cells from detrimental structural and functional changes. Some proteins, presumably dehydrins (DHNs-LEA D11 family), a group of thermostable, highly hydrophilic proteins, are involved in the control and protection of the normal functional conformations of enzymes and some structural proteins (Close 1997; Cellier et al. 1998; Rinne et al. 1999). Furthermore, dehydrins may also act as free radical scavengers and sequester ions (Collet et al. 2004; Hara et al. 2004).

The majority of resurrection plants originate from the southern hemisphere and they have received much attention and multi-disciplinary investigation regarding diverse and numerous physiological, ultrastructural, biochemical and molecular analyses (Bartels and Salamini 2001; Bartels 2005; Farrant et al. 2003; Kranner et al. 2002; Oliver et al. 2000; Vicré et al. 2004; Moore et al. 2009). Among the rare angiosperm resurrection plants of the northern hemisphere are two endemic and preglacial relict species of the genus *Ramonda*—*R. serbica* and *R. nathaliae* (Košanin 1921, Stevanović et al. 1991). They grow on the shallow soil in crevices of the northern rocky slopes of gorges and canyons of the Balkan Peninsula, from the foothills to the upper alpine belts. They tolerate in their restricted ecological niches relatively short, but severe periods of water loss, from early summer to late autumn when rainfall is often very scarce.

Being homoiochlorophyllous, both species preserve more or less of their chlorophyll through a dehydration/rehydration cycle that enables them to rapidly resume photosynthesis upon rehydration, but also makes them more susceptible to ROS formation (Dražić et al. 1999; Quartacci et al. 2002). These plants need effective protection against the free radical attack experienced either during dehydration or immediately upon rehydration. Although phylogenetically closely related, these plants differ in their ecological requirements (Stevanović et al. 1991) and ploidy levels (Šiljak-Yakovlev et al. 2008). Protective systems involved in the response to dehydration/rehydration, such as changes of membrane lipids (Quartacci et al. 2002), ascorbate and glutathione contents (Sgherri et al. 2004), antioxidative enzyme activities (Veljović-Jovanović et al. 2006; Veljović-Jovanović et al. 2008), have been investigated in *R. serbica* but not in *R. nathaliae*. The objective of this work was to analyse some oxidative/antioxidative events in leaves of *R. nathaliae* during dehydration and rehydration. Changes in pigment content, antioxidative enzyme activities and dehydrin expression have been investigated in relation to changes in levels of lipid peroxidation and ion leakage, and, for the first time in resurrection plants, accumulation of hydrogen peroxide.

## Materials and methods

### Plant material and treatment

Specimens of *R. nathaliae* were taken from their natural habitat in Jelašnica gorge (SE Serbia). Fifteen plants were harvested together with the attached layers of soil. After collection, the plants were acclimated in Belgrade Botanical garden for 4 weeks under full watering until the beginning of experiments. To study dehydration, plants were subjected to drought by withholding watering over a period of 14 days, and were kept at room temperature and with ambient photoperiod. After 14 days of withholding water, leaves were completely desiccated and plants were in a state of anabiosis. After 2 weeks in anabiosis the plants were rehydrated until they recovered their initial hydrated state. Rehydration was achieved by spraying the plants with water. For analysis, samples of leaves were taken from fully hydrated control plants (C), from plants in different stages of dehydration, after 7 days (D<sub>1</sub>—initial stage), 10 days (D<sub>2</sub>—middle stage) and 14 days (D<sub>3</sub>—desiccated state), and upon re-watering, after 6 (R<sub>1</sub>), 12 (R<sub>2</sub>), 24 (R<sub>3</sub>) and 48 (R<sub>4</sub>) h. They were immediately frozen in liquid nitrogen. In order to avoid variation due to differences in the age and position of the leaves, all analyses

were performed on the fully expanded leaves comparable in size and position in the plants.

#### Relative water content

Relative water content (RWC) was determined at each sampling time during the dehydration-rehydration cycle ( $D_1$ – $R_4$ ) according to the formula (Barrs and Weatherly 1962).

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

Leaf dry weight was measured after oven drying at 105°C for 24 h, and the saturated weight after incubation of the leaves in moist filter paper for 24 h in Petri dishes at room temperature.

#### Lipid peroxidation

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction, as described by Heath and Packer (1986). The crude extract was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95°C for 30 min and then rapidly cooled in an ice-bath. After centrifugation at 3,500×g for 10 min, the absorbance of the supernatant was monitored at 532 nm. The MDA content in the reaction mixtures was calculated per mg of dry weight, and the level of lipid peroxidation was expressed relative to control plant (100%).

#### Electrolyte leakage

Plasma membrane permeability during the dehydration-rehydration cycle was evaluated from the amount of electrolytes released from leaf discs rinsed in distilled water at 25°C. Conductivity of the sample medium was measured each hour (Conductivity meter HI 8733 (Hanna, Tokyo)), according to the manufacturer's protocol. The total electrolyte of samples was obtained after boiling for 5 min. Results were expressed as percentage of total electrolyte.

