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Activity of δ-aminolevulinic acid dehydratase at *Ramonda nathaliae* and *Ramonda serbica* plants during dehydration and rehydration

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Introduction: Ramonda nathaliae and *Ramonda serbica* are resurrection plants belonging to homoiochlorophyllous desiccation-tolerant angiosperms. Chlorophyll biosynthesis is one of the most important metabolic pathways to tolerate desiccation in these plant species. *Materials and methods:* To better understand the early pathway steps of chlorophyll biosynthesis, we have analyzed the enzyme δ-aminolevulinic acid dehydratase (ALA-D) and contents of δ-aminolevulinic acid (ALA) and total chlorophyll as a final product during dehydration and rehydration stages for these plant species. *Results:* Our results showed that the activity of ALA-D in *R. nathaliae* and *R. serbica* plants rapidly decreased during dehydration and in the final stage of desiccation the activity of this enzyme was decreased by 79% and 86%, respectively. After rehydration of plants, the ALA-D activity was fully restored. In contrast, the ALA content of both plant species significantly increased during desiccation and decreased after 48 hr of rewatering. In each stage of dehydration or rehydration, a significant negative correlation was established between ALA-D activity and ALA content in both plant species. *Conclusions:* Total chlorophyll content was preserved more in *R. nathaliae* than in *R. serbica* during desiccation. Moreover, ALA-D activity was decreased to a minimal level but preserved its function during desiccation, and this suggests one possible mechanism of desiccation tolerance to retain the chlorophyll of these plant species.

Original Article

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INTRODUCTION

Ramonda nathaliae and *Ramonda serbica* are endemic and relict plant species of the Balkan Peninsula, which belong to a small group of angiosperms known as resurrection plants that can tolerate extreme dehydration. In the territory of Kosovo, *R. nathaliae* grows in a few populations in the Sharri Mountains, and *R. serbica* in some populations in the Sharri Mountains and in the Albanian Alps (Gashi et al., 2015). They are mainly found on the shaded, northern, chiefly limestone slopes in mountain zones with relatively high humidity and their populations decrease during summer months when the weather is hot and dry.

Desiccation tolerance occurs throughout the plant kingdom; this process is widespread and is found in most taxonomic groups ranging from mosses and pteridophytes to dicots but is not observed in gymnosperms (Alpert, 2000; Gaff & Oliver, 2013). Desiccation tolerance of the vegetative tissues in vascular plants has been demonstrated in some 350 species, making up less than 0.2% of the total flora (Porembski & Barthlott, 2000) but the list is constantly being extended. These desiccation-tolerant plants are also referred to as resurrection plants because they can fully restore their metabolism upon rehydration (Toldi et al., 2009). Desiccation-tolerant plants are capable of withstanding severe water loss to almost complete desiccation while maintaining some metabolic functions and maintaining the ability to quickly restore normal physiological activity upon rehydration (Schwab et al., 1989).

Resurrection or dessication-tolerant plants are usually divided into two groups: homoiochlorophyllous plants, which retain their chlorophyll during drying, and

poikilochlorophyllous plants, which lose chlorophyll on drying (Tuba et al., 1998). Although poikilochlorophyllous plants dismantle chlorophyll on drying, they are still able to resynthesize it following rehydration (Gaff, 1989; Gaff & Oliver, 2013; Navari-Izzo & Rascio, 1999). In homoiochlorophyllous plants, during dehydration, the amounts of chlorophyll are comparable to those of fresh tissue (Navari-Izzo et al., 1994). Typical examples of homoiochlorophyllous plants are *R. serbica* and *R. nathaliae*. These species are good examples of ecophysiological divergence in terms of plant–water relationship and adaptations to their habitat's water regime. Under conditions of water deficit in the habitat, these plants gradually wilt and pass to anabiosis (Gashi et al., 2013). Resurrection plants in general contain non-rigid cell walls and, as a consequence, their leaf surface decreases during dehydration due to cell wall folding. This mechanism minimizes drought-induced mechanical stresses on the plasma membrane, maintaining cell-to-cell connections and allowing a rapid recovery upon rewatering (Farrant, 2000).

