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Photosynthetic activity and antioxidative defense during cold and freezing stress of the resurrection plants *Ramonda nathaliae* and *Ramonda serbica*

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ABSTRACT

Ramonda nathaliae and *Ramonda serbica* belong to the group of resurrection plants, which are capable of restoring normal physiological activity after the desiccation period. Understanding photosynthetic apparatus's response to harsh environmental conditions and evaluating antioxidative levels is essential since it can reveal the mechanisms of their stress tolerance and the strategies for enhancing cold and freezing tolerance. This study examines how cold and freezing temperatures impact photosynthetic activity, chlorophyll fluorescence, total flavonoid and phenol content, antioxidant capacity, as well as the levels of photosynthetic and stress-induced proteins in Ramonda species leaves during autumn and winter in the natural condition. Based on the results obtained, the greatest reduction in CO_2 assimilation occurred during freezing stress in comparison to both cold stress and control conditions. The quantum efficiency of photosystem II was slightly affected during cold temperatures but gradually decreased when the temperatures dropped to freezing, reaching minimum values in the desiccated leaves. Significant enhancement in the total phenolic and flavonoid content, as well as increased antioxidant capacity in both Ramonda species during desiccation under freezing stress, helps to protect plants against oxidative damage. Low temperatures decreased the abundance of fundamental photosynthetic proteins, whereas the content of some light-harvesting complex (LHC) proteins was increased. The accumulation of early light-inducible proteins (ELIPs) and dehydrins suggested their role in the acquisition of freezing tolerance. The results show that Ramonda species have developed some biochemical and physiological adaptations that enhance their ability to preserve their photosynthetic processes during cold and freezing temperatures. Findings from this study may contribute to the development of breeding strategies in agriculture, particularly for cold-prone regions, to enhance crop resilience and productivity under challenging environmental conditions.

1. Introduction

Ramonda species of the Gesneriacae family are a unique plants known for their ability of "resurrection" and their high poikilohydric status. The resurrection plants *Ramonda nathaliae* Pančić & Petrović and *Ramonda serbica* Pančić are endemic species from the tertiary period found in the Balkan Peninsula (Gashi et al., 2012). Known for their exceptional desiccation tolerance, a rare trait in flowering plants, these species can survive nearly complete dehydration of their vegetative tissues until they rehydrate (Lyall and Gechev, 2020; Legardón and García-Plazaola, 2023). These plants exhibit notable ecophysiological adaptations that help them manage water relations in response to habitat water availability. During water scarcity, they slowly wilt and enter anabiosis (Gashi et al., 2013). According to Weber (2004), most desiccation-tolerant gesneriads are ancient species found in mountainous, typically north-facing limestone regions, where they have adapted to survive drought and cold stress. Cells in plants and seeds that can tolerate desiccation need core protective strategies to endure dehydration and subsequent rehydration (Oliver et al., 2020).

Resurrection plants are generally categorized as homoiochlorophyllous species, which can keep chlorophyll (Chl) throughout desiccation, and poikilochlorophyllous species, which degrade Chl and

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must resynthesize it upon rehydration (Tuba et al., 1998). These plants face various stresses, including metabolic, oxidative, structural, and mechanical challenges (Oliver et al., 2020). Adaptation and resistance in plants against cold temperatures are crucial for surviving under freezing conditions (Chen et al., 2016). Research on the biochemical or physiological adaptions involved in the revival of angiosperms from freezing temperatures, which may be accompanied by desiccation, is limited. Another species from the Balkan gesneriads of resurrection plants, *Haberlea rhodopensis* Friv., belongs to the same family, Gesneriaceae. Various studies have demonstrated its unique ability to survive freezing temperatures, and it is also classified among the homoiochlorophyllous plants (Georgieva et al., 2021; Mihailova et al., 2023a). Recently, a study conducted under "ex-situ" conditions demonstrated and proposed that *R. nathaliae* and *R. serbica* are considered tolerant to cold temperatures and freezing. However, this tolerance may vary under the natural growth conditions of their respective habitats (Mihailova et al., 2023a).

The cold acclimation of resurrection plants in autumn is an effective defense mechanism that enables them to survive the freezing temperatures of winter. This annual cycle in perennial plants reflects their transition from cold to frost tolerance and their deacclimatization in spring (Mihailova et al., 2020). It has been demonstrated that the protective machinery of resurrection plants, including anthocyanins and other phenolic compounds, the accumulation of certain antioxidants, and leaf folding, directly contribute to reducing absorbed radiation, which has a significant impact in mitigating the damage due to oxidative stress (Farrant and Moore, 2011). In terms of photosynthesis, cold stress can lead to photoinhibition, which subsequently reduces the photosynthetic systems ability to absorb and capture light, along with the efficiency of electron transfer and carbon dioxide fixation (Li et al., 2024). The impact of temperatures under 0°C can have opposing effects, as ice formation primarily occurs in the intercellular spaces, but the intracellular fluid itself is less directly affected due to its lower freezing point, making these temperatures less harmful (Thomashow, 1998).

Efficient recovery of resurrection species following rehydration is facilitated by physiological and biochemical processes that occur during dehydration or as a response to low temperature stress, allowing smoother recovery once favorable conditions return (Oliver et al., 2020; Georgieva et al., 2022). Harsh environmental conditions, particularly drought and low temperatures, can trigger a rise in antioxidants, especially those with low molecular weight, enhancing the activity of antioxidant enzymes (SOD, CAT, APX, and GR). In plants, these enzymes play a crucial function as a defense mechanism against reactive oxygen species (Pogany et al., 2016; Gołebiowska-Pikania et al., 2017). In our earlier study we demonstrated that plants can withstand harsh winter conditions and recover quickly in spring due to reversible adjustments in photosynthesis, modifications in the pigment protein complexes, as well as enhanced energy dissipation as heat (Mihailova et al., 2020). On the other hand, studies on *R. myconi* (L.) Rchb. have revealed that common feedback to both desiccation and low temperatures include mechanisms that protect chloroplasts, such as increased thermal energy dissipation, an increased carotenoid-to-chlorophyll ratio, and de-epoxidation within the xanthophyll cycle. Furthermore, specific responses, such as elevated antioxidant activity, activation of secondary metabolic pathways and accumulation of different stress-induced proteins, are observed in response to desiccation. These adaptive strategies help the plant cope with environmental stress and contribute to its survival in harsh conditions (Farrant and Moore, 2011; Fernández-Marín et al., 2020). The complexity of tolerance to desiccation relies on many factors, with the modulation of gene expression playing a crucial role during dehydration and the subsequent recovery process (Dinakar and Bartels, 2013). Along with dehydration, a challenge for resurrection plants is the process of rehydration. The initial stages of rehydration after water uptake are critical factors that can increase stress, potentially leading to degenerative changes in plant cells and irreparable damage (Oliver et al., 2000). One of the protective mechanisms of these plants is their remarkable ability to produce antioxidants during periods of stress, which helps mitigate the effects of oxidative stress caused by ROS (Dinakar et al., 2012). In resurrection plants, during stress caused by both low positive and subzero temperatures, maintaining high levels of antioxidant enzymes and polyphenols is essential to the defense mechanisms of plants (Georgieva et al., 2021; Mihailova et al., 2023a). The rate of CO_2 assimilation was found to be significantly low in *R. myconi* (Fernández-Marín et al., 2020) and decreases with decreasing RWC in *H. rhodopensis* (Georgieva et al., 2007), as a consequence of stomatal closure and reduced photochemical efficiency. While these plants' tolerance to environmental stresses may have potential applications in agricultural improvement, their challenging indoor gardening and limited outdoor accessibility pose significant barriers to research (Legardón and García-Plazaola, 2023).

We hypothesize that both *R. nathaliae* and *R. serbica* can withstand cold and freezing stress, but their responses may vary due to differences in the habitats and environmental conditions in which they grow. Since *R. nathaliae* is exposed to drier conditions in the summer and grows in more open areas with less vegetation (Stevanović et al., 1991), it is more resistant to dehydration compared to *R. serbica*, which thrives in more moist environments with greater vegetation cover (Gashi et al., 2013).

Therefore, our aim was to investigate the photosynthetic response and protective mechanisms involved in the cold and freezing tolerance of *R. nathaliae* and *R. serbica* resurrection plants, as well as any eventual differences between the two species. To achieve this, we conducted various measurements under natural environmental conditions or collected samples for further laboratory analysis, focusing on: (a) the impact of cold and freezing temperatures on the photosynthetic apparatus and chlorophyll fluorescence induction; (b) the amount of secondary metabolites (total phenols and flavonoids); (c) antioxidant activity (FRAP and TAC) and scavenging capacity (DPPH and ABTS); and (d) changes in the levels of key photosynthetic and stress-induced proteins through SDS-PAGE and Western blot analysis.

2. Materials and Methods

2.1. Study area and Plant material

For this study, we have used plants of *Ramonda nathaliae* located in Glloboqicë (UTM-zone 34, WGS84, X=0515118 and Y=4669685), and *Ramonda serbica* located in Radac (UTM-zone 34, WGS84, X=0443216 and Y=4731643), Kosovo. This population of *R. nathaliae* grows on the northern, north-western and occasionally western slopes of the Mt. Luboteni massif, at an altitude around 950 meters above sea level, where the dominant plant is *Ostrya carpinifolia* and in this case plant association is *Ramondo-Ostryetum carpinifoliae* (Berisha et al., 2019*)*. On the other hand, *R. serbica* with its habitat in Radac grows in the dolomite rocks, mainly on the foothills (around 500 meters above sea level), northern and north-eastern exposition (Gashi et al., 2013; Gashi et al., 2015), part of the plant community *Musco-Ramondaetum serbicae* (Jankovic and Stevanović, 1980). Photosynthetic and fluorescence measurements of leaves of both Ramonda species were carried out in natural environmental conditions, whereas leaves of the same sizes from the middle of the rosette were collected and stored in liquid nitrogen for further biochemical analysis. All analyses were conducted during cold acclimation (November 2023: 01.11, 15.11, 23.11 and 30.11; T1, T2, T3 and T4, respectively), after exposure to freezing stress (December 2023 and January 2024: 14.12, 28.12, and 21.01; T5, T6 and T7, respectively), during freezing-induced desiccation (January and February 2024: 30.01, 03.02 and 11.02; T8, T9 and T10, respectively), and after rehydration from anabiosis - Control (June: 06.06.2024; C) (Fig. 1). Overall, the average minimum temperatures for each measurement period were as follows: T1-T4 ranged from 10°C to 0°C, T5-T7 from 0°C to -5°C, T8-T10 from -5°C to -10°C, and C from 20°C to 25°C (Fig. 2 and 3).

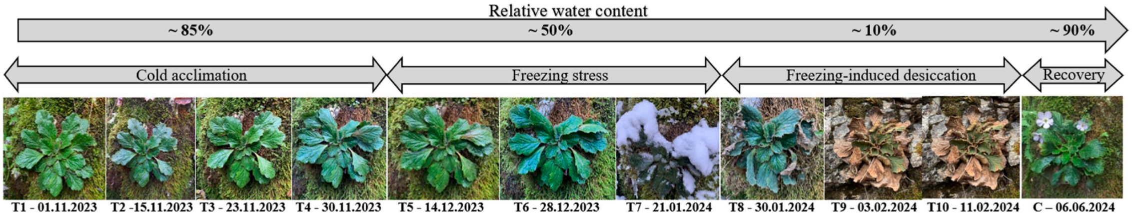

Fig. 1. *Ramonda serbica* plant in natural habitat (Radac), during cold acclimation, freezing stress, freezing-induced desiccation, and after anabiosis. Relative water content throughout each stage.T1-T10: periods of measurements; C: control.