#### In situ histochemical detection of hydrogen peroxide

Hydrogen peroxide ( $H_2O_2$ ) in leaves was localized in situ according to Thordal-Christensen et al. (1997) enabling its accumulation to be detected visually. The method is based on  $H_2O_2$  catalyzed polymerization of 3,3'-diaminobenzidine (DAB). Once DAB encounters  $H_2O_2$ , it undergoes oxidative polymerization to produce a dark brown precipitate. Leaves were detached from each control and treated

plants, placed in 1 mg ml<sup>-1</sup> 3,3'-diaminobenzidine-HCl pH 3.8 (Sigma, MO, USA; #D-8001). A low pH is necessary to solubilize DAB. The leaves were incubated for 8 h in the dark and then immersed in boiling ethanol (96%) for 20 min., for easier visualization of the brown polymerization product. The DAB, polymerized in the presence of  $H_2O_2$ , was visualized as a brown precipitate in the leaves. Plants were treated with 10 mM ascorbic acid or 5 mM hydrogen peroxide, respectively prior to infiltration of DAB, as negative and positive controls.

#### In situ histochemical detection of $O_2^{\bullet-}$

The histochemical detection of  $O_2^{\bullet-}$  was performed by vacuum infiltrating leaves directly with 6 mM nitroblue tetrazolium (NBT). NBT reacts with superoxide producing a dark blue insoluble formazan compound (Flohe and Otting 1984; Beyer and Fridovich 1987; Maly et al. 1989). Chlorophyll was partially removed by rinsing leaves in 80% ethanol for 10 min at 70°C. As no reaction was detectable with NBT, the control test with 1 mM tetramethylpyrazine was not performed.

#### Pigment content

Pigments (chlorophyll, carotenoids, anthocyanins) were extracted from 0.5 g of plant leaves by homogenization in ethanol in the dark. Extracts were centrifuged for 10 min at 3,500×g. Supernatants were analysed spectrophotometrically and the pigment contents were calculated according to Lichtenthaler (1987) and expressed as mg per g of dry weight (dw).

#### Preparation of extracts for assaying enzyme activities

Frozen leaves were ground in liquid nitrogen and the powder suspended in extraction buffer—50 mM potassium phosphate, pH 7.0, and 0.1 mM EDTA, containing 5% (w/v) PVP. The homogenates were centrifuged at 15,000×g for 20 min and the supernatant fraction used for the assays. All steps were carried out at 4°C. Protein concentration in the extracts was determined according to Bradford 1976, using a *BioRad* assay kit with bovine serum albumin as standard.

#### Enzyme assays

*Catalase* (EC 1.11.1.6) activity was assayed in a reaction mixture (1.5 ml) composed of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 2 mM  $H_2O_2$  and 20 μl crude extract. The reaction was started by adding  $H_2O_2$  and the activity was followed by monitoring the decrease in absorbance at 240 nm as a consequence of  $H_2O_2$

consumption (Aebi 1984). Catalase activity was expressed as  $\Delta A_{240} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

*Ascorbate peroxidase* (EC 1.11.1.11) activity was assayed as described by Nakano and Asada (1981), using a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.5 mM ascorbate, 10 mM  $\text{H}_2\text{O}_2$  and 20  $\mu\text{l}$  crude extract.  $\text{H}_2\text{O}_2$  dependent oxidation of ascorbate was followed by monitoring absorbance decrease at 290 nm. Ascorbate peroxidase activity is expressed as  $\Delta A_{290} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

Total soluble *peroxidase* (POD) activity was determined in the crude extract by measuring the increase in absorbance at 470 nm, due to the formation of tetraguaiacol, in a reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.1 mM guaiacol and 20  $\mu\text{l}$  crude extract. The reaction was started by adding  $\text{H}_2\text{O}_2$  (final concentration 10 mM). POD activity was expressed as  $\Delta A_{470} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

*Superoxide dismutase* (EC 1.15.1.1) activity was measured according to Beuchamp and Fridovich (1971). Crude extract (50  $\mu\text{l}$ ) was added to the reaction mixture (1.5 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 13 mM methionine, 2  $\mu\text{M}$  riboflavin and 75  $\mu\text{M}$  nitro blue tetrazolium (NBT). Riboflavin was added last and the tubes were shaken. The reaction was started by exposing the mixture to a cool white fluorescent light. After 15 min. the light was switched off, the tubes were mixed and the absorbance measured at 560 nm. SOD activity was expressed as  $\Delta A_{560} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

*Glutathione reductase* (EC 1.6.4.2) was measured according to Foyer and Halliwell (1976). The assay medium contained 0.025 mM phosphate buffer pH 7.8, 0.5 mM GSSG, 0.12 mM NADPH- $\text{Na}_4$  and 50  $\mu\text{l}$  of extract. NADPH oxidation was determined at 340 nm. Activity was expressed as  $\Delta A_{340} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

#### Western blot analysis of dehydrins

Proteins were resolved by 12% SDS-PAGE and then transferred to a PVDF membrane (Millipore) using Biometra semi-dry transblotter. The cathode transfer buffer contained 25 mM Tris-HCl pH 9.4, 40 mM glycine and 20% (v/v) methanol. The anode transfer buffer I contained 300 mM Tris-HCl pH 10.4, and 20% (v/v) methanol, the anode transfer buffer II contained 25 mM Tris-HCl pH 10.4 and 20% (v/v) methanol. Proteins were transferred for 40 min at room temperature and a constant current of 5 mA/cm<sup>2</sup> membrane. The membranes were then blocked overnight with 5% dried non-fat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blots were used for immunochemical detection of dehydrins.