In recent years, *Ramonda* species have become important models in physiological studies of desiccation tolerance. Special attention has been given to oxidative damage during desiccation of *R. serbica* and *R. nathaliae* and to the adaptive features necessary to preserve cell membrane integrity (Quartacci et al., 2002), antioxidative capacity (Jovanoviç et al., 2011; Sgherri et al., 2004), photosynthetic activity (Tuba et al., 1998), CO_2 fixation and chlorophyll *a* fluorescence (Degl'Innocenti et al., 2008), genome size variation and polyploidy (Gashi et al., 2013; Siljak-Yakovlev et al., 2008), osmotic adjustment (Zivkoviç et al., 2005), seed germination (Gashi et al., 2012), and *in vitro* cultivation from seeds of *Ramonda* plants (Daskalova et al., 2012; Dontcheva et al., 2009; Gashi et al., 2015).

Plant growth, development, photosynthesis, and plant productivity are severely affected by environmental stresses, and also during the desiccation period of resurrection plants. In line with this, it is important to study the role of the enzyme δ-aminolevulinic acid dehydratase (ALA-D) because its activity is fundamental for biosynthesis of tetrapyrroles, such as porphyrins, hemes, and chlorophyll (Tanaka & Tanaka, 2007). This enzyme catalyzes the condensation of two molecules of δ-aminolevulinic acid (ALA), yielding the monopyrrole product porphobilinogen (PBG). It is a metalloenzyme-containing sulfhydryl (-SH) groups and zinc, which are essential for its activity (Rocha et al., 1995). Notably, its cysteinyl residues are highly sensitive to heavy metals (Jaffe, 2000), molecular oxygen, and cellular oxidative stress that induce disulfide bond formation and enzyme inhibition (Noriega et al., 2007). Therefore, the sulfhydryl enzyme ALA-D, used together with other parameters, can play an important role as a marker of oxidative stress and impairment of metabolic processes.

To our knowledge, relatively few studies have been carried out to characterize chloroplast structure and chlorophyll synthesis of resurrection *Ramonda* plant species and *Haberlea rhodopensis*. Most published data describe the size, shape, and number of chloroplasts or chloroplast structure in the palisade and spongy parenchyma layers of *H. rhodopensis* leaves during drought (Georgieva et al., 2010; Nagy-Deri et al., 2011) or chlorophyll content and chlorophyllase activity during the dehydration and rehydration cycles of *R. serbica* and *R. nathaliae* (Drazic et al., 1999; Gashi et al., 2013). The aim of this study is to determine the importance of early steps of chlorophyll biosynthesis during the dehydration and rehydration cycles of resurrection plants, including the response of ALA-D activity to water deficit and differences between *R. nathaliae* and *R. serbica* with respect to contents of aminolevulinic acid (ALA) and chlorophyll content during this period.

MATERIALS AND METHODS

Plant material

R. nathaliae and *R. serbica* plants of about the same age were collected from their natural habitat together with the layer of soil on which they grew. *R. nathaliae* plants were collected from Glloboçica (Kosovo), near the border with Macedonia, and *R. serbica* plants from the Sharri Moutains, near the city of Prizren (Kosovo). Plants were harvested together with the attached layers of soil. After collection, the plants were acclimated for 2 weeks at greenhouse under full watering until the beginning of the experiments. One set of plants was maintained in a fully hydrated condition, whereas the other was dehydrated by withholding water, leaving the soil to dry naturally in the pot. Air temperature was about 24 °C, relative humidity was around 80%, and maximum photosynthetic photon flux density was about 70 $\mathrm{mmol \cdot m^{-2} \cdot s^{-1}}$ of diffuse natural light. Briefly, samples of similar weight were obtained from comparable-sized leaves. After 14 days of withholding water, leaves of both species were completely desiccated and plants were in the state of anabiosis. After 5 days in anabiosis, 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 (D1), 10 (D2), and 15 (D3) days; and upon rewatering, after 6 (R1), 12 (R2), 24 (R3), and 48 (R4) hr.