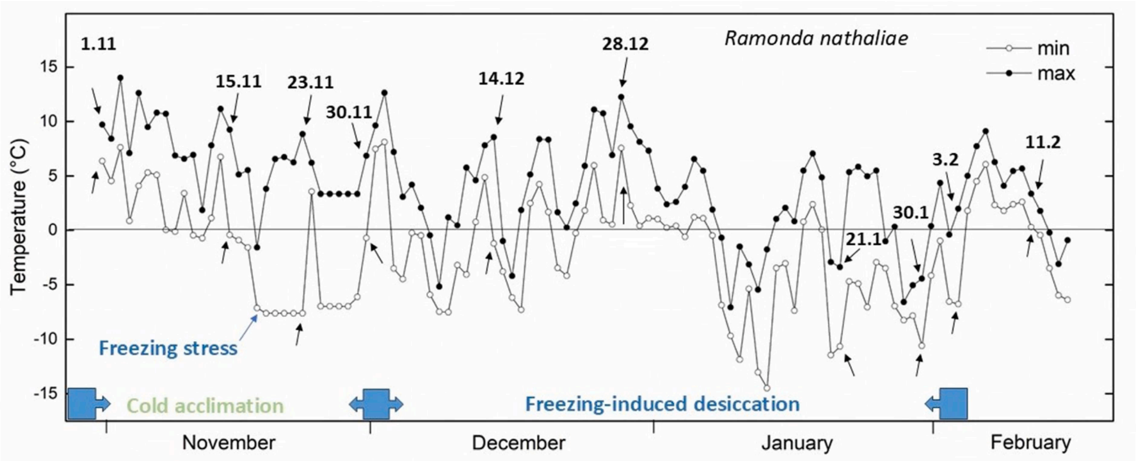

Fig. 2. Changes in the minimum and maximum daily temperatures (from November to February) during the autumn/winter season of 2023–2024 in Glloboqica, the habitat of *R. nathaliae*, are presented. The numbers and arrows indicate the specific dates of measurements and sampling under natural environmental conditions.

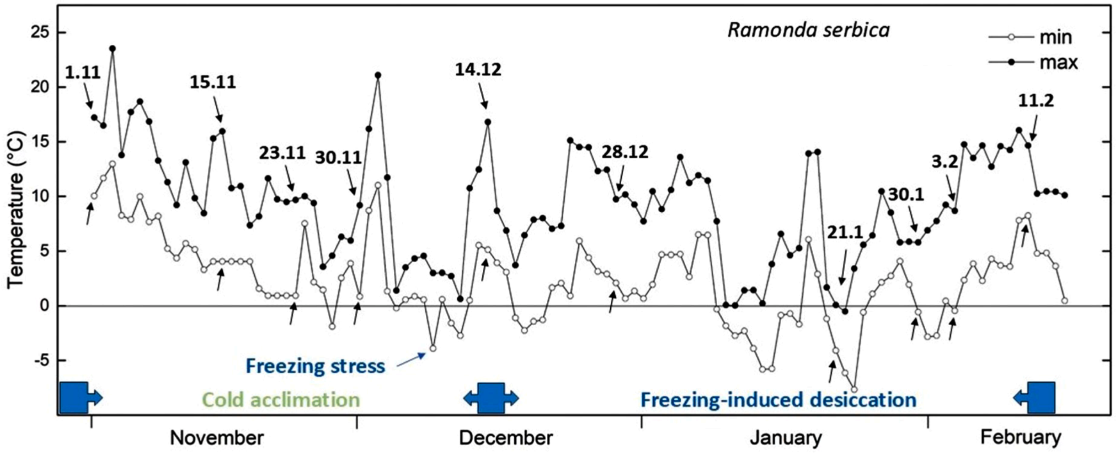

Fig. 3. Changes in the minimum and maximum daily temperatures (from November to February) during the autumn/winter season of 2023–2024 in Radac, the habitat of *R. serbica*, are presented. The numbers and arrows indicate the specific dates of measurements and sampling under natural environmental conditions.

2.2. *Measure of relative water content (RWC)*

RWC was measured on five leaves from the middle of the rosette of plants at each time period of the study (T1-T10 and Control). Gravimetric analysis was used to determine RWC, where leaves were weighed before (fresh weight) and after oven-drying at 80°C (dry weight) to a constant mass. The RWC percentage was calculated as the water content of dehydrated tissue relative to fully hydrated tissue, using the formula: RWC (%) = (fresh weight - dry weight)/ (turgid weight - dry weight) × 100 (Gashi et al., 2012). Turgid weight was measured on leaves maintained for 24 h at room temperature in the dark floating on water.

2.3. *Photosynthetic measurments*

2.3.1. *Leaf gas exchange rate*

Measurements were performed under natural environmental conditions using a CIRAS-4 infrared gas analyzer (PP System, USA) equipped with PLC4 Universal Leaf Cuvettes and an integrated LED Light Unit (RGBW-FR). All measurements were performed on the middle-aged leaves of the rosette, analyzing approximately ten different individual plants, each with three repetitions. The recorded parameters included: CO_2 assimilation (A), stomatal conductance (g_s), transpiration rate (E), vapor pressure deficit (VPD), water use efficiency (WUE) and intercellular CO_2 concentration (C_i). The experimental setup was standardized with a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1}, leaf temperature at 25°C, relative air humidity of 80 %, and CO_2 concentration at 400 μmol mol^{-1}.

2.3.2. Chlorophyll fluorescence

The maximal photochemical efficiency of photosystem II (F_v/F_m), photochemical efficiency under far-red illumination ($F_{v'}/F_{m'}$), electron transport rate (ETR), quantum yield of photosystem II (PSII) electron transport during light exposure (ϕPSII), quantum yield of light-induced non-photochemical quenching (ϕNPQ) and quantum yield of non-regulated heat dissipation (ϕNO) were measured using the CIRAS-4 system, equipped with an integrated Chlorophyll Fluorescence Module (CFM-4). This module utilizes pulse amplitude modulated (PAM) fluorometer to measure chlorophyll fluorescence. Prior to exposure to the saturating pulse and actinic light, leaves were dark-adapted for 20 minutes. The minimal fluorescence level in darkness (F_0) was recorded when all PSII reaction centers were fully oxidized and open to photochemistry. A saturating flash (6000 μmol m^{-2} s^{-1} for 0.7 seconds) was then applied to obtain maximal fluorescence (F_m), where all photosystem II reaction centers were reduced and thus closed to photochemistry. Using F_0 and F_m, the maximum quantum efficiency of PSII (F_v/F_m) was calculated as (F_m-F_0)/F_m. Upon switching on actinic light (200 μmol m^{-2} s^{-1}), a sequence of saturating pulses measured maximum fluorescence in the light-adapted state ($F_{m'}$), allowing calculation of the quantum yield of PSII photochemistry (ϕPSII). This ϕPSII value indicates the portion of light absorbed by PSII that is utilized for photochemistry, serving as a basis for estimating the electron transport rate (ETR) as ϕPSII × incident PAR × 0.5 × leaf absorption, which reflects the plants photosynthetic capacity (Baker, 2008).

2.4. Secondary metabolites and antioxidant capacity

For the analysis of the secondary metabolites and antioxidant activity, leaves from the middle of the rosette of *R. nathaliae* and *R. serbica* were collected at different stages: during the vegetation period (control: June), during cold acclimation (November), after exposure to freezing temperatures (December to the end of January), and during freezing-induced desiccation (end of January-February). During the extraction procedure, 150 mg of dried leaves were carefully weighed, finely ground, and homogenized using a homogenizer (Benchmark model) under the following settings: cycle 05, speed 4350, and time 40 seconds. The homogenization was carried out with 5 ml of 80 % methanol to ensure thorough mixing and efficient extraction of the desired compounds from the plant material. This method helps in obtaining a homogenized mixture for further processing and analysis.

2.4.1. Determination of total phenolic content (TP)

TP content was assessed following Singleton and Rossi's method (1965) with slight modification using Folin-Ciocalteu reagent and Na_2CO_3. As a standard gallic acid was used to genetrate the standard curve. For each sample, 50 μl of methanolic extract was combined with 100 μl of Folin-Ciocalteu reagent (diluted in a 1:6 ratio with distilled water) and allowed to react for 3 minutes, after which 100 μl of sodium carbonate was added. Absorbance was measured at 765 nm.

2.4.2. Determination of total flavonoid content (TF)

TF was quantified using a colorimetric method adapted from Makris et al. (2007), with sodium nitrate as the reagent. Specifically, 50 μl of the methanolic extract was mixed with 500 μl of water, followed by the addition of 100 μl of 5 % $NaNO_2$. The mixture was incubated at 50°C for 5 minutes. Results were reported as milligram catechin equivalents per milligram of dry weight. Absorbance was measured at 510 nm.

2.4.3. Reducing ferric capacity (FRAP)

FRAP was quantified using the method according to Oyaizu (1986). 100 μl methanolic extract was taken and mixed with 1000 μl 0.2 M sodium phosphate buffer, then 1000 μl $K_3[Fe(CN)_6]$ 1 % were added and incubated for 20 minutes in water bath at a temperature of 50 °C. A standard curve was generated using ascorbic acid as a standard. Absorbance was read at 700 nm.

2.4.4. Determination of DPPH radical scavenging capacity

The determination of DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity was performed following the method of Brand-Williams et al. (1995). For this assay, 10 μl of the methanolic extract was combined with 2990 μl of 80 % methanol to reach a total volume of 3000 μl. Next, 2000 μl of 0.25 mM DPPH solution (prepared with 0.0098 g DPPH per 100 ml methanol) was added, followed by mixing on a vortex. Samples were then incubated for 30 minutes in the dark. A standard curve was created using trolox as a reference standard, and absorbance was measured at 517 nm.

2.4.5. Determination of total antioxidant capacity (TAC)

TAC was assessed using the phosphomolybdenum method as described by Prieto et al. (1999) with a slight modification (Gashi et al., 2024). In this procedure, 50 μl of the methanolic extract was mixed with 5000 μl of phosphomolybdate reagent, prepared by combining equal volumes of 0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate, each prepared in separate containers. The samples were incubated for 60 minutes at 95°C. A standard curve was created using ascorbic acid as the standard, and absorbance was measured at 695 nm.

2.4.6. Determination of ABTS radical scavenging activity

ABTS was performed according to Re et al. (1999). A standard curve was generated using trolox as a standard. Absorbance was read at 734 nm.