Dehydrins were detected according to the method of Close et al. (1993). The membranes were incubated in 1:1,000 dilution of rabbit anti-dehydrin antibodies in blocking solution (5% non-fat milk in TBST) for 1 h. The antibody was developed against the conserved consensus sequence TGEKKGIMDKIKEKLPQGH and kindly provided by dr Timothy Close (Department of Biochemistry and Plant Science, University of California, Riverside, USA). The blots were washed 3 times for 5 min each in TBST and incubated with 1:15,000 dilution of secondary antibody—goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma). After washing, the signal was detected using AP buffer and NBT/BCIP as a substrate.

#### Statistics

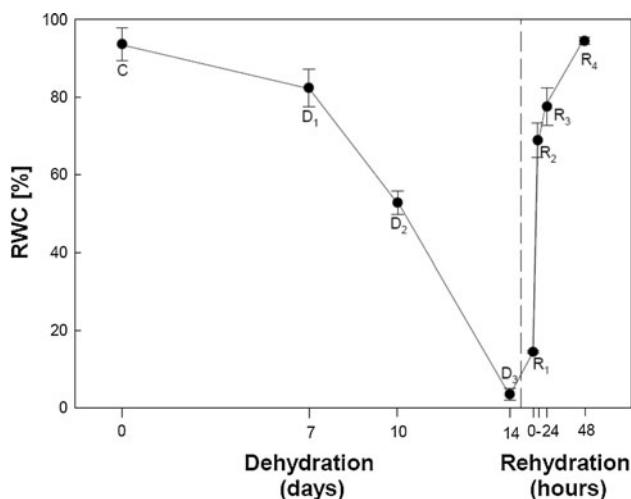
All analyses were performed in three replicates, each of them originated from different plant. All data were subjected to statistical analysis using the SigmaStat program. Comparisons with  $P < 0.05$  were considered significantly different. In all the Figures, the spread of values is shown as error bars representing standard errors of the means.

#### Results

Fully hydrated plants of *R. nathaliae* were subjected to slow dehydration by withholding water for 14 days and, after 2 weeks of anabiosis, rehydration. During dehydration, plant leaves exhibited the changes of shape and colour characteristic of resurrection plants—hanging down, inward folding of the adaxial side of the leaf, completely dry and brown at the end of dehydration. At the same time the relative water content (RWC) decreased from 94% in fully hydrated plants to 3.6% in the desiccated state. Kinetics of water loss are shown on Fig. 1. According to the RWC and rate of water loss, four important points were selected during dehydration and assigned as: C, control fully hydrated plant (RWC 94.5%), D<sub>1</sub> (after 7 days of dehydration, RWC 82.4%), D<sub>2</sub> (after 10 days of dehydration, RWC 54.9%), and D<sub>3</sub> (after 14 days of dehydration, RWC 3.6%). On rewatering, the RWC was restored rapidly, the leaves started to unfold within the first 6 h following initial rehydration, when their relative water content reached 14.5% (R<sub>1</sub>). Their RWC continued to increase to 68.9% (R<sub>2</sub>), 77.6% (R<sub>3</sub>) and 94.5% (R<sub>4</sub>), in leaves harvested at 12, 24 and 48 h of rewatering (Fig. 1).

#### Lipid peroxidation and ion leakage

Levels of lipid peroxidation showed significant, but transient increases twice during the dehydration-rehydration cycle, being 1.5-fold higher in D<sub>2</sub> and 1.2-fold higher in R<sub>2</sub> (RWC



**Fig. 1** Changes of the relative water content (RWC) in leaves of *R. nathaliae* plants subjected to dehydration and rehydration. C control; D<sub>1</sub>, dehydrated for 7 days; D<sub>2</sub>, dehydrated for 10 days; D<sub>3</sub>, dehydrated for 14 days; R<sub>1</sub>, rehydrated for 6 h; R<sub>2</sub>, rehydrated for 12 h; R<sub>3</sub>, rehydrated for 24 h; R<sub>4</sub>, rehydrated for 48 h. Data points represent the average of three different measurements ± standard errors based on three replicates

54.9%) and (RWC 68.9%) than in control plants (Fig. 2). In both completely desiccated (D<sub>3</sub>) and well re-watered plants (R<sub>3</sub> and R<sub>4</sub>) these values were close to control level. Electrolyte leakage showed the same pattern, thus corresponding with the level of lipid peroxidation and being high in both D<sub>2</sub> (RWC 52.8%) and R<sub>2</sub> (RWC 68.9%) (Fig. 2).