Determination of relative water content (RWC)

RWC of *Ramonda* leaves was determined at each sampling time during the dehydration and rehydration cycles (D1–R4). Leaves of comparable size (12 replicates) were selected from near the middle of rosettes. Fresh weight was measured by weighing them immediately after harvest and dry weight (DW) after oven drying for 48 hr at 80 °C to a constant mass. Turgid weights (TW) were measured after the leaves had been immersed in distilled water for 24 hr at 20 °C in the dark. The above determination was conducted according to Sgherri et al. (1994). RWC was calculated using the equation:

$$\mathrm{RWC} = [(\mathrm{FW} - \mathrm{DW})/\mathrm{TW} - \mathrm{FW}] \times 100.$$

δ-*Aminolevulinic acid dehydratase (ALA-D) activity*

The enzyme was assayed colorimetrically using modified Ehrlich reagent to estimate the amount of PBG formed. Extraction and assay of ALA-D was carried out according to the procedure described in Jain and Gadre (2004). The enzyme was extracted with ice–cold 50 mM Tris-HCl buffer (pH 8.4) containing 0.2% of Triton ×100. Briefly, samples of similar weight were obtained from comparable-sized leaves. Three leaves of similar ages, comparable in size, were taken from each of five plants (15 leaves in total) from the middle of the rosette and each of them (about 500 mg) was ground to a fine powder under ice–cold temperature in a mortar. Immediately after grinding, 1 g of polyvinylpolypyrrolidone was mixed with the powdered tissue to prevent phenolic oxidation. After filtration, the homogenate was centrifuged at 15,000 × *g* for 20 min at 4 °C. The pellet was discarded, and the supernatant was used as the enzyme source. One ml of enzyme was incubated with 0.27 ml of 1 mg/ml ALA, 1.35 ml of 50 mM Tris-HCl buffer (pH 8.5), and 0.08 ml of 0.02M $MgCl_2$. The reaction was initiated by adding the extract at time zero. After 1 hr of gentle shaking (150 rpm) at 37 °C, the reaction was stopped by adding 0.3 ml of 3 M trichloroacetic acid, followed by centrifugation at 5,000 × *g* for 10 min. For PBG estimation, the supernatant was mixed with Ehrlich reagent (prepared freshly by dissolving 1.0 g of 4-dimethyl aminobenzaldehyde in 30.0 ml of glacial acetic acid and 8.0 ml of 70% perchloric acid, and then made up to 50.0 ml with glacial acetic acid) in a ratio of 1:1 (v/v); absorbance was measured at 553 nm after 15 min against zero time control. One unit of enzyme activity was defined as 1 nmol of PBG formed per hour. Protein content was estimated by the method of Lowry et al. (1951).

Aminolevulinic acid (ALA) content

This was determined according to the method of Tewari and Tripathy (1998). Three leaves of similar ages, comparable in size, were taken from five plants from the middle of the rosette and were homogenized using 1.0 ml of 1M Na-acetate buffer (pH 4.6). After centrifugation at 15,000 × *g* for 15 min at 4 °C, ALA of the supernatant was condensed into PBG using ethylacetoacetate: 0.7 ml of supernatant, 0.8 ml of distilled water, and 0.1 ml of ethylacetoacetate mixture were kept in a boiling water bath for 10 min. After cooling, an equal volume of Ehrlich reagent was added and the colored complex formed was read for absorbance at 553 nm. Amount of PBG formed was calculated using a standard curve of ALA and the results were expressed in μM ALA g^{-1} fresh leaf mass (FM).

Total chlorophyll determination

Three leaves of similar ages, comparable in size, were taken from each of five plants (15 leaves in total) from the middle of the rosette to determine total chlorophyll content. Chlorophyll was extracted with 80% acetone. Chlorophyll contents were calculated using absorbance values at 663 and 645 nm by a UV-Vis spectrophotometer (*SECOMAM – Anthelie* Advanced *5*, Champigny sur Marne, France). The new extinction coefficients and reevaluated equations of Porra et al. (1989) were applied. Total chlorophyll content was expressed as mg/g FM.

Data analysis

The experiment was performed in a randomized design with three replicates from five plants. Differences among parameters and between the stages of dehydration–rehydration were tested using SPSS 17 statistical program (SPSS Statistics for Windows, version 17.0, SPSS Inc., Chicago, IL, USA). Statistical analyses of all data were performed using one-way analysis of variance, and mean comparison was performed with Duncan's multiple range test at 5% level of significance.