2.5. SDS-PAGE and Western blot analysis of total leaf proteins and thylakoid proteins

Total leaf proteins were extracted in sample buffer (50 mM Tris-HCl, pH 6.8, 2 % SDS, 2 % β-mercaptoethanol and 10 % glycerol) and the protein content was determined according to Bradford (1976). Thylakoid membranes were prepared as described by Georgieva et al. (2009). After, thylakoid samples were solubilized in sample buffer. Both type of samples were separated on SDS-PAGE (SE260 Mighty Small II, Hoefer, Holliston, MA, USA) according to Laemmli (1970), modified by adding 8.7 % glycerol to stacking and separating gels using a constant current of 20 mA per gel. Equal amount of total leaf proteins (30 μg) or thylakoid samples (corresponding to 2 μg Chl) were loaded on gels. TE70X (Hoefer, Holliston, MA, USA) was used for semi-dry transfer of the proteins on nitrocellulose membrane for 90 min at a current of 1.5 mA cm^{-2}. ROTI®Mark WESTERN PLUS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used for monitoring electrophoresis separation, transfer efficiency and detection of molecular weights on X-ray film. Resulted membranes were tested with the following primary antibodies: ELIP (AS06 147A), dehydrin K-segment (AS07 206A), PsaB (AS10 695), D2 (AS06 146), PsbO (AS06 142-33), PetB (AS18 4169), Lhcb2 (AS01 003) and Lhca2 (AS01 006) (Agrisera, Vännäs, Sweden). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was used (AS09 602, Agrisera, Vännäs, Sweden). The resulting bands were visualized by enhanced chemiluminescence (ECL) and signals were recorded on X-ray Blue films (Carestream Dental LLC, Atlanta, GA, USA). Films were scanned using an Epson Perfection V850 PRO scanner (Seiko Epson Corporation, Suwa, Japan) and densitometry of bands was made by Gel-Pro Analyzer software (Media Cybernetic, Rockville, MD, USA).

2.6. Statistical analyses

Data were presented as arithmetic mean and standard error (±). Differences between periods of cold and freezing conditions as well as control were tested with the statistical program SPSS 25.0. Statistical analysis of variance for all data were done with one way ANOVA and Multivariate Tests, with comparisons made using Duncan's Multiple Range Test at a 5 % significance level ($P \leq 0.05$).

Changes in the investigated photosynthetic proteins were statistically compared by the Fisher least significant difference test at $P \leq 0.05$ following ANOVA. A statistical software package (Statgraphics Plus, version 5.1 for Windows, The Plains, VA, USA) was used.

3. Results and discussion

3.1. Relative water content

Relative water content (RWC) is a critical physiological parameter to assess the physiological resilience of resurrection plants, particularly in their ability to survive extreme dehydration and later rehydrate when water becomes available again. During the cold acclimation period (T1-T4, November) plants were exposed to positive temperatures range from 10°C to 0°C (Figs. 1, 2, 3), thus both plants maintained a relatively high RWC with a slight decrease observed in *R. nathaliae* at the end of November (77.38 %) (Fig. 4). The leaves of both Ramonda species exhibited very good physiological condition throughout the entire cold acclimation period. When plants faced negative temperatures (from -5°C to -10°C) during January and February (T8-T10, Figs. 2, 3), RWC significantly decreased and reached minimum values (10.91 % *R. serbica* and 8.06 % *R. nathaliae*) in February. Overall, *R. serbica* maintained a higher RWC compared to *R. nathaliae* during each stage, thus dehydration occurred more slowly. Both plants recovered their RWC after rehydration (*R. nathaliae* 91.58 % and *R. serbica* 93.05 %) in June.

On the other hand, during the summer period, Gashi et al. (2013) reported that under experimental conditions, RWC decreased in leaves of *R. serbica* and *R. nathaliae* during desiccation, reaching about 5.88 % in *R. serbica* and about 7.87 % in *R. nathaliae*. A reduction in RWC in *R. serbica* plants of up to 4.2 % as a result of desiccation was also found by Sgherri et al. (2004). In our previous study, similar results for RWC were observed during cold acclimation of *R. serbica* and *R. nathaliae*, but in *ex situ* conditions, where values of RWC ranged between 76.9-85.5 %, with a significant decrease of 12.6 % in leaves of *R. serbica* and 8.9 % in leaves of *R. nathaliae* throughout the freezing-induced desiccation period (Mihailova et al., 2023a). A decreased RWC due to sub-zero temperatures in the leaves of *H. rhodopensis* was also found in *ex situ* conditions (Mihailova et al., 2020). Our findings showed that both plant species are capable of maintaining relatively high water content until temperatures drop below 0 °C. Low RWC value can trigger several protective mechanisms in resurrection plants, including the production of antioxidants to prevent cellular damage and also the downregulation of photosynthesis, thus preventing damage from excessive light energy, which cannot be used efficiently due to limited water availability. In agreement with our previous results we also found that the cold acclimation period didn't affect the water content in both Ramonda species, however, when plants were exposed to –10°C, dehydration was induced (Mihailova et al., 2023a). Overall, dehydration was slower in *R. serbica* leaves compared to leaves of *R. nathaliae*. This might be due to differences in environmental conditions that these two species are exposed to, considering that *R. nathalie* is exposed to harsher environmental conditions compared to *R. serbica*. In addition, *R. nathaliae* inhabits more open and drier habitats whereas *R. serbica* prefers cooler habitats with higher humidity, protected by the tree canopy (Gashi et al., 2013).

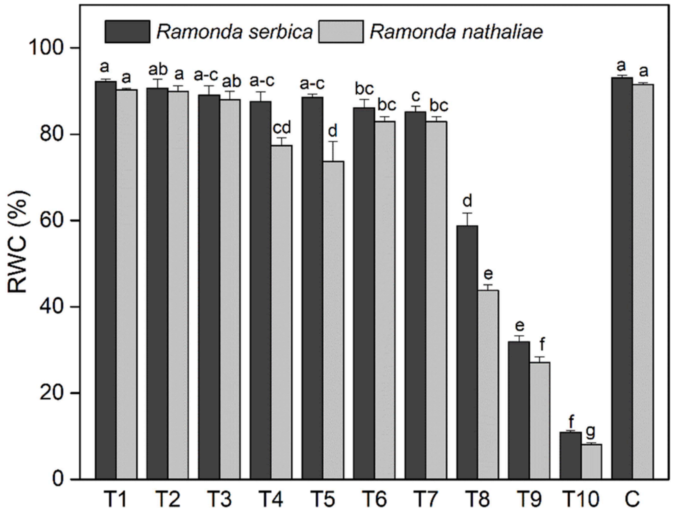

Fig. 4. Relative water content (RWC) of the leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C; June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

3.2. Photosynthetic performance

3.2.1. Photosynthetic activity throurghout cold acclimation, freezing stress, freezing-induced desiccation, and after anabiosis

To better understand the response of resurrection plants of Ramonda species in facing cold and freezing stress in natural conditions, we have investigated their photosynthetic activity during autumn, winter, and spring. Due to their features of being sessile organisms, plants are exposed to daily abiotic stresses that can cause detrimental effects on photosynthetic structure (Guidi et al., 2019).

Our results for both Ramonda plant species showed that the stomatal conductance declined throughout the cold acclimation period, however freezing stress had a significant impact on reducing the stomatal conductance, and it was stronger in *R. serbica* (Fig. 5). Additionally, throughout freezing-induced desiccation, when the RWC decreased to 10.91 % (*R. serbica)* and 8.06 % (*R. nathaliae)*, stomatal conductance was significantly reduced, especially in *R. serbica*, with values of about 35.23 mmol m^{-2} s^{-1}. Water uptake amid the desiccation phase was reduced due to soil freezing conditions, and in response to that, stomata closure was important to protect plants against dehydration and the possibility of evaporation in case stomata would be opened. When plants were fully rehydrated after anabiosis (*R. serbica* and *R. nathaliae*: 93.05 % and 91.58 % RWC, respectively), stomatal conductance was restored optimally, especially in *R. serbica* with the higher values of 299.75 mmol m^{-2} s^{-1}. Based on our results we can presume that *R. nathaliae* showed less fluctuations of stomatal conductance during cold acclimatization and freezing stress period and was able to maintain it more stable and had higher values during the freezing-induced desiccation compared with *R. serbica*. Therefore, we hypothesize that the harsher environmental conditions to which *R. nathaliae* plants are exposed might have improved their protective mechanisms against freezing conditions. Strong inhibition of stomatal conductance was also observed in another resurrection plant *H. rhodopensis*, a close relative to Ramonda species (Gesneriaceae), during the desiccation period (Georgieva et al., 2010). In agreement with our results, a strong inhibition of stomatal conductance in Ramonda plants, in *ex situ* conditions, when the temperature dropped to -10°C was found by Mihailova et al. (2023a). As temperature rises, the viscosity of water decreases by approximately 20 % for every 10°C increase, thus enhancing mesophyll conductance (von Caemmerer et al., 2015). A reduction in stomatal size was observed in Arabidopsis plants grown in water scarcity (Doheny-Adams et al., 2012). In *Barbacenia purpurea*, a poikilochlorophyllous resurrection plant, photosynthesis was reduced due to stomatal closure and also declines in mesophyll conductance in response to severe water stress (Nadal et al., 2023). The

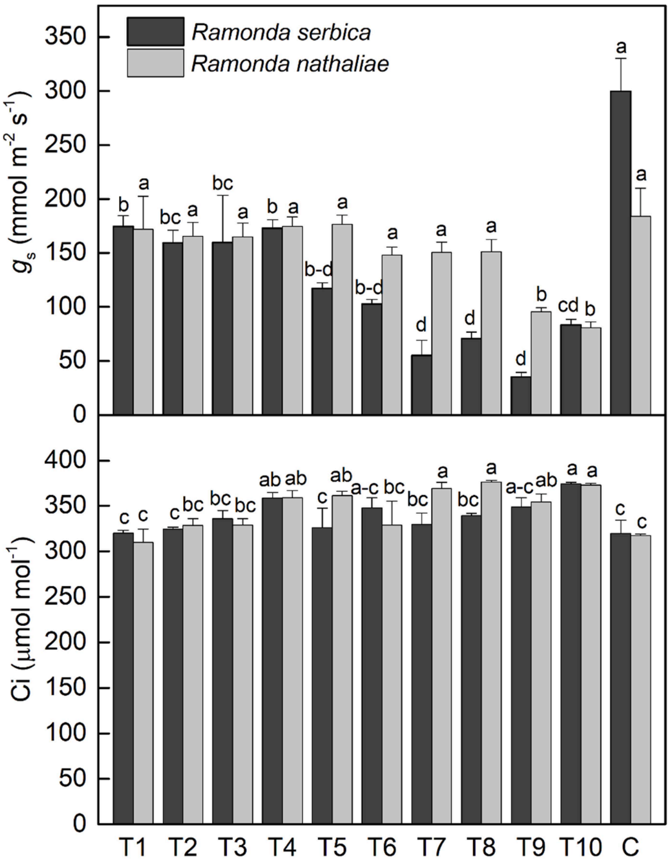

Fig. 5. Changes in stomatal conductance (*gs*) and sub-stomatal CO_2 (Ci) concentration in leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C; June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

most important factors regulating the processes related to stomatal aperture involve hormones, Ca^{2+} levels, and guard cell turgor (Lawson et al., 2014; Assmann, 1999). Furthermore, abscisic acid appears to have a crucial role in the monitoring of cold or prolonged water shortage stress (Agurla et al., 2018).