**H<sub>2</sub>O<sub>2</sub> accumulation in *R. nathaliae* leaves**

Hydrogen peroxide accumulated in leaves of *R. nathaliae* during dehydration (Fig. 3I). The presence of hydrogen peroxide is evident in the initial phase of dehydration and accumulated further, reaching its maximum level in D<sub>2</sub>. Brown precipitates, reflecting H<sub>2</sub>O<sub>2</sub> accumulation, were

not detected in fully dehydrated plants. Hydrogen peroxide accumulation was not observed after 24 h of rehydration. The orange-brown staining, characteristic for the presence of H<sub>2</sub>O<sub>2</sub>, was absent in the leaves of the plants pretreated with 10 mM ascorbic acid (Fig. 3II).

**O<sub>2</sub><sup>•-</sup> accumulation in *R. nathaliae* leaves**

Formation of formazan deposits was not detected in any phase of dehydration-rehydration cycle indicating that superoxide production was not significantly higher than the rate of its detoxification (Fig. 3IV). This result corresponds to the increased activity of superoxide dismutase as detected during both dehydration and rehydration phases, attesting the necessity for superoxide scavenging.

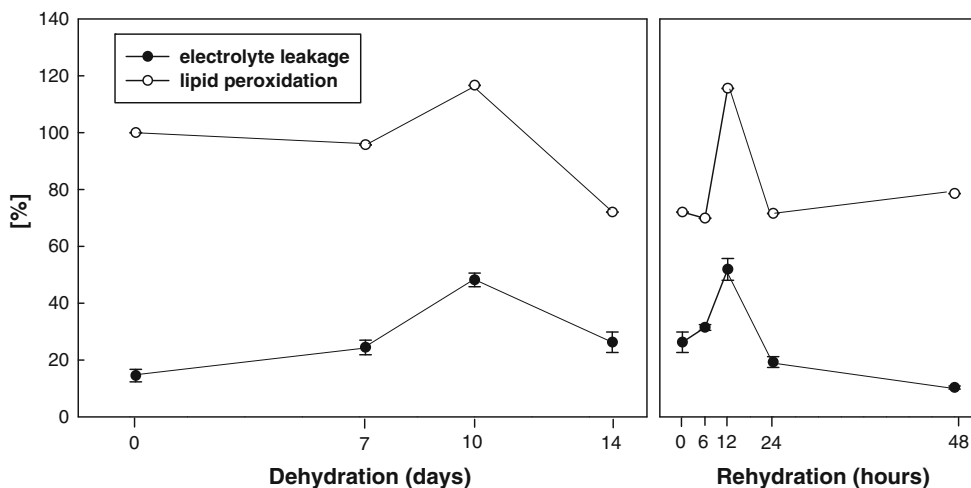
**Pigment content**

Amounts of chlorophyll *a*, chlorophyll *b* and carotenoids decreased during dehydration and reached 33, 30 and 36% lower values, respectively, in respect to the control (Fig. 4). Upon rehydration, their amounts gradually increased and returned to near control levels in fully rehydrated plants. In contrast, the content of anthocyanins increased during plant desiccation, reaching values almost twofold higher in D<sub>2</sub> than in the control plant. In the course of the first 12 h of rewatering their amount decreased, after which it started to increase continuously with further plant rehydration.