RESULTS AND DISCUSSION

Kinetics of the RWC

RWC of *R. nathaliae* and *R. serbica* leaves during desiccation continuously decreased from 96–98% in the fully hydrated plants (C) to around 10% in the desiccated ones (D4) (Fig. 1). There was no statistical difference between the two *Ramonda* species for RWC in the fully hydrated plants (C). For both *Ramonda* species, RWC showed a remarkable decline, especially in the initial stage of dehydration (D1) after 7 days, where the RWC decreased to around 64% in *R. nathaliae* and 58% in *R. serbica.* After 10 (D2) and 15 days (D3) of dehydration, RWC decreased to around 20% and 10%, respectively, in both *Ramonda* species. Upon rehydration, plants of *Ramonda* species showed a rapid recovery of their water content, and within the first 6 hr, the RWC in *R. nathaliae* plants reached around 40% (R1), but a little less in *R. serbica* (~30%). The RWCs for *R. nathaliae* and *R. serbica* continued to increase to 90%–92% (R4) in leaves harvested at 48 hr of rewatering (Fig. 1). Rehydration was restored more rapidly in *R. nathaliae* plants than in *R. serbica*. Our RWC results showed that dehydration of *R. nathaliae* and *R. serbica* leaves was very slow, especially in the first stage. Similar results reported by other authors, Degl'Innocenti et al. (2008) and Gashi et al. (2013), in their investigations of *R. serbica* showed that the RWC decreased from values around 97% in fully hydrated control plants to values of only 7% after 2 weeks of dehydration. In addition, the RWC in *R. nathaliae* decreased from 98% to 7.8% during dehydration (Gashi et al., 2013). Kinetics of RWC during the dehydration–rehydration cycle were reported by other authors in *R. serbica* (Augusti et al., 2001; Quartacci et al., 2002; Sgherri et al., 2004; Veljovic-Jovanoviç et al., 2006) and *H. rhodopensis* (Djilianov et al., 2011; Georgieva et al., 2010; Mihailova et al., 2011; Nagy-Deri et al., 2011).

ALA-D activity

Throughout the dehydration–rehydration cycle, leaves of control fully hydrated plants (C) and leaves of plants after rewatering (R4) showed significant differences in the activity of ALA-D between *R. nathaliae* and *R. serbica* plants (Table 1). ALA-D activity was significantly higher in leaves of control fully hydrated plants (C) in *R. serbica* compared