Our data indicate a slight elevation of sub-stomatal CO_2 (C_i) concentrations in both species of *R. serbica* and *R. nathaliae* throughout the cold acclimation period and freezing-induced desiccation, compared to the control (Fig. 5). We assume that this might be due to the inhibition of stromal enzymes, such as rubisco, responsible for carbon fixation, whose activity was reduced during cold and freezing temperatures but not fully inhibited. As a result, a low amount of CO_2 was incorporated in active sites of rubisco, thus impacting photosynthesis by reducing carbon fixation, and as a result, more CO_2 was accumulated in the sub-stomatal spaces of the leaf. Elevated sub-stomatal CO_2 levels under cold stress trigger photoprotective mechanisms in some resurrection plants, mitigating the risk of photooxidative damage and improving freezing resistance (Buckley et al., 2017). We can presume that the decrease in stomatal conductance due to stomata closure in both Ramonda plants during freezing stress and freezing-induced desiccation might be triggered due to slight increase in sub-stomatal CO_2 concentration (T10, Fig. 5). Furthermore, our results showed that sub-stomatal CO_2 (C_i) concentrations were similar in both Ramonda species after anabiosis (June) and they were comparable with those from the cold acclimation period (November). An elevated CO_2 concentration (C_i) that occurs in the intercellular space can trigger an anion channels to open. As a consequence, depolarization of the plasma membrane in guard cells takes place, thus initiating stomatal closure (Driesen et al., 2020). A notable increase in sub-stomatal CO_2 (C_i) concentration under cold stress was observed in a cold-resistant cotton seedling of *Gossypium hirsutum* L. (Li et al., 2024). Our results showed a negative correlation between sub-stomatal CO_2 (C_i) concentrations and CO_2 assimilation (A), meaning that there were lower concentrations of sub-stomatal CO_2 (C_i) and higher assimilation (A), after the recovery phase (June) compared to cold acclimatization, freezing stress, and freezing-induced desiccation periods. When plants are fully hydrated, there is an optimal stomatal conductance of CO_2 in the mesophyll part of the leaf, thus more CO_2 will be fixed by rubisco which indicates that more CO_2 will be incorporated in photosynthesis, but at the same time, less CO_2 is left in sub-stomatal spaces.

Our results showed that CO_2 assimilation (A) was significantly reduced in both Ramonda species during cold acclimation, compared to control (June). It decreased more as a result of freezing temperatures reaching the lowest values in the desiccated state (0.58 µmol CO_2 m^{-2} s^{-1} *in R. serbica* and 0.68 µmol CO_2 m^{-2} s^{-1} in *R. nathaliae*) (Fig. 6). We found no significant changes in CO_2 assimilation during freezing stress and freezing-induced desiccation among the two species. Elevated assimilation (A) after anabiosis (June) was positively correlated with an

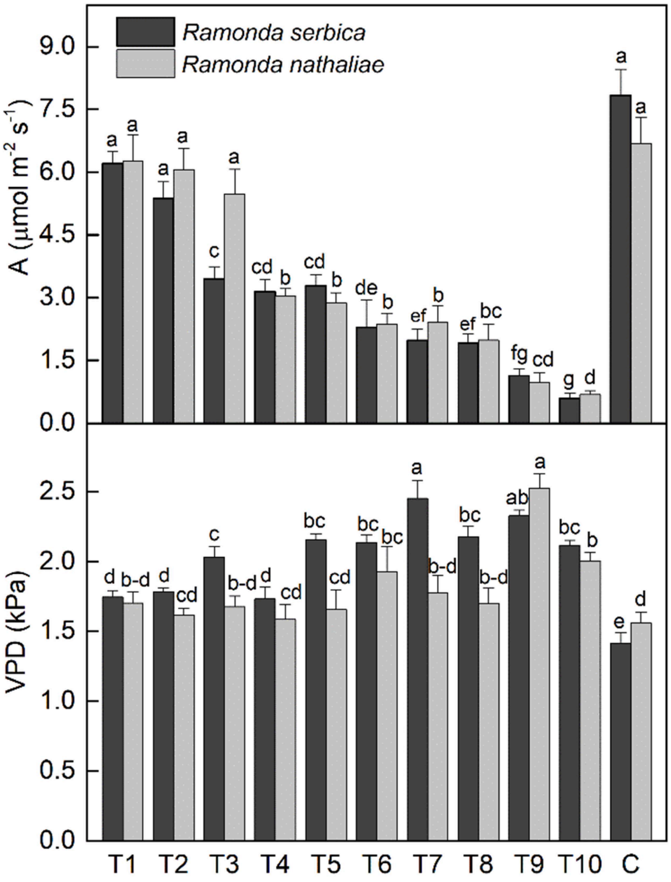

Fig. 6. Changes in assimilation (A) and vapor pressure deficit (VPD) in leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C, June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

enhancement of stomatal conductance in both Ramonda species, which was in agreement with the results observed by Degl'Innocenti et al. (2008) in leaves of *R. serbica*. The decline in stomatal conductance (g_s) led to a further decline in CO_2 assimilation (A), particularly throughout the freezing period and freezing-induced desiccation period. A similar correlation was described by Noormets et al. (2001). A significant impact of freezing-induced desiccation on CO_2 assimilation was observed when temperatures decreased to -8 °C in Radac (*R. serbica*) and -11 °C in Glloboqica (*R. nathaliae*) reducing the relative water content to 10.91 % in *R. serbica* and 8.05 % in *R. nathaliae*. The reduction of CO_2 assimilation (A) during the freezing stress and especially during freezing-induced desiccation can be interpreted by the fact that when plants face water scarcity, they minimize stomatal conductance, a mechanism that prevents dehydration. As a consequence, stomatal closure could impact CO_2 assimilation by reducing its concentration inside the leaves. In addition, we assume that freezing temperatures might have reduced the activity and ability of Rubisco to bind CO_2, thus reducing assimilation. Georgieva et al. (2007) showed that during the initial stage of desiccation in *H. rhodopensis*, the decrease in CO_2 assimilation was primarily due to stomatal closure. Consequently, the CO_2 assimilation rate declined in parallel with stomatal conductance (g_s). This indicates that stomatal regulation plays a crucial role in limiting water loss and maintaining homeostasis during the early phases of desiccation, highlighting a vital adaptive mechanism in stress-tolerant plants. Similar to our results, a study done on *R. serbica* showed that the CO_2 assimilation rate was significantly reduced throughout dehydration, however, recovery was accomplished with rehydration (Degl'Innocenti et al., 2008).

Higher values of VPD in both Ramonda species were recorded in the course of freezing stress and when the desiccation period started (T7, Fig. 6), however, there was no significant increase during the cold acclimatization period (T1-T4, Fig. 6). After the period of anabiosis (June, Control) the VPD in both plants was decreased. A higher value of VPD was recorded during the freezing-induced desiccation period in *R. nathaliae,* reaching values of 2.53 kPa (T7, Fig. 6). In the other resurrection plants, *Selaginella bryopteris*, due to the high value of VPD, net photosynthesis was decreased marginally (Soni et al., 2012).

The results of our study revealed that WUE was reduced during cold acclimation only in *R. serbica*. When plants were exposed to freezing, fluctuations in WUE were recorded in both species. In *R. serbica,* in the early stage of freezing stress (T5) plants showed higher values of WUE compared to *R. nathaliae* whose WUE was significantly lower (Fig. 7). Water use efficiency was reduced during the late freezing-induced desiccation period (T10, Fig. 7), however, it increased after the recovery from anabiosis (June). Plant water use efficiency represents the balance of carbon uptake through photosynthesis and water loss through stomata and it is an essential indicator for investigating plant water use approaches (Rahman et al., 2019). Therefore, WUE is often considered a key factor influencing agricultural output and drought resilience (Blum, 2009). As we found in this study, Ramonda plants were able to reduce their stomatal conductance against cold and freezing stress by closing their stomata, decreasing their transpiration rate thus enhancing their water use efficiency. This response suggests their enhanced mechanisms to conserve water while maintaining essential metabolic functions in cold and freezing environmental conditions. Our data show a relationship between WUE and temperatures with some exceptions. In *R. nathaliae,* during the final stages of freezing-induced desiccation (T10, Fig. 7) where temperatures decreased to -10 °C, WUE also was reduced. After the recovery of plants (C, June) where temperatures increased WUE also increased. Leaves under water scarcity conditions, exposed to drought stress respond differently in terms of water use efficiency and this is associated with the physiological processes regulating CO_2 and H_2O gradients, between the air and leaf (Hatfield et al., 2019).

Transpiration rate (E) was not significantly affected during cold acclimatization compared to the control in June (Fig. 7). However, freezing stress reduced its values (T9 and T10, Fig. 7) and it was stronger in *R. serbica* than in *R. nathaliae*. The period of freezing-induced desiccation caused the most substantial drop in transpiration rate. After anabiosis (June), the transpiration rate was higher in the leaves of both species. In addition to that, *R. serbica* performed a higher transpiration rate (3.62 mmol m^{-2} s^{-1}) compared to *R. nathaliae* (2.98 mmol m^{-2} s^{-1}). Comparable to our results, the decline in transpiration rate was found during the desiccation of *H. rhodopensis* (Georgieva et al., 2010). In another study, a decrease in the photosynthetic transpiration rate was found in wheat leaves exposed to low temperatures (Zhang et al., 2022). We assume that this significant decrease in transpiration rate in both species of Ramonda during the freezing-induced desiccation period might be due to the reduction of the amount of water in the leaves and its transport through the xylem. In addition, lowering the transpiration rate due to stomata closure in response to water shortage and freezing stress might be one of the protecting strategies of these plants to prevent dehydration and avoid damage to photosynthetic apparatus from ROS generation. The results with the resurrection plant *R. myconi* showed that transpiration caused a decrease in RWC due to a lack of water availability (Kampowski et al., 2018).

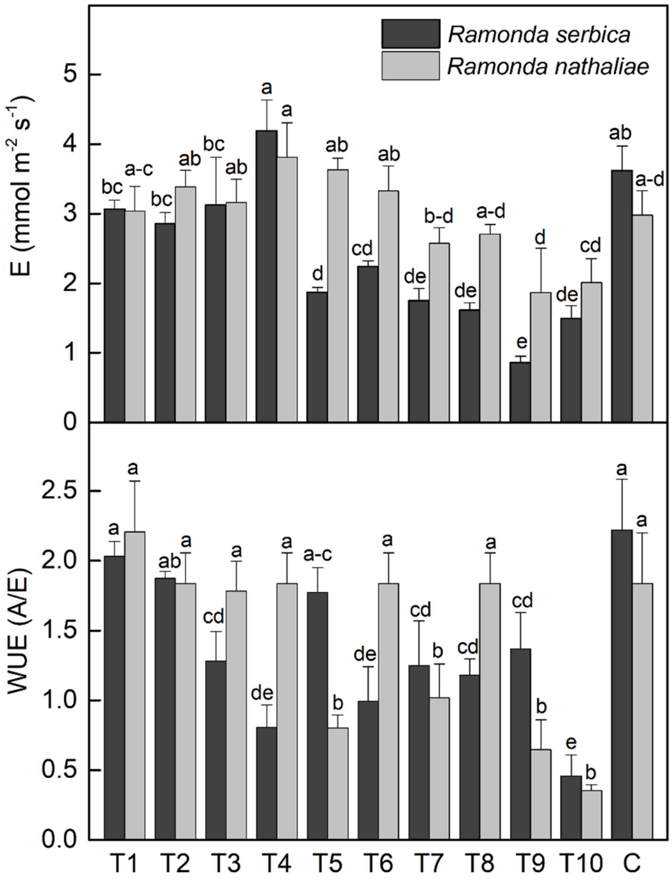

Fig. 7. Changes in transpiration (E) and photosynthetic water use efficiency (WUE) in leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C, June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