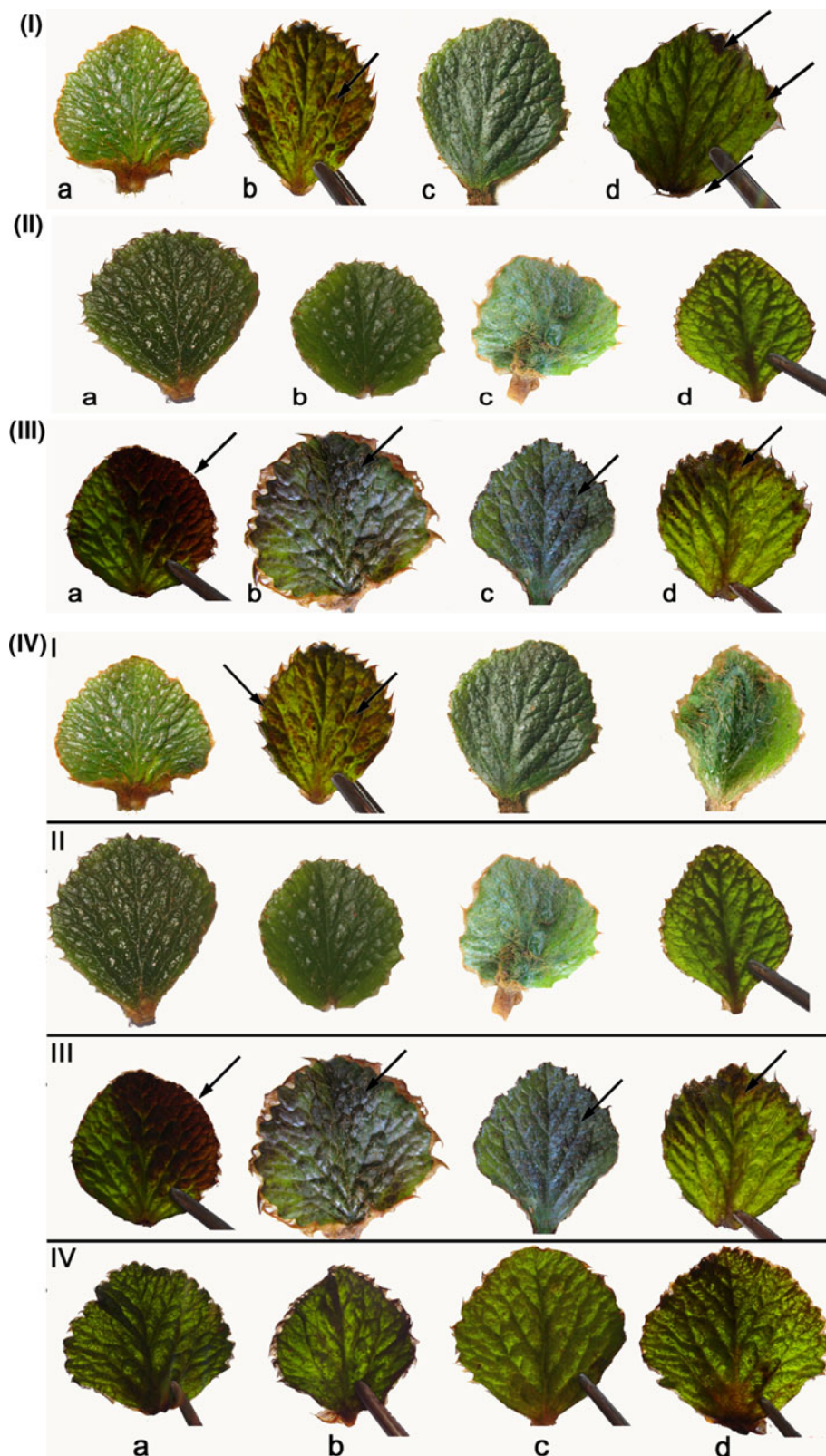
**Activity of antioxidative enzymes**

The dynamic changes in antioxidative enzyme activities were found to depend on the plant hydration status. The activities of class III peroxidases (PODs), APX and GR showed similar trends during the first 7 days of dehydration, reaching 3.8-fold, 9.1-fold and 5.6-fold higher values,

**Fig. 2** Changes in ion leakage and lipid peroxidation in the leaves of *R. nathaliae* during dehydration and rehydration. The measurements of ion leakage were performed after 1 h (filled circle). Level of lipid peroxidation was determined as MDA content (open circle). The values were expressed as percentage relative to the controls ± standard errors based on three replicates. Significant differences *P* < 0.05



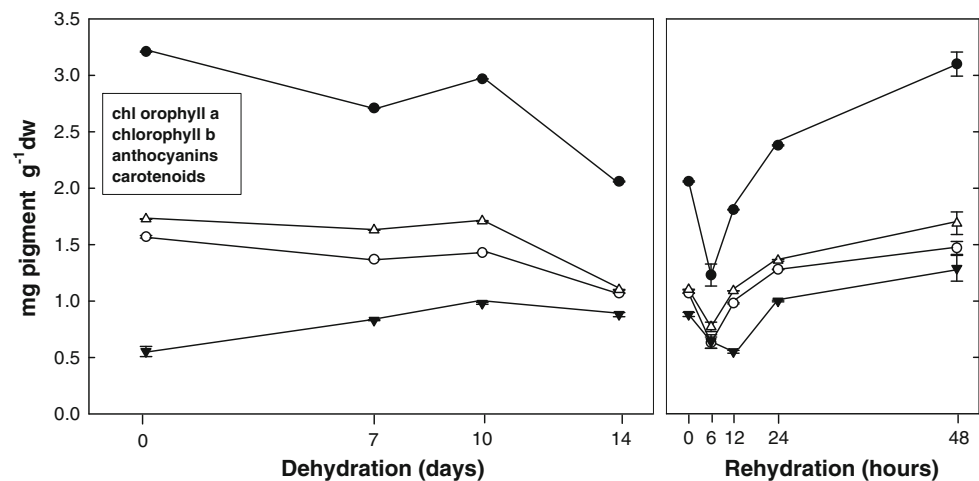
**Fig. 3** **a** control plant; **b** middle phase of dehydration—D2; **c** desiccated state—D3; **d** rehydrated plant. In situ histochemical detection of hydrogen peroxide by DAB staining (I); absence of DAB precipitates in ascorbate pretreated leaves (II); induction of DAB staining in  $H_2O_2$  pretreated leaves. *Arrows* indicate precipitates due to DAB staining. Absence of blue formazan deposits in leaves indicates the absence of superoxide accumulation in leaves (IV)



respectively, than in control plants (Fig. 5a, b). All these activities were reduced further, to near control level, in fully desiccated leaves (D<sub>3</sub>). The water influx upon

rewatering triggered significant increases in APX and GR activities during the first 12 h, to levels 2.8- and 6.4-fold higher than control. The activities of these enzymes then