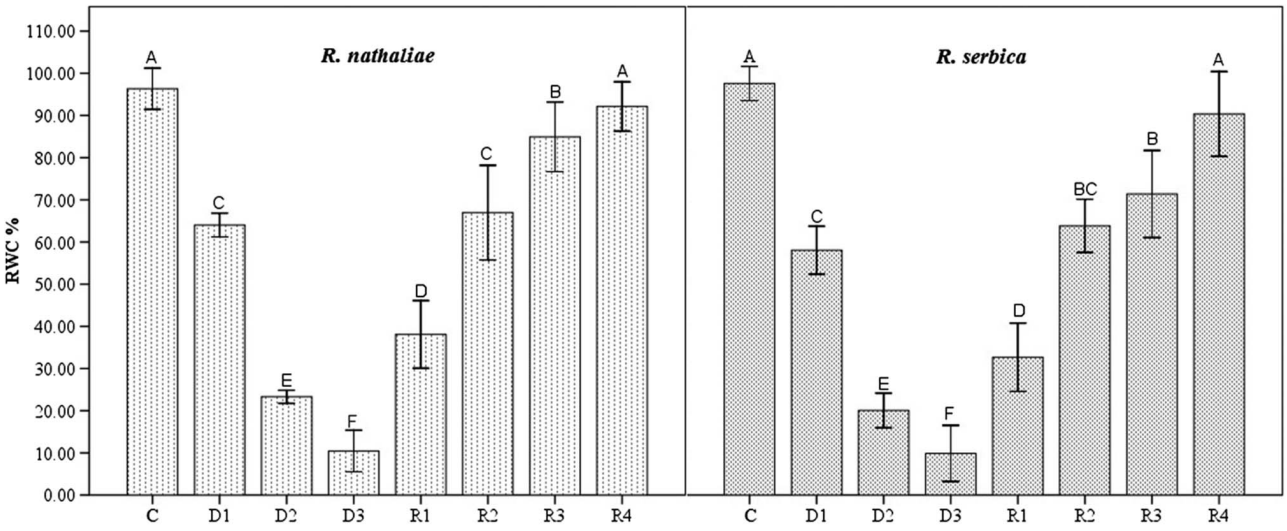

Fig. 1. Relative water content (RWC) of *R. nathaliae* and *R. serbica* leaves during dehydration and rehydration cycles. Different letters indicate significant differences at $p = .05$ by one-way ANOVA with Duncan's multiple range tests. Control plants (C), plants in different stages of dehydration, after 7 (D1), 10 (D2), and 15 (D3) days, and upon rewatering, after 6 (R1), 12 (R2), 24 (R3), and 48 (R4) hr

with *R. nathaliae.* Enzyme activity of ALA-D rapidly decreased during dehydration, especially on the first stage of dehydration (D1) in both *Ramonda* species. During first stage of dehydration (D1), the ALA-D activity in *R. nathaliae* plants decreased from 0.78 to 0.25 μmol of PGB mg protein$^{-1} \cdot$ hr^{-1} and in *R. serbica* plants from 1.38 to 0.41 μmol PGB mg protein$^{-1} \cdot$ hr^{-1} (Table 1). ALA-D activity decreased about 70% in the first stage of dehydration (D1) and by 80%–86% during final stages of dehydration (D2–D3) in both *Ramonda* species (Table 1). The conservation of other enzyme activities involved in photosynthesis or carbohydrate metabolism in resurrection plants during dehydration has also been reported from other authors (Schwab & Gaff, 1990; Zhang et al., 2016).

Our results of ALA-D activity for control plants showed that *R. serbica* plants have higher activity of this enzyme than *R. nathaliae* (Table 1). We presume that these plant species have preserved the natural and molecular mechanisms of adaptation to their environment. *R. serbica* usually grows in more favorable habitats with mosses, which preserve the soil humidity, often sheltered by forest canopy, whereas *R nathaliae* thrives under open, drier, and warmer habitats (Stevanovic et al., 1991). Mosses are efficient in absorbing moisture and simultaneously they play the role of an insulating buffer that prevents rapid evaporation from the shallow soil (Rakic et al., 2009). These ecological differences show that *R. nathaliae* is a more xerophilous species than *R. serbica* (Gashi et al., 2013; Rakic et al., 2009). Therefore, the higher ALA-D activity in *R. serbica* relative to *R. nathaliae* control plants (C) could be a result of these natural conditions. However, ALA-D activity in *R. nathaliae* plants during dehydration, especially after 15 days (D3), is conserved more than in *R. serbica* plants (Table 1). It may be assumed that the relatively higher percentage of ALA-D activity in *R. nathaliae* plants depends on RWC, because this plant is more resistant during water deficit. 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 closely related plants *R. nathaliae* and *R. serbica*.

In specimens of both species studied, the enzyme activity was not completely inhibited during anabiosis; this might be a consequence of the magnesium (Mg) content in the leaf during this stage. According to Zivkoviç et al. (2005), in *R. serbica* plants during the final stage of dessication, the Mg concentration was increased in comparison with control plants. In green plants, the ALA-D enzyme requires Mg for activity (Jaffe, 2000), and the increased concentration of Mg in leaves causes increased ALA-D activity (Osmani et al., 2018). On the other hand, it is well known that, in resurrection plants, decreases in the free amino acid pool during water stress might result from a lower rate of amino acid biosynthesis as well as from enhanced degradation (Rakic et al., 2014; Schiller et al., 1998; Zivkoviç et al., 2005). The inhibition of ALA-D activity in both *Ramonda* species during dehydration might also result from the lower amino acid content, which affects enzyme biosynthesis.