3.2.2. Photochemical efficiency during cold acclimation, freezing stress, freezing-induced desiccation, and after anabiosis

The ratio F_v/F_m is considered to be an important parameter in plants indicating the activity of photosystem II under cold stress conditions (Mishra et al., 2011). Our data show that the maximum quantum efficiency of PSII, slightly declined during the cold acclimation of *R. serbica* and *R. nathaliae* (Fig. 8). Under these conditions the F_v/F_m values decreased to 0.75 (*R. serbica*) and 0.65 (*R. nathaliae*), indicating that the photosynthetic apparatus remained functional. However, we found that F_v/F_m significantly declined as the temperature in their habitat decreased to freezing especially in *R. nathaliae*, reaching the value of 0.40 as a result of freezing-induced desiccation (T10, Fig. 8). Thus, we could conclude that lowering photochemical efficiency in response to freezing stress might be a strategy for *R. serbica* and *R. nathaliae* to avoid the overexcitation of the photosynthetic apparatus. Upon rehydration (June) when temperatures increased and relative water content reached a value of 93.05 % in *R. serbica* and 91.58 % in *R. nathaliae* the maximal photochemical efficiency of PSII increased to 0.82 in *R. serbica* and 0.81 in *R. nathaliae.* Similarly, it was found that the maximum efficiency of PSII, wasn't impacted during cold acclimatization of Ramonda species under *ex situ* conditions, but was strongly reduced when plants were exposed to subzero temperatures (Mihailova et al., 2023a). The study of Fernández-Marín et al. (2018) confirmed that freezing conditions can trigger a reduction in F_v/F_m in the leaves of *R. myconi*. A notable decline in F_v/F_m under cold stress was also observed in a cold-resistant cotton seedling of *Gossypium hirsutum* L. (Li et al., 2024). Drought stress can also induce a significant reduction of the F_v/F_m ratio in *R. serbica* and in *R. nathaliae* (Gashi et al., 2013). In addition, it has been shown that in both Ramonda plants F_v/F_m was not affected during the early stage of dehydration, compared to severely desiccated leaves, where F_v/F_m was pronouncedly reduced (Rakić et al., 2015).

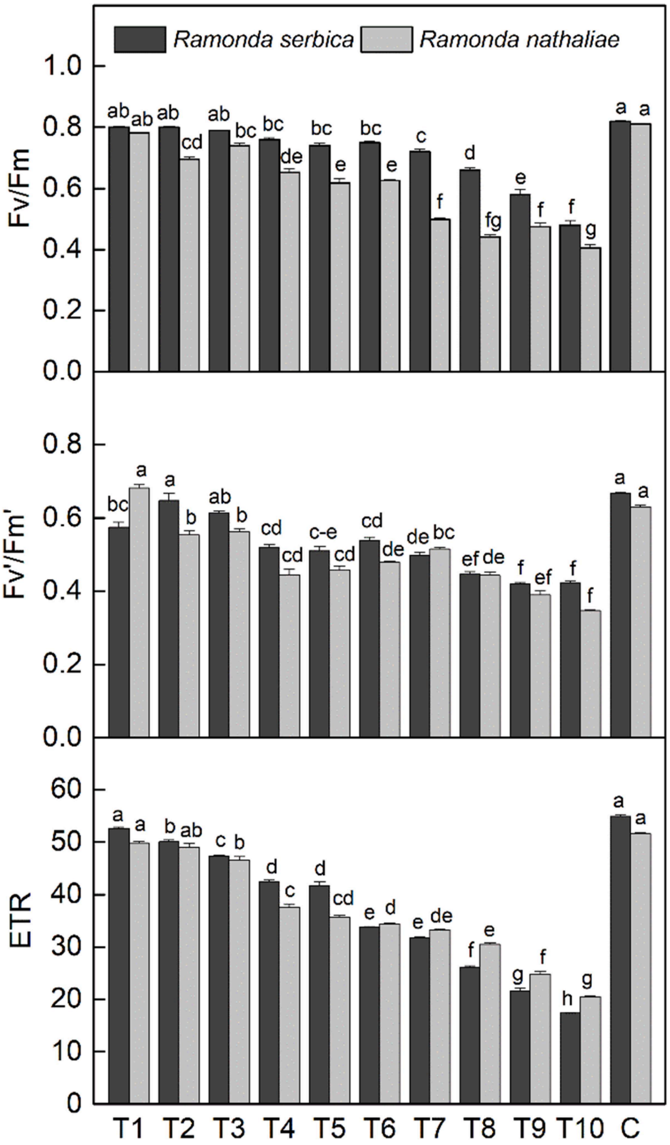

Fig. 8. Changes in maximal photochemical efficiency of PSII (Fv/Fm), photochemical efficiency of PSII, sample illuminated by far-red light (Fv′/Fm′), and thylakoid electron transport rate (ETR) in leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C; June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

Similar to F_v/F_m, the photochemical efficiency in light-adapted state, F_v'/F_m' declined during exposure of both Ramonda plants to freezing temperatures and after freezing-induced desiccation (Fig. 8). Recovery of *R. serbica* and *R. nathaliae* after anabiosis (June) was accompanied by an increase of F_v'/F_m'. We assume that the induction of the protective mechanisms throughout cold acclimatization and freezing period, such as antioxidant accumulation and increased synthesis of stress-related proteins (Mihailova et al. 2023a) could protect the photosynthetic apparatus of *R. serbica* and *R. nathaliae* from irreversible damage. Downregulation of photosynthesis in wintering plants is thought to be an essential strategy that limits the efficiency of photosynthetic apparatus, avoiding any damage (Míguez et al., 2015).

Estimation of the electron transport rate (ETR) in *R. serbica* and *R. nathaliae* showed that ETR gradually declined during the cold acclimation (T1-T4, Fig. 8), it was stronger at freezing temperatures (December till end of January: T5-T10, Fig. 8), reaching the lowest values in desiccated plants (17.40 in *R. serbica* and 20.54 in *R. nathaliae*). After recovery from anabiosis (June), ETR was restored in both plants and showed similar values with the initial stages of the cold acclimation. The reduction of ETR during exposure of plants to freezing stress could be due to slow down or inhibition of biochemical reactions in photosynthetic apparatus at subzero temperatures. In addition, freezing induces the generation of ROS, thus furthermore damaging the photosynthetic system, reducing markedly ETR. Similar to our results, a low rate of ETR during freezing-induced desiccation in *R. serbica* and *R. nathaliae* in *ex situ* conditions was observed by Mihailova et al. (2023a). Degl'Innocenti et al. (2008) found that the electron transport rate (ETR) decreased markedly in *R. serbica* in *ex situ* conditions during severe dehydration.

The results presented in Fig. 9 show that regardless of some decline in the quantum yield of PSII ($\phi PSII$), the main part of energy needed for excitation during cold acclimatization was used for photochemical reactions. When plants were exposed to freezing temperatures $\phi PSII$ further decreased and the lowest values were detected after freezing-induced desiccation. As previously mentioned, the significant reduction of $\phi PSII$ as a result of severe water loss can be regarded as a protective mechanism for PSII. In this case, light energy cannot be used for photochemical reactions and is instead dissipated, primarily as heat, to prevent potential damage to the photosynthetic system. Following anabiosis (June), the quantum yield of PSII ($\phi PSII$) was recovered, reaching a higher value in *R. serbica* compared to *R. nathaliae.* A strong reduction of the quantum yield of PSII in a resurection plant of *H. rhodopensis* and Ramonda species exposed to -10°C was previously shown in *ex situ* experiments (Mihailova et al. 2020; 2023a). Comparable to our results, the reduction of ϕPSII throughout severe dehydration in both Ramonda species in *ex situ* conditions was confirmed by Rakić et al. (2015). Low temperatures impair the transfer of energy through PSII, hence diminishing electron transport ability (Paredes and Quiles, 2015).

The decline in ϕPSII values during cold acclimation was associated with an increase of $\phi(NO)$ and $\phi(NPQ)$ (Fig. 9). The increase of $\phi(NO)$

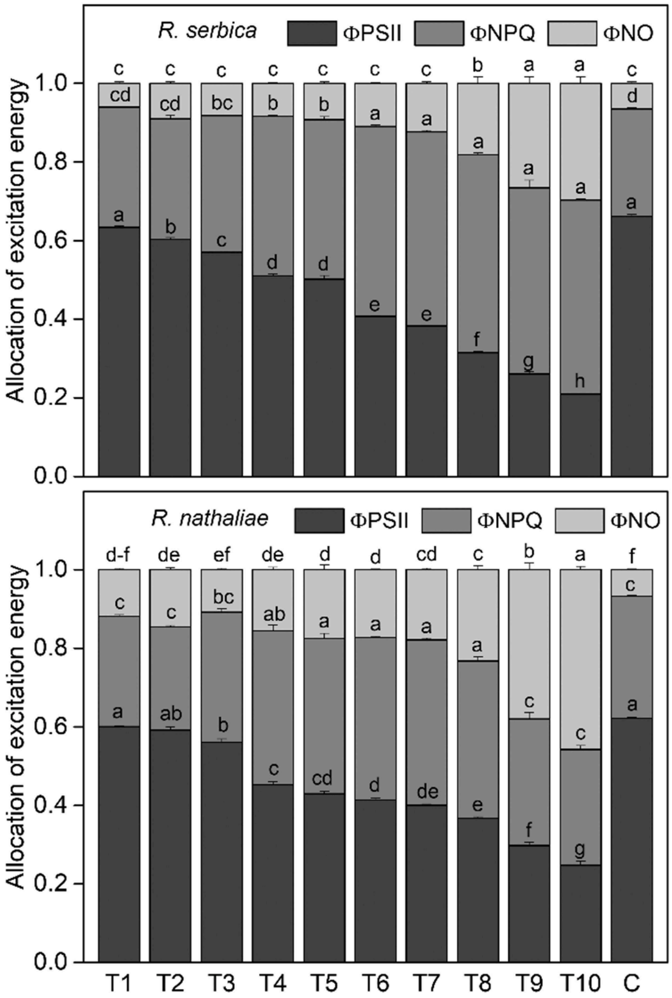

Fig. 9. Changes in the quantum yield of PSII electron transport during illumination (ϕPSII), the quantum yield of non-regulated heat dissipation and fluorescence emission (ϕNO), and the quantum yield of light-induced non-photochemical fluorescence quenching (ϕNPQ) in leaves of *R. serbica* and *R. nathaliae* during exposure to cold acclimation (T1-T4; November), freezing stress (T5-T7; December) and freezing-induced desiccation (T8-T10; January–February), as well after anabiosis (C; June) in natural environmental conditions. The average minimum temperatures recorded were as follows: T1–T4: 10 to 0°C, T5–T7: 0 to -5°C, T8–T10: -5 to -10°C, and C: 20 to 25°C. The values are presented as mean ±SE. Identical letters within a graph indicate no significant differences, as determined by Duncan's Multiple Range Test at the 5 % significance level ($P \leq 0.05$) following ANOVA analysis.

continued during the freezing stress and reached maximum values during the freezing-induced desiccation period (T10, Fig. 9). Thermal energy dissipation of excessive excitation energy during environmental stress is one of the main protective mechanisms against photoinhibition and oxidative stress. In fact, with the inhibition of photochemical activity during the severe freezing-induced desiccation of *R. serbica* and *R. nathaliae*, the contribution of ϕ(NO) increased. After rehydration (June), both species showed a similar reduction of ϕ(NO), indicating that a higher amount of energy was used for photochemistry and successful recovery after anabiosis. Since *R. nathaliae* mostly inhabit rocky habitats exposed to high radiation, the most efficient mechanism to dissipate the excess light energy into heat is considered to be non-photochemical quenching (ϕNPQ). This mechanism is activated in *R. serbica* to control the generation of ROS, thus protecting membrane structure (Degl'Innocenti et al., 2008). Meanwhile, ϕ(NO) in severely desiccated leaves becomes the most important mechanism of energy dissipation, hence protecting them effectively against overexcitation (Mihailova et al., 2023a). Thermal energy dissipation is the main photoprotective mechanism that helps plants to survive against excessive light absorption, which is associated with the accumulation of zeaxanthin (Fernández-Marín et al., 2018).