**Fig. 4** Changes in chlorophyll a (filled circle), chlorophyll b (open circle), carotenoid (open triangle) and anthocyanin contents (filled inverted triangle), in the leaves of *R. nathaliae* during dehydration and rehydration. Pigment contents were expressed as mg per g of dry weight (mg pigment  $\text{g}^{-1}$  dw)  $\pm$  standard errors based on three replicates



decreased rapidly. PODs activity increased during rehydration, by 24 h (R<sub>3</sub>) reaching 10-fold higher activity than in control, and then decreased. SOD activity increased slightly during the first 10 days of dehydration and then returned to near control level in D<sub>3</sub> (Fig. 5b). During rehydration, its activity increased steadily. On the other hand, CAT activity showed no significant changes during plant desiccation, but upon rewatering its activity increased rapidly during the first 6 h, reaching a value about 15-fold higher than control. This high activity was retained during further rehydration. (Fig. 5a).

#### Expression of dehydrins

Dehydrins in *R. nathaliae* leaves during dehydration and rehydration were detected by western blot, using dehydrin-specific antibodies (Fig. 6). Dehydrins (MW 25 kDa) were abundant in fully hydrated control plants and new dehydrin polypeptides (MW 24 and 26 kDa) appeared after 7 days of dehydration. The appearance and accumulation of 28 kDa dehydrins, together with further accumulation of 24, 25 and 26 kDa dehydrins in fully desiccated leaves (D<sub>3</sub>), were the most prominent changes in dehydrin expression during dehydration. These dehydration-specific dehydrins gradually decreased during rehydration, although accumulation of 24 kDa dehydrin is evident during 24 h of rehydration.

#### Discussion

Two closely related endemorelict homiochlorophyllous resurrection plants of the genus *Ramonda* inhabit refugial habitats in the Balkan peninsula and are subject to short but repetitive periods of environmental drought stress. The polyploid *R. serbica* has until now received more attention. The diploid *R. nathaliae*, probably the evolutionary older species (Šiljak-Yakovlev et al. 2008), is restricted to habitats

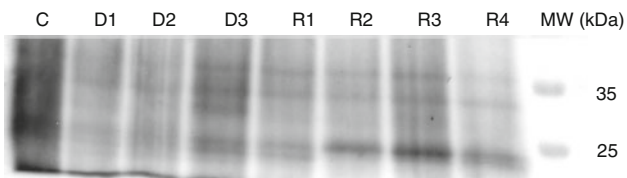
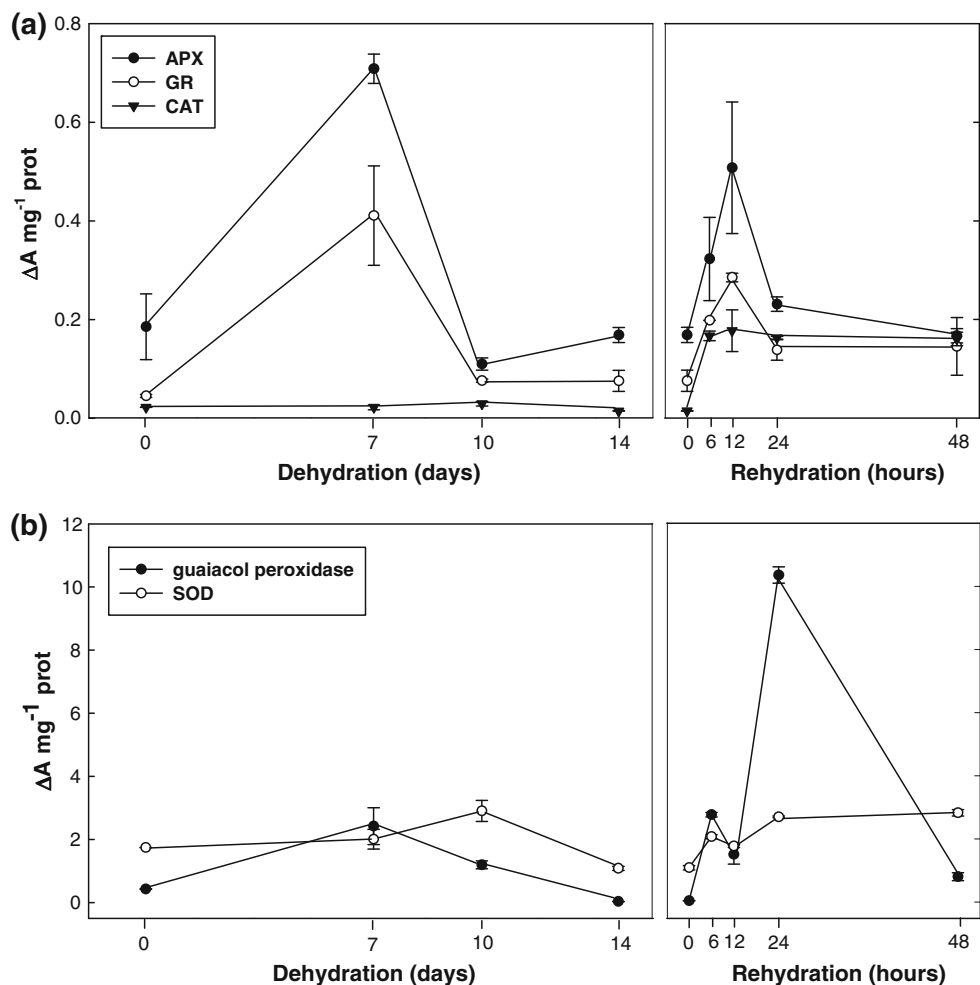
with least competition and consequently exposed to even harsher environmental conditions where the drought is prolonged and more severe (Stevanović et al. 1991).