After 12 hr of rehydration (R2), ALA-D activity in both studied species was significantly increased and after 48 hr of rehydration (R4), it reached the highest value, which was the same as in control plants (Table 1). In *R. nathaliae* plants, ALA-D activity increased more rapidly after 6 hr of rehydration than in *R. serbica*. In this case, the ALA-D activity of *R. nathaliae* during rehydration may be a result of RWC in this plant, which restored water more rapidly after rehydration (Fig. 1). Moreover, a positive significant association was established between ALA-D activity and RWC in leaves of *R. nathaliae* and *R. serbica* plants (Fig. 2). Rehydration of leaves induced ALA-D resynthesis or restored ALA-D activity in both *Ramonda* species, and the ALA-D resynthesis rate depending on previous hydration of the plant. This indicates that water is a basic factor during reconstruction of the photosynthetic apparatus.

Table 1. δ-Aminolevulinic acid dehydratase (ALA-D) activity, δ-aminolevulinic acid (ALA), and total chlorophyll contents in leaves of *R. nathaliae* and *R. serbica* species during a dehydration and rehydration cycles

	ALA ($\mu mol \cdot mg^{-1}$ FM)	ALA-D (μmol PGB mg $protein^{-1} \cdot hr^{-1}$)	Inhibition of ALA-D activity (%)	Total chlorophyll ($mg \cdot g^{-1}$ FM)
R. nathaliae				
C	$24.32^{D} \pm 3.85$	$0.78^{A} \pm 0.09$	$0^{E} \pm 0$	$5.78^{A} \pm 0.48$
D1	$75.39^{B} \pm 7.14$	$0.25^{D} \pm 0.09$	$68^{B} \pm 2$	$5.65^{AB} \pm 0.32$
D2	$76.68^{B} \pm 7.81$	$0.22^{D} \pm 0.05$	$72^{AB} \pm 1$	$5.01^{BC} \pm 0.21$
D3	$155.29^{A} \pm 10.94$	$0.17^{E} \pm 0.04$	$79^{A} \pm 3$	$3.91^{D} \pm 0.18$
R1	$65.79^{BC} \pm 8.98$	$0.34^{C} \pm 0.08$	$55^{C} \pm 2$	$4.28^{C} \pm 0.11$
R2	$52.30^{C} \pm 5.60$	$0.36^{C} \pm 0.05$	$52^{C} \pm 1$	$4.69^{C} \pm 0.16$
R3	$28.61^{D} \pm 3.71$	$0.53^{B} \pm 0.09$	$32^{D} \pm 2$	$5.03^{BC} \pm 0.22$
R4	$27.04^{D} \pm 4.18$	$0.79^{A} \pm 0.10$	$0^{E} \pm 0$	$5.81^{A} \pm 0.31$
R. serbica				
C	$22.94^{E} \pm 2.25$	$1.38^{A} \pm 0.08$	$0^{E} \pm 0$	$5.61^{A} \pm 0.24$
D1	$47.72^{C} \pm 8.35$	$0.41^{C} \pm 0.10$	$70^{B} \pm 2$	$5.12^{B} \pm 0.21$
D2	$67.22^{B} \pm 13.97$	$0.30^{D} \pm 0.03$	$78^{AB} \pm 2$	$4.35^{CD} \pm 0.24$
D3	$140.25^{A} \pm 15.66$	$0.20^{E} \pm 0.09$	$86^{A} \pm 3$	$4.03^{D} \pm 0.27$
R1	$52.68^{BC} \pm 9.20$	$0.32^{D} \pm 0.05$	$73^{B} \pm 2$	$4.32^{B} \pm 0.34$
R2	$38.28^{CD} \pm 4.22$	$0.47^{D} \pm 0.04$	$66^{C} \pm 1$	$4.77^{C} \pm 0.22$
R3	$30.87^{D} \pm 3.36$	$0.89^{B} \pm 0.16$	$35^{D} \pm 1$	$5.15^{B} \pm 0.32$
R4	$24.55^{E} \pm 3.18$	$1.32^{A} \pm 0.04$	$0^{E} \pm 0$	$5.70^{A} \pm 0.31$

Note. Control plants (C), plants in different stages of dehydration, after 7 (D1), 10 s (D2), and 15 (D3) days, and upon rewatering, after 6 (R1), 12 (R2), 24 (R3), and 48 (R4) hr. Means and standard errors (±) are presented. Means in each column followed by same letters are not significantly different at $p = .05$ by one-way ANOVA with Duncan's multiple range tests. FM: fresh leaf mass.