3.2.3. *Differences in the main photosynthetic proteins*

Changes in the main photosynthetic proteins content from thylakoid membranes of *R. serbica* and *R. nathaliae* were estimated by immunoblot analysis. Cold acclimatization and freezing-induced desiccation decreased the abundance of the PSII and PSI reaction center proteins D2 and PsaB, the 33 kDa protein of oxygen-evolving complex of PSII, PsbO, and the component of cyt b_6f complex, cyt b_6 in both Ramonda species (Fig. 10, Table 1). The decrease in protein abundance was more pronounced in *R. serbica*. The main difference between the two Ramonda plants could be seen in the content of light-harvesting complex (LHC) proteins. After a slight decline at the beginning of cold acclimation (T1), the abundance of the component of the PSII antenna Lhcb2 was increased when plants were exposed to freezing stress (T5, T6) and it was higher in *R. serbica* compared to *R. nathaliae*. Freezing-induced desiccation (T8-T10) decreased the content of Lhcb2, but its level remained close to control values. The amount of PSI antenna protein Lhca2 declined throughout cold acclimation period and freezing-induced desiccation in *R. serbica*, but its content increased in *R. nathaliae*, reaching 140 % in dried plants. As other plants, *R. serbica* and *R. nathaliae* decreased their photosynthetic activity during stress to maintain the stability between light energy absorption and energy usage. Overall, the low temperatures in the course of cold acclimation and freezing-induced desiccation of *R. serbica* and *R. nathaliae* led to a decrease of most of the investigated proteins, except for the LHC proteins. Protein decline could be a result of generated ROS as both Ramonda species are homoiochlorophyllous resurrection species that can preserve their chlorophyll content during desiccation (Georgieva et al., 2009) and potentially generate singlet oxygen and induce photooxidative damage (Aro et al., 1993). In resurrection species the content of reaction centre proteins D1, D2, PsaA/B and cyt b_6f components and PsbO decreased during dehydration induced by drought (Georgieva et al. 2009; Charuvi et al., 2015) or low temperature (Mihailova et al., 2020). Decreased protein and transcript levels of reaction centers protein of PSII and PSI, oxygen-evolving complex components and Calvin cycle enzymes were observed in many resurrection species during desiccation induced by drought or low temperature (Deng et al., 2003; Gechev et al., 2013; Charuvi et al. 2015; Ma et al., 2015 Mihailova et al., 2020; Vidović et al., 2022). These low levels of genes and proteins lead to inhibition of photosynthesis during drought that reduce the amount of produced ROS, thus protecting homoiochlorophyllous resurrection species from photoinhibition (Dinakar et al., 2012). The increased abundance of LHC coincides with the raised levels of NPQ in *R. serbica* and *R. nathaliae* during cold acclimatization, freezing stress and freezing-induced desiccation. The higher amount of Lhca2 in *R. nathaliae* and Lhcb2 in both Ramonda species could be a compensatory replacement mechanism. It had been suggested that the decline of abundance of one LHC is leading to accumulation of another (Ruban et al., 2006). Dehydration of *Craterostigma pumilum* and sun *H. rhodopensis* plants also demonstrated similar pattern of change in the amount of the LHC proteins (Charuvi et al., 2015; Mihailova et al., 2016). Our previous results demonstrated that freezing-induced desiccation in *H. rhodopensis* did not change significantly the total amount of LHCII and LHCI antenna proteins, but shifted the stoichiometry of LHC proteins (Mihailova et al., 2020).

3.3. *Antioxidative defense throughout cold acclimation, freezing stress, freezing-induced desiccation, and after anabiosis*

3.3.1. *Total phenolic and flavonoid content*

A significant enhancement in flavonoids and total phenols content was observed throughout the cold acclimatization, freezing stress, and

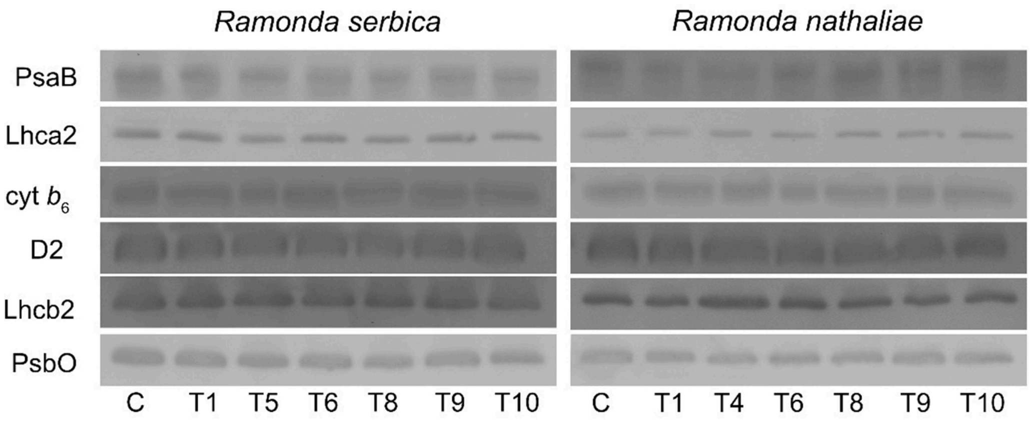

Fig. 10. Western blots of photosynthetic proteins in thylakoid membranes of *R. serbica* and *R. nathaliae* control plants (C; June) and after cold acclimation (T1, T4; November), exposure to freezing stress (T5, T6; December) and freezing-induced desiccation (T8-T10; January–February) in natural environmental conditions. The average minimum temperatures recorded were as follows: C: 20 to 25°C,T1–T4: 10 to 0°C, T5–T6: 0 to -5°C, and T8–T10: -5 to -10°C. Proteins were probed with antibodies against: PsaB (PSI-B core subunit of photosystem I), Lhca2, PetB (cytochrome *b*6 subunit of cytochrome *b6f* complex), PsbD (D2), Lhcb2 and PsbO (33 kDa of the oxygen-evolving complex of PSII).

Table 1

Changes in the main photosynthetic proteins of *Ramonda serbica* and *Ramonda nathaliae* in control plants (C) and after cold acclimation (T1, T4; November), exposure to freezing stress (T5, T6; December) and freezing-induced desiccation (T8-T10; January-February) in natural environmental conditions. The average minimum temperatures recorded were as follows: C: 20 to 25°C, T1-T4: 10 to 0°C, T5–T6: 0 to -5°C, and T8–T10: -5 to -10°C.

Protein	Abundance (%)						
			Ramonda serbica				
	C	T1	T5	T6	T8	T9	T10
PsaB	**100**^{a}	**66**±3.4^{def}	**67**±4.2^{def}	**56**±4.3^{fg}	**50**±4.1^{g}	**61**±3.6^{defg}	**60**±4.5^{efg}
Lhca2	**100**^{cd}	**86**±5.0^{ef}	**59**±1.7^{i}	**72**±3.3^{gh}	**68**±2.6^{hi}	**76**±2.0^{fgh}	**66**±1.7^{hi}
cyt b_6	**100**^{a}	**83**±8.4^{b}	**54**±4.1^{fgh}	**76**±1.3^{bc}	**61**±7.4^{defg}	**56**±3.8^{efgh}	**68**±5.3^{cde}
PsbD (D2)	**100**^{a}	**71**±2.5^{bc}	**70**±0.9^{bc}	**65**±4.6^{bcd}	**48**±3.4^{e}	**76**±5.5^{b}	**74**±7.1^{bc}
Lhcb2	**100**^{de}	**88**±3.9^{ef}	**163**±6.5^{a}	**145**±4.2^{b}	**112**±6.7^{d}	**91**±6.8^{ef}	**85**±3.7^{fg}
PsbO	**100**^{a}	**93**±7.6^{a}	**74**±6.1^{c}	**62**±3.8^{cde}	**51**±4.4^{e}	**51**±5.1^{de}	**53**±2.0^{de}
			Ramonda nathaliae				
	C	T1	T4	T6	T8	T9	T10
PsaB	**100**^{a}	**63**±1.2^{def}	**79**±4.7^{bc}	**72**±4.7^{cd}	**84**±3.7^{b}	**71**±6.2^{cde}	**82**±6.1^{bc}
Lhca2	**100**^{cd}	**82**±0.7^{fg}	**97**±1.1^{de}	**98**±2.3^{cd}	**109**±9.0^{bc}	**111**±3.1^{b}	**145**±8.1^{a}
cyt b_6	**100**^{a}	**64**±2.5^{cdef}	**62**±4.6^{def}	**47**±4.1^{h}	**57**±4.5^{efgh}	**48**±3.0^{gh}	**70**±1.4^{cd}
PsbD (D2)	**100**^{a}	**71**±1.2^{bc}	**70**±0.6^{bcd}	**53**±4.0^{e}	**58**±3.6^{de}	**64**±6.3^{cd}	**71**±5.3^{bc}
Lhcb2	**100**^{de}	**73**±2.9^{g}	**129**±8.7^{c}	**102**±3.8^{de}	**94**±4.0^{ef}	**82**±2.4^{fg}	**91**±8.2^{ef}
PsbO	**100**^{a}	**89**±7.0^{ab}	**75**±8.1^{bc}	**72**±7.5^{c}	**68**±3.8^{c}	**65**±2.2^{cd}	**62**±1.6^{cde}

Abundance of proteins is presented in percent of the control values. Values are given as mean ± SE. The same letters within a graph indicate no significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA. The changes in the content of the respective protein between *R. serbica* and *R. nathaliae* were statistically compared.

freezing-induced desiccation in both Ramonda species compared to control plants (June) (Table 2). Our results are in agreement with those obtained on *H. rhodopensis* demonstrating a notable enhancement in total flavonoids as a result of freezing-induced desiccation at 8 % RWC (Georgieva et al., 2021). This might be due to protection mechanisms against ROS and photoinhibition when plants are exposed to freezing-induced desiccation. Desiccation-induced leaf folding is associated with the accumulation of total phenols and anthocyanins (Farrant and Moore, 2011). Similar to our results, an increase in polyphenolic synthesis upon cold acclimation (Georgieva et al., 2021) and during desiccation in leaves of *H. rhodopensis* was found (Georgieva et al., 2017). The increase in phenolic acids during dehydration may also protect Ramonda membranes from desiccation-induced damage (Sgherri et al., 2004). The other studies also confirmed the higher phenolic content in desiccated leaves of *R. serbica* compared to hydrated leaves (Vidović et al., 2022). In *Selaginella lepidophylla*, phenolics, and flavonols accumulation was reported in desiccated tissues (Yobi et al., 2013). The increased accumulation of total phenols during cold stress plays a role as free radical scavengers and helps the plant mitigate oxidative stress (Kumar et al., 2023). In plants, the production of flavonoids and their accumulation is initiated by abiotic stimuli, resulting in the modification of pathways related to stress response. Additionally, they regulate redox homeostasis and cellular defense mechanisms, hence improving their chances of surviving in cold and freezing conditions (Shomali et al., 2022).