Here we present results of the study performed in *R. nathaliae* analyzing the changes in hydrogen peroxide accumulation, lipid peroxidation levels and ion leakage together with the changes in the leaf pigment content, antioxidative enzymes activity as well as the expression of dehydrins. This combination of elements playing in the dehydration/rehydration stress response was chosen in order to investigate possible relationships and interactions between them, in the time-related course of events during the dehydration-rehydration cycle.

Three phases were analyzed in the dehydration process, based on the morphological changes of the leaves which correspond to the decrease in RWC: initial (D<sub>1</sub>, RWC up to 80%), middle (D<sub>2</sub>, RWC up to 50%) and late (anabiotic) phase (D<sub>3</sub>, RWC below 5%) (Fig. 1). The initial phase of dehydration is accompanied by a mild increase in hydrogen peroxide production and, subsequently, an increase in lipid peroxidation and ion leakage, suggesting the beginning of the oxidative damage in this phase (Figs. 2, 3).

*R. nathaliae* leaves were stained with DAB for in situ detection of hydrogen peroxide (Fig. 3). Increased H<sub>2</sub>O<sub>2</sub> accumulation was observed in leaves dehydrated for 7 and 10 days, suggesting the dehydration induced rapid, time-dependent production of H<sub>2</sub>O<sub>2</sub> which brought about an oxidative burst in the middle phase at the 50% RWC. However, in fully dehydrated plants (D<sub>3</sub>) H<sub>2</sub>O<sub>2</sub> accumulation was not observed and at the same time there is a drop in ion leakage and lipid peroxidation levels, indicating that potent protective mechanisms are activate at the time of the oxidative burst (Fig. 5). To our knowledge, the generation of hydrogen peroxide in leaves of resurrection plants during dehydration has not previously been investigated. During rehydration, a significant, but transient, increase in lipid peroxidation and ion leakage closely accompanied

**Fig. 5** Changes in specific activity of antioxidative enzymes in the leaves of *R. nathaliae* during dehydration and rehydration. **a** activities of APX (filled circle), GR (open circle) and CAT (filled inverted triangle); **b** activities of SOD (open circle) and PODs (filled circle). Enzyme activities are expressed as  $\Delta A \text{ mg}^{-1}$  of proteins. Values are mean  $\pm$  standard error based on three replicates



**Fig. 6** Western blot analysis of dehydrin expression during dehydration and rehydration of *R. nathaliae*. Total soluble proteins (50  $\mu\text{g}$ ) from each sample of *R. nathaliae* leaves during dehydration ( $D_1$ – $D_3$ ) and rehydration ( $R_1$ – $R_4$ ) were resolved on 12% polyacrylamide gel, transferred to PVDF membrane and dehydrins were detected using anti-dehydrin antibodies. C-fully hydrated plants (control). MW molecular weight standards

with the rise in antioxidative enzymes could be explained by re-establishment of metabolic activities.

There is a marked involvement of antioxidative enzymes in maintaining redox homeostasis during dehydration-rehydration processes in *R. nathaliae*. The time courses of different antioxidative enzyme activities revealed a sequential involvement of these enzymes. Thus, during the initial phase of dehydration, APX and GR are the first enzymes to show markedly increased activities (Fig. 5a). In the early stages of

dehydration, increased activity of GR could lead to enhanced production of reduced glutathione that serves as a thiol cell buffer. In addition, a similar profile of APX activity indicates that the glutathione-ascorbate cycle might be playing a major role in redox homeostasis during the initial dehydration. PODs activity was also increased suggesting their important role in ROS detoxification (Fig. 5b). The dominance of these enzymes over CAT activity (Fig. 5a) makes them the major scavengers of hydrogen peroxide in *R. nathaliae* cells during the first 10 days of dehydration.