ALA and chlorophyll contents

The ALA and chlorophyll contents in leaves of *R. nathaliae* and *R. serbica* in fully hydrated plants (C) showed no significant differences between species (Table 1). The ALA contents were significantly higher (155.29 $\mu mol \cdot mg^{-1}$ FM and 140.25 $\mu mol \cdot mg^{-1}$ FM, respectively) during the final stage of desiccation (D3), whereas the lowest contents were found in fully hydrated plants (C: 24.32 $\mu mol \cdot mg^{-1}$ FM and 22.94 $\mu mol \cdot mg^{-1}$ FM, respectively) and in the final of stage of rehydration (R4: 28.61 $\mu mol \cdot mg^{-1}$ FM and 24.55 $\mu mol \cdot mg^{-1}$ FM, respectively). When leaves of both *Ramonda* species had lost more than 50% water during dehydration, the ALA content was increased marginally; moreover, ALA-D activity and chlorophyll decreased significantly during this period. Furthermore, there was an association between inhibition of ALA-D activity and increase of ALA content in leaves of *R. nathaliae* and *R. serbica* during dehydration (Table 1). This negative correlation between ALA-D activity and ALA content assumes that the ALA-D enzyme catalyzes the asymmetric condensation of two molecules of ALA to PBG, and this process results in a decrease of ALA content. On the other hand, ALA accumulation in the chlorophyll and heme pathways leads to generation of reactive oxygen species as well as cellular oxidative stress (Noriega et al., 2007). In this condition, detoxification processes are activated, especially enzymatic and non-enzymatic antioxidants, as has been previously seen for *R. serbica* (Sgherri et al., 2004; Veljovic-Jovanoviç et al., 2006) and *R. nathaliae* (Jovanoviç et al., 2011), and for another Balkan resurrection plant from the *Gesneriaceae* family, *H. rhodopensis* (Djilianov et al., 2011). However, in the state of anabiosis (D4), the very slow, almost suspended metabolism of these plants accompanied by decreased ALA-D activity was followed by an increase of ALA content (Table 1), which may constitute a reservation that allows *R. nathaliae* and *R. serbica* plants to tolerate oxidative damage during dehydration and even more during rehydration.

Chlorophyll contents in *R. nathaliae* and *R. serbica* also significantly decreased after 15 days of dehydration (D3), but the decline was found to be less severe than for ALA contents (Table 1); increase in ALA content was more substantial than the decrease in chlorophyll. This implies that, apart from PBG synthesizing capacity, other steps of the chlorophyll biosynthetic pathway are also being affected by desiccation or water deficit. The finding is supported by the activity of ALA-D because we also found an association between ALA-D activity and total chlorophyll content during the dehydration/rehydration cycle.

The total chlorophyll content in *R. nathaliae* and *R. serbica* slightly decreased during the initial stage of dehydration (D1); after the RWC decreased more than 50% in leaves of both *Ramonda* species, the chlorophyll content significantly decreased. The fact that chlorophyll contents decreased during desiccation, especially during the final stage of dehydration (D3) in both *Ramonda* species, is in agreement with the results of some other authors. Gashi et al. (2013) reported that chlorophyll contents of *R. nathaliae* and *R. serbica* were significantly decreased during desiccation; Degl'Innocenti et al. (2008) reported that chlorophyll contents of *R. serbica* during anabiosis reached the lowest value, in comparison with fully hydrated plants.

After 48 hr of rehydration, ALA and total chlorophyll contents at *R. nathaliae* and *R. serbica* were significantly increased and reached the control (fully hydrated plant) values (Table 1). After rehydration, homoiochlorophyllous