3.3.2. *Antioxidant activity (FRAP and TAC) and scavenging capacity (DPPH and ABTS)*

To have a better understanding of how *R. serbica* and *R. nathaliae* cope against cold and freezing conditions a set of antioxidant parameters was determined. We found that in both species, the total antioxidant capacity (TAC) and reducing power of antioxidants (FRAP) increased during cold acclimatization, freezing stress and freezing-induced desiccation compared to the control (Table 2). This enhancement could indicate an adaptive strategy in response to the drop in temperature and increased degree of stress. A stronger response to freezing stress and

Table 2

Secondary metabolites, antioxidant activity, and scavenging capacity in leaves of *R. serbica* (*RS*) and *R. nathaliae* (*RN*) during cold acclimation (T1-T4; November), freezing stress (T5-T7; December), freezing-induced desiccation (T8-T10; January-February), and after recovery from anabiosis (C; June) in natural conditions. The average minimum temperatures recorded were as follows: C: 20 to 25°C, T1-T4: 10 to 0°C, T5–T6: 0 to -5°C, and T8–T10: -5 to -10°C.

	TP (mg GAE/g DW)		TF (mg CE/g DW)		FRAP (µmol AAE/g DW)		DPPH (µmol TE/g DW)		TAC (mg AAE/g DW)		ABTS (µmol TE/g DW)	
	RS	*RN*	*RS*	*RN*	*RS*	*RN*	*RS*	*RN*	*RS*	*RN*	*RS*	*RN*
C	**24.88**^{H}	**33.11**^{E}	**16.62**^{D}	**10.95**^{H}	**159.22**^{F}	**107.70**^{G}	**196.46**^{C}	**143.63**$^{D-E}$	**23.16**^{G}	**21.03**^{G}	**94.71**^{E}	**109.89**^{F}
	±0.97	±1.39	±1.70	±1.10	±19.25	±17.74	±16.29	±8.32	±0.98	±0.72	±9.48	±7.69
T1	**34.63**^{G}	**33.89**^{E}	**29.15**^{C}	**22.71**^{G}	**187.73**^{F}	**321.82**^{F}	**222.03**^{C}	**107.16**^{E}	**34.92**^{F}	**33.18**^{F}	**113.19**^{E}	**236.61**^{F}
	±1.49	±3.55	±2.48	±0.37	±1.87	±8.23	±27.14	±6.72	±1.22	±0.79	±3.19	±20.35
T2	**45.74**$^{D-F}$	**36.30**^{E}	**29.30**^{C}	**24.46**^{G}	**401.48**^{E}	**306.37**^{F}	**231.81**^{C}	**196.84**^{C}	**37.29**^{F}	**39.65**^{E}	**129.03**^{E}	**230.01**^{F}
	±1.02	±1.89	±0.52	±0.79	±13.26	±19.72	±13.46	±18.09	±0.51	±1.16	±7.66	±36.27
T3	**38.04**^{G}	**45.04**^{D}	**32.04**^{C}	**50.36**$^{E-F}$	**510.52**^{D}	**631.80**^{E}	**400.54**^{B}	**300.43**^{C}	**37.69**^{F}	**54.78**^{D}	**312.51**^{D}	**519.09**^{E}
	±0.80	±0.58	±0.63	±1.46	±22.88	±16.67	±47.92	±25.73	±1.46	±0.44	±56.31	±36.12
T4	**48.80**$^{C-D}$	**49.64**^{D}	**46.34**^{B}	**55.88**^{E}	**699.75**^{C}	**798.45**^{D}	**540.89**^{A}	**321.11**^{C}	**49.06**^{D}	**52.81**^{D}	**378.51**^{D}	**668.25**^{D}
	±0.67	±1.18	±1.87	±1.70	±36.63	±15.01	±32.77	±39.18	±1.09	±1.43	±76.80	±52.35
T5	**42.57**^{F}	**49.67**^{D}	**43.85**^{B}	**65.90**^{D}	**726.00**^{C}	**1059.97**^{C}	**641.10**^{A}	**310.39**^{C}	**46.21**$^{D-E}$	**63.57**$^{B-C}$	**582.45**^{C}	**708.51**^{D}
	±1.50	±0.99	±0.42	±1.44	±31.79	±11.19	±24.97	±23.39	±0.83	±0.54	±20.43	±27.44
T6	**53.99**^{B}	**63.75**^{B}	**48.77**^{B}	**78.99**^{C}	**794.78**$^{B-C}$	**1096.50**^{C}	**551.04**^{A}	**503.10**^{B}	**42.33**^{E}	**61.30**^{C}	**607.53**^{C}	**887.37**^{C}
	±1.74	±1.95	±2.28	±2.01	±32.23	±20.52	±32.72	±25.11	±1.70	±0.80	±80.36	±97.47
T7	**49.80**^{C}	**67.18**$^{A-B}$	**45.77**^{B}	**48.42**^{F}	**840.83**^{B}	**782.85**^{D}	**627.37**^{A}	**476.03**^{B}	**63.41**^{C}	**66.11**^{B}	**794.97**^{B}	**917.07**^{C}
	±1.69	±1.26	±2.10	±1.00	±44.19	±25.99	±13.21	±16.05	±2.98	±1.56	±46.12	±43.94
T8	**45.04**$^{E-F}$	**58.03**^{C}	**45.76**^{B}	**104.68**^{B}	**779.78**$^{B-C}$	**1433.10**^{A}	**568.69**^{A}	**538.26**^{B}	**70.86**^{B}	**77.53**^{A}	**907.83**^{B}	**1447.05**^{A}
	±0.44	±1.51	±1.74	±2.79	±46.27	±13.94	±20.49	±48.72	±1.29	±1.34	±13.07	±11.74
T9	**47.61**$^{C-E}$	**55.60**^{C}	**79.32**^{A}	**117.46**^{A}	**852.00**^{B}	**1287.22**^{B}	**623.05**^{A}	**677.19**^{A}	**77.70**^{A}	**81.00**^{A}	**911.79**^{B}	**1136.19**^{B}
	±0.96	±0.68	±7.27	±2.38	±77.74	±34.04	±35.03	±24.53	±0.98	±1.90	±18.73	±23.46
T10	**63.32**^{A}	**69.71**^{A}	**45.43**^{B}	**67.08**^{D}	**1098.98**^{A}	**1070.25**^{C}	**613.27**^{A}	**510.06**^{B}	**78.61**^{A}	**67.39**^{B}	**1330.89A**	**1367.19**^{A}
	±1.12	±1.45	±1.52	±5.18	±15.01	±92.86	±62.19	±14.99	±1.08	±3.16	±15.13	±31.22
F	71.04	60.43	34.74	222.21	63.76	162.77	29.20	50.24	172.94	159.72	92.30	115.56
$p < 0.05$	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

TP (Total phenols), TF (Total flavonoids), FRAP (Ferric Reducing Antioxidant Power Assay), DPPH (Radical Scavenging Assay), TAC (Determination of Total Antioxidant Capacity), ABTS (Radical Scavenging Assay). T1-T10: periods of measurements; C: control. Values are given as mean ± SE. The same letters within a graph indicate no significant differences assessed by Duncan's Multiple Range Tests at the 5 % level of significance ($P \leq 0.05$) after performing ANOVA.

higher values of the total reducing the power of antioxidants were recorded in *R. nathaliae* during the early stage of the freezing-induced desiccation period (T8) compared to *R. serbica*. It has been demonstrated that the antioxidant activity of enzymes remains elevated after a freezing period and freezing-induced desiccation, suggesting their crucial function in combating oxidative stress (Georgieva et al., 2021). Nearly all overwintering plants have evolved so-called cold acclimation to adjust in response to low temperature, enhancing their cold tolerance by accumulating compatible osmolytes and increasing levels of antioxidants (Xin and Browse, 2000). Studies on *Oryza sativa* L. also revealed that antioxidant enzyme activity is crucially enhanced at low temperatures (Guo et al., 2022). However, other studies showed that the antioxidant activity of enzymes in plants facing cold stress at first increases, but decreases later on (Hongtao et al., 2017).

Similar to antioxidant activity, the results regarding both radical-scavenging assays (DPPH and ABTS) indicate that the activity increased due to cold aclimatization, freezing stress and reaching the highest values during freezing-induced desiccation (Table 2). We assume that these periods with subzero temperatures during the night/day when plants had to cope with severe stress might have triggered a strong antioxidative response. The strongest capacity to scavenge the DPPH radical was pronounced in *R. nathaliae* and was higher (677.19 µmol TE/g DW) during freezing induced-desiccation period (T9), compared to *R. serbica* (627.05 µmol TE/g DW). This indicator suggests that the kinetics of radical scavenging reactions in Ramonda plants might be different. Our results indicates a negative correlation between DPPH, ABTS, and RWC in leaves of *R. serbica*, with some exceptions. Georgieva et al. (2022) confirmed a slightly higher metabolic activity in partially dehydrated leaves and higher molecular mobility compared to fully hydrated leaves of *H. rhodophensis* in response to low temperatures in *ex situ* conditions. Similar to our results, reversible oscillations during different stages of stress and after recovery of DPPH and FRAP were also observed in *H. rhodopensis* extracts (Moyankova et al., 2014). Moreover, the activity of antioxidant enzymes has been shown to remain high after freezing stress and freezing-induced desiccation, demonstrating their important role in overcoming oxidative damage (Georgieva et al., 2021). We assume that the decrease of some parameters regarding antioxidant activity and scavenging capacity of *R. serbica* and *R. nathaliae* during the freezing-induced desiccation period might be because of a temporary increase in the temperature during the time of plant material collection in both localities Radac and Glloboqicë, which could frequently happen under natural conditions (Figs. 2, 3). Nevertheless, freezing stress followed by a rapid decline in relative water content led to an accumulation of excessive oxygen radicals (ROS) that might have disrupted the equilibrium between ROS production/ROS scavenging, thus weakening their ability to remove reactive oxygen species during freezing stress in Ramonda plants studied. Overall, our results suggest that this enhancement in oxidative response during cold acclimation and freezing-induced desiccation actively contribute to the induction of the desiccation adaptive response in *R. serbica* and *R. nathaliae.*

The role of cold acclimation period for plants in response to oxidative stress was demonstrated in *Camelina sativa* L. by Soorni et al. (2022). Non acclimated plants showed a higher antioxidant activity (glutathione peroxidase, superoxide dismutase, and catalase), compared to those acclimated. In addition, higher expression of CBF genes (*CsCBF1-3, CsICE1*, and *CsCOR6.6*) was recorded in the non-acclimated plants. In another study, under cold conditions, Triticum aestivum L. cultivars showed significant reduction in leaf Chl contents, and significant increace in catalase activity. Moreover, cold increased the level of genes expression of TaADC transcripts (Gholizadeh et al., 2024).