The increase in SOD activity (Fig. 5b), that coincides with decrease in APX, GR and PODs activities in  $D_2$ , could lead to enhanced generation of  $\text{H}_2\text{O}_2$  and subsequent increase in lipid peroxidation and ion leakage, as was indeed observed (Figs. 2, 3, 5). In severe water loss ( $D_3$ ) hydrogen peroxide is not detected and lipid peroxidation and ion leakage are brought to near control levels. Also, it is remarkable that in the fully dehydrated state ( $D_3$ ), the antioxidative activity of all investigated enzymes was close to the control levels, indicating their resistance to breakdown, although in conditions of nearly complete water loss. It can be assumed that some other powerful protective mechanisms are operative.



Changes in the content of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) on the one hand and anthocyanins on the other, showed an inverse relationship (Fig. 4). Photosynthetic pigments were only partially broken down during dehydration, in agreement with results previously obtained in both *Ramonda* species as well as in other homoiochlorophyllous resurrection plants (Dražić et al. 1999; Tuba 2008). The persistently increased level of anthocyanins during dehydration indicates their important antioxidative role in the first line of antioxidative defence, as suggested by Larson 1988 and Smirnov 1993.

Presence of dehydrins appears closely related to desiccation tolerance (Bartels 2005). Two proteins that resemble dehydrins were detected in well-watered leaves in the drought-tolerant resurrection plant *Craterostigma plantagineum*, while changes in plant hydration status led to the appearance of new proteins (Schneider et al. 1993). Our results indicate that in *Ramonda nathaliae* the presence of dehydrins and their variations during dehydration-rehydration could be both constitutive and inducible. Thus, dehydrins are already present in fully hydrated leaves and new classes emerge and accumulate during the late phase of dehydration, suggesting that they are required for normal plant metabolism, and that their diversification and accumulation could be elicited in dehydration. Dehydrins could be responsible for water retention, for slowing down dehydration and might exercise a direct protective role for proteins. The possible free radical scavenging activity was reported for *Citrus unshiu* dehydrin CuCOR19 (Hara et al. 2004). Emergence of the new dehydrins during dehydration in *R. nathaliae* coincides with decline in both antioxidative enzyme activities (Fig. 5a, b, d) and hydrogen peroxide content (Fig. 3). The dehydrin of 28 kDa molecular mass is induced during the late dehydration phase and could be seen as a marker of the fully dehydrated state. This dehydrin could be responsible for protection of cell structures in conditions of significant water loss. The different pattern of dehydrins during rehydration suggests new roles for dehydrins, like chaperone-like activity in refolding and repair of proteins.

Comparison of the responses of *R. nathaliae* to dehydration and to rehydration reveals the different involvement and activities of particular defence players. The most prominent difference between dehydration and rehydration, regarding the involvement of antioxidative enzymes, was the significant increase in CAT activity during rehydration. The peak of APX and GR activities reached in R<sub>2</sub> (Fig. 5a) could be related to regeneration of reduced ascorbate and glutathione pools during the first 12 h of plant rehydration. Thus, it was shown for its closely related species *R. serbica* that the total pools of ascorbate and glutathione in the initial rehydration state are mainly in their oxidized forms (Sgherri et al. 2004). Decrease in hydrogen peroxide and

lipid peroxidation during rehydration correlated with increased SOD, CAT and PODs activities (Fig. 5a, b), suggesting that coordinated activity of these enzymes is responsible for ROS detoxification. Repair of membranes (decreasing ion leakage) could be assigned, at least in part, to prominent POD activities (Figs. 2, 5b).

Accumulated knowledge about the mechanisms of resurrection strategies in plants supports the conclusion that each plant has its own specificity in antioxidative response (Farrant et al. 2007). This might also be the case in the close related plants *R. nathaliae* and *R. serbica*. The involvement and kinetics of antioxidative enzyme activities during dehydration/rehydration of *R. nathaliae* differ slightly from those observed previously during dehydration/rehydration of *R. serbica*. In addition, the investigations in *R. serbica* have added valuable information about phenolic acids, demonstrating the important role of polyphenols in antioxidative protection (Sgherri et al. 2004; Veljović-Jovanović et al. 2006).

The time course of oxidative events and antioxidative system activity in *R. nathaliae* suggests an ROS-based regulation of defence response in a cascade of events and a sequential prevalence of different compounds in this dynamic network. The critical and the least stable periods appear to be the middle phase of dehydration, between days 7 and 10, and the first 12 h upon rehydration, when occurs an enhanced oxidative status at the RWC values of 50–70%. It is noteworthy that dehydrins are present in control plants, but specific new classes emerge and accumulate in the late dehydration in a profound lack of water where enzymatic activity is downgraded to nearly control levels. The intense enzymatic activity in the first hours upon rehydration attests enzymes' preservation. The precise interaction and triggering in this highly reproducible sequence of events is a major challenge for further investigation.

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