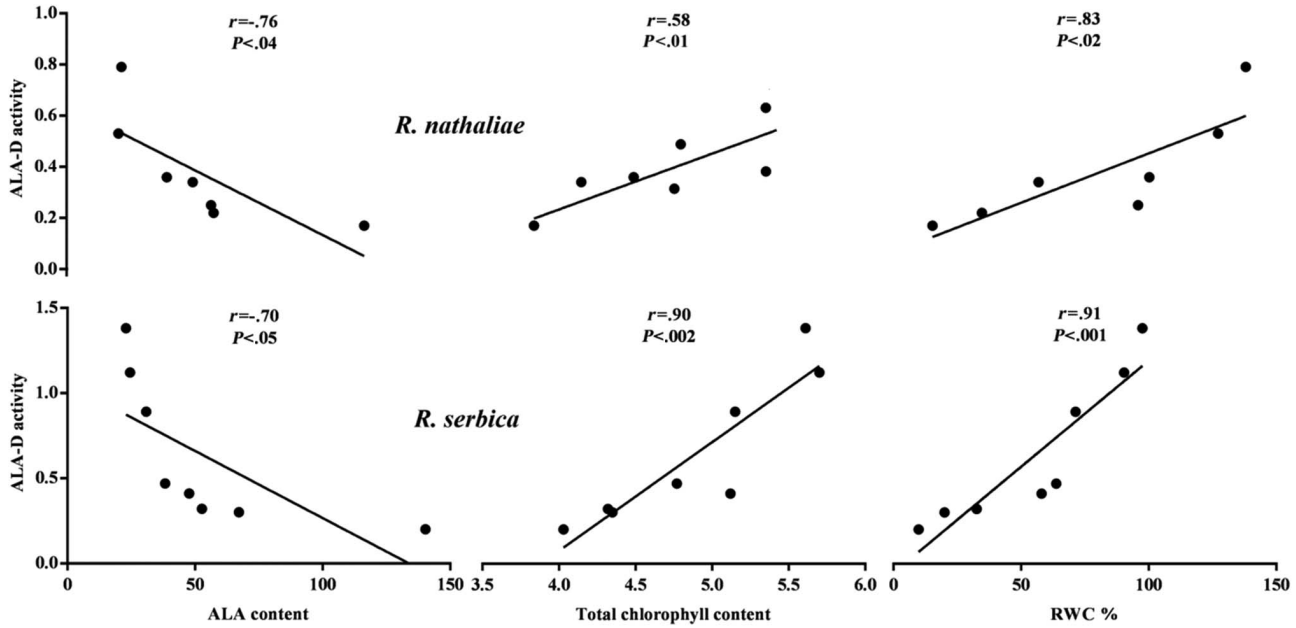

Fig. 2. Correlation between ALA-D activity and ALA content, total chlorophyll content, and relative water content (RWC) in *R. nathaliae* and *R. serbica* during dehydration and rehydration cycles

resurrection plants increase chlorophyll content. During rehydration, chlorophyll resynthesis and chlorophyll amounts gradually increase and return to near control levels in fully rehydrated plants of *R. serbica* (Augusti et al., 2001; Degl'Innocenti et al., 2008; Gashi et al., 2013), *R. nathaliae* (Gashi et al., 2013; Jovanoviç et al., 2011), and *H. rhodopesis* (Georgieva et al., 2010; Péli et al., 2012). According to previous investigations (Augusti et al., 2001; Drazic et al., 1999; Gashi et al., 2013; Markovska et al., 1994), *R. serbica* and *R. nathaliae* plants are homoiochlorophyllous desiccation-tolerant angiosperms because they retain most of their chlorophyll content during desiccation. We presume that a key factor during chlorophyll biosynthesis is the activity of ALA-D during the dehydration/rehydration cycle, which contributes to preserving the early steps of chlorophyll synthesis.

CONCLUSIONS

The results showed that *R. serbica* plants have higher ALA-D activity in control plants than *R. nathaliae*. We presume that this difference obtained from their natural and molecular mechanisms of adaption. On the other hand, ALA-D activity in *R. nathaliae* plants during dehydration, especially after 15 days of desiccation, was preserved more than in *R. serbica* plants. Furthermore, the ALA-D activity was decreased to minimal levels but its function was preserved during desiccation in the two studied species and after rehydration the ALA-D activity was rapidly restored as well as to control plant levels. It is clear that full recovery of the enzyme activity a few hours after rehydration indicates that both *Ramonda* species have enzyme preservation during dehydration cycle. In addition, two factors may contribute to a particularly efficient protection of ALA-D activity: (a) the RWC content in leaves and (b) Mg and amino acid contents during the dehydration stage.

Moreover, the results of this study suggest that resurrection plants *R. nathaliae* and *R. serbica* have developed a specific desiccation tolerant molecular mechanism to control early stages of chlorophyll biosynthesis during the dehydration/rehydration cycle.

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