3.4. Accumulation of stress-induced proteins

3.4.1. Dehydrins

Protein abundance of dehydrins, a LEA2 group stress-induced proteins, throughout cold acclimatization, freezing stress, and freezing-induced desiccation in natural environmental conditions of *R. serbica* and *R. nathlaiae* was assessed by Western blot using particular antibodies raised against the conserved K-segment of dehydrins. Immunoblot signals showed the presence of the proteins with apparent molecular weight between 20–50 kDa in all investigated samples in both *R. serbica* and *R. nathaliae* plants (Fig. 11). The protein pattern of dehydrins differed in both Ramonda species. The main bands detected were located around 50 and 30 kDa, and 50 and 37 kDa in *R. serbica* and *R. nathlaiae*, respectively. Small bands were monitored around 12-13 kDa in both plant species in all investigated samples. Freezing-induced desiccation of *R. nathaliae* increased the content in the main bands and the appearance of new dehydrin bands around 30 and 20 kDa, and a very faint band around 40 kDa. The protein pattern of dehydrins in *R. serbica* did not change significantly during cold acclimatization and freezing-induced desiccation, despite some faint bands could be detected around 40 and 20 kDa.

Our results confirmed previously obtained data about *R. serbica* and *R. nathaliae* and the role of dehydrins in acquisition of tolerance to low temperatures (Mihailova et al., 2023a). Accumulation of dehydrins in the vegetative tissues is a common mechanism to overcome desiccation after drought and low temperatures of resurrection species from Gesneriaceae family (Mladenov et al., 2022; Pantelić et al., 2022; Georgieva et al., 2022; Mihailova et al., 2023a). Dehydrins are protective proteins stabilizing the cellular macromolecules and acting as ROS scavengers (Graether and Boddington, 2014). Dehydrins decrease the temperature of membrane state transition and stabilize their structure, interact with dehydrated parts of protein molecules and DNA, and are able to bind ROS, thus protecting membrane integrity and cell macromolecules alleviating oxidative damage during drought and low temperature stresses (Sun and Lin, 2010; Thalhammer and Hincha, 2014; Andersson et al., 2020; Szlachtowska and Rurek, 2023). Pantelić et al. (2022) characterized the structure of the LEA proteins and the levels of gene expression of LEA genes of *R. serbica*. Using transcriptome *de novo* assembly the authors annotated 318 LEA proteins and predicted the presence of globular domains in LEA2 group to which dehydrins belongs. According to the authors, *R. serbica* LEA2 proteins have the highest potential of antiradical scavenging. The protein pattern of dehydrins from *R. serbica* and *R. nathaliae* in this study slightly differed from previous results, which may be due to different combinations of dehydrin genes or posttranslational modifications (Vaseva et al., 2014; Mladenov et al., 2022). Xiao et al. (2015) demonstrated that the length and severity of the seasonal dehydration periods of *Boea hygrometrica* reflect the different expression of LEA genes. It was shown previously that some of the dehydrin proteins have ecotype-level differences in *H. rhodopensis* (Mihailova et al., 2023b).

3.4.2. ELIPs

The accumulation and protein pattern of ELIPs (early light-inducible proteins) in *R. serbica* and *R. nathaliae* species throughout exposure to low temperatures was investigated by Western blot (Fig. 12). The protein pattern of ELIPs was similar in both Ramonda plants during cold acclimatization and freezing-induced desiccation. Freezing-induced desiccation increased the relative abundance of ELIP proteins. One main (13-14 kDa) and several minor ELIP bands could be identified and their molecular weight ranged between 13 to 19 kDa in both plants. ELIPs were present also in control plants (15 and 19 kDa bands), but in very low quantity. The latest stages of cold acclimatization period, freezing stress and freezing-induced desiccation increased the accumulation of ELIPs in *R. serbica* and *R. nathaliae*, especially the bands around 13-14 kDa.

ELIPs are chl a/b binding proteins form the LHC superfamily that enhance non-photochemical quenching and avoid photodamage of photosynthetic apparatus (Król et al. 1999). It was suggested that ELIPs maintain low level of free chlorophyll molecules during stress and this chlorophyll was released in the process of degradation of other pigment-binding proteins (Hutin et al., 2003; Skotnicová et al., 2021). Moreover, Liu et al. (2020a) reported that ELIP are related also to the accumulation of carotenoid under high-light stress in the desert moss *Syntrichia caninervis*. Cold acclimatization and freezing-induced desiccation of *R. serbica* and *R. nathaliae* induced the accumulation of several ELIP proteins. This accumulation coincided with the increase of non-photochemical processes in PSII, thus suggesting the protective role of ELIP in photoprotection and freezing tolerance of those resurrection species. Higher protein and transcript content of ELIPs are shown to accumulate during desiccation induced by drought or low temperatures in different resurrection plants (Alamillo and Bartels, 2001; Xiao et al., 2015; Georgieva et al., 2022, 2023, Mihailova et al., 2023a). Previously, we found that ELIP transcript abundance was higher in dried *H. rhodopensis* leaves after freezing-induced desiccation compared to desiccation induced only by drought (Mihailova et al., 2022). A *Chlamydomonas reinhardtii* mutant, overexpressing *ELIP3* gene, survived longer periods of high light stress and low temperatures (Lee et al., 2020). Duplication of ELIP genes in the resurrection plants' genome is confirmed (Xiao et al. 2015; VanBuren et al., 2019). Transcript accumulation of dehydrins and ELIP (Xiao et al., 2015; Yobi et al., 2017), and antioxidants (Yobi et al., 2017) support the important role of those proteins in the protection of photosynthetic apparatus of resurrection plants. It had been demonstrated that the build-up of non-reducing sugars and stress-induced proteins lead to stabilization of macromolecules in the process of dehydration (Crowe et al., 1996; Hoekstra et al.,

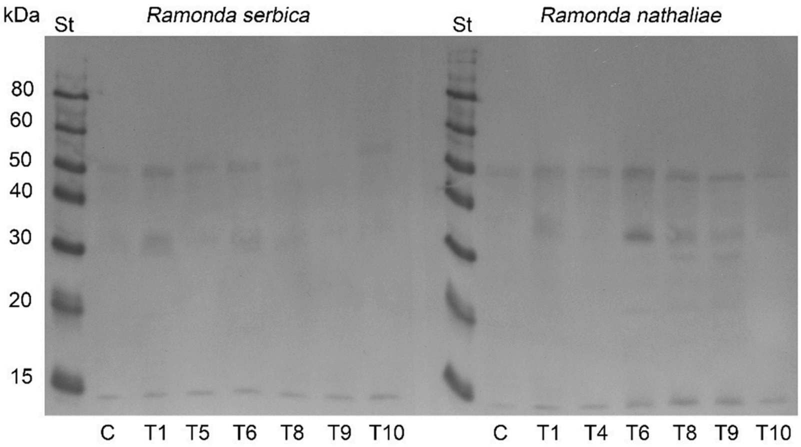

Fig. 11. Protein pattern of dehydrins (K-segment), detected by Western blot in total leaf proteins of *R. serbica* and *R. nathaliae* control plants (C; June) and after cold acclimation (T1, T4; November), exposure to freezing stress (T5, T6; December) and freezing-induced desiccation (T8-T10; January–February) in natural environmental conditions. The average minimum temperatures recorded were as follows: C: 20 to 25°C, T1-T4: 10 to 0°C, T5–T6: 0 to -5°C, and T8–T10: -5 to -10°C. 30 μg protein was applied per lane. St: ROTI®Mark WESTERN PLUS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

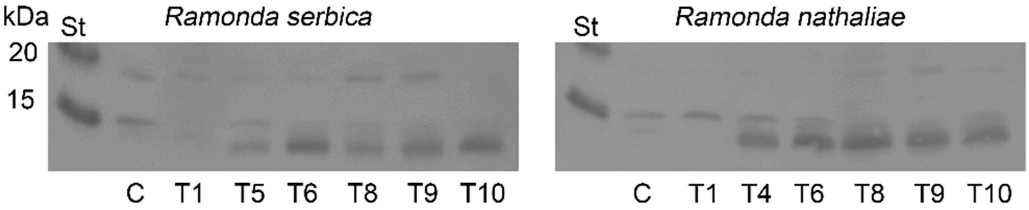

Fig. 12. Protein pattern of ELIPs, detected by Western blot in total leaf proteins of *R. serbica* and *R. nathaliae* control plants (C; June) and after cold acclimation (T1, T4; November), exposure to freezing stress (T5, T6; December) and freezing-induced desiccation (T8-T10; January–February) in natural environmental conditions. The average minimum temperatures recorded were as follows: C: 20 to 25°C, T1-T4: 10 to 0°C, T5–T6: 0 to -5°C, and T8–T10: -5 to -10°C. 30 μg protein was applied per lane. St: ROTI®Mark WESTERN PLUS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2001).

4. Conclusions

This study provides valuable insight into how cold and freezing stress can alter photosynthetic activity and the importance of antioxidant protection of the resurrection plants of *R. nathaliae* and *R. serbica* to cope with cold and freezing conditions in their natural habitats. The ability of these plants to desiccate as a result of freezing stress and survive harsh winter conditions in a dry state in their habitats has been confirmed. Our results indicated that during cold acclimation and freezing-induced desiccation, damage caused to the photosynthetic apparatus in *R. nathaliae* and *R. serbica* remained at a repairable level, with physiological integrity preserved due to elevated antioxidative mechanisms. At low and subzero temperatures both plants exhibited a sharp decline in net photosynthetic rate (A), resulting in an accumulated intercellular CO_2 concentration. To cope with cold and freezing stress Ramonda plants were able to reduce their stomatal conductance by closing their stomata, decreasing their transpiration rate thus enhancing their water use efficiency. This response suggests their enhanced mechanisms to conserve water while maintaining essential metabolic functions. The reduction of the quantum yield of PSII indicates a protective measure to avoid overexcitation and photodamage. The enhancement of non-photochemical quenching during cold acclimatization was crucial in protecting PSII from photoinhibition, while during freezing-induced desiccation the proportion of non-regulated thermal energy dissipation of excessed excitation energy increased. The reduction in the amount of core proteins of photosystem II and photosystem I, and the enhancement of some light-harvesting complex (LHC) proteins confirmed the obtained fluorescent data. An enhanced acumulation of antioxidants and protective proteins during freezing stress and freezing-induced desiccation are some of the strategies used by *R. serbica* and *R. nathaliae* to survive stress conditions. Understanding the mechanisms of resurrection plants to survive cold and freezing stress may reveal agricultural and ecological strategies aimed at mitigating the effects of climate change on global vegetation.

The future perspective of these model plant species must focus on analyzing their metabolomics and exploring their potential use as a priming agent (biostimulants) for crop plants to enhance resistance to drought and low temperatures.

CRediT authorship contribution statement

Fitim Kastrati: Writing – original draft, Methodology, Formal analysis. **Bekim Gashi:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation. **Gergana Mihailova:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Katya Georgieva:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Eva Popova:** Writing – review & editing, Formal analysis. **Erzë Çoçaj:** Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.100741.

Data availability

No data was used for the research described in the article.

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