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DEHYDRATION-RELATED CHANGES OF PEROXIDASE AND POLYPHENOL OXIDASE ACTIVITY IN FRONDS OF THE RESURRECTION FERN *ASPLENIUM CETERACH* L.

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Abstract - Asplenium ceterach belongs to a group of poikilohydric ferns and it can recover uninjured from an almost completely dehydrated state. In our study, short term dehydration (24h) at four different water potentials, resulted in moderate water loss (partial desiccation) in fern tissue. The main phenolic acids represented in *A. ceterach* were chlorogenic (CGA) and caffeic acid (CA) and their content decreased during the dehydration process. For the first time, peroxidase (POD) and polyphenol oxidase (PPO) isoforms were determined in the rustyback fern. The results exhibit the presence of numerous anionic POD isoforms, with pI ranging from 4.4 to 5.8, but none of the cationic isoforms was detected. Two PPO isoforms were identified, one anionic with pI 6.3 and one cationic with pI of about 9.0. Short-term dehydration brought about a remarkable increase in POD and PPO activity using CGA as a substrate. Changes in enzyme activity and content of substrates during dehydration may play an important role in the adaptation of the rustyback fern to water deficit, and increase the overall plant resistance to stress conditions.

Key words: Asplenium ceterach, dehydration, peroxidase, polyphenol oxidase

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INTRODUCTION

Certain plant species, termed desiccation-tolerant or resurrection plants, have evolved the remarkable ability to withstand extreme dehydration and rapid rehydration of vegetative tissues without cell damage. The mechanisms by which such rapid and ecologically beneficial changes in cellular activity are achieved in resurrection plants are not well understood. The number of environmental stresses, including water-deficit stress, could disrupt normal metabolism in plants and cause (initiate) oxidative damage as a secondary effect (Smirnoff, 1993). Oxidative stress is a result of a drying-induced disturbance of electron transport in chloroplasts and mitochondria accompanied by the production of reactive oxygen species (ROS), and therewith lead to cell and tissue damage. However, desiccation-tolerant plants have a well-developed defense system that involves the reduction of metabolic activities, thereby slowing down the production of ROS (Navari-Izzo et al., 1997; Sgherri et al., 1996). In angiosperms these protection mechanisms, among the rest, include controlled loss/retention of chlorophyll (Proctor & Tuba, 2002; Farrant et al., 2003) and the accumulation of various antioxidant protectants for the quenching the ROS (Kranner & Birtić, 2005). Thus, plant cells utilize an integrated system of enzymatic and non-enzymatic antioxidants to ensure the efficient removal of ROS under both normal and adverse environmental conditions (Grace et al., 1998).

Plants also contain a number of natural secondary products with antioxidant properties, including different phenolics (Larson, 1995, Rice-Evans et al., 1997). There is no universal pattern for phenolic compound activity during the dehydration of different plants and organs (Bagniewska-Zadworna et al., 2007). Enzymes, such as polyphe-

nol oxidase (PPO) and peroxidase (POD), may oxidize phenolics and thus take part in the regulation of the phenolic concentration in plants. Recent studies have also indicated that phenol oxidizing enzymes may participate in the response to various abiotic stresses including drought (Sofo et al., 2005; Veljović-Jovanović et al., 2006, 2008). PPO is a copper-containing protein widely distributed in the plant kingdom that catalyzes the oxygen-dependent oxidation of monophenols or *o*-diphenols to *o*-quinones. The *o*-quinones are highly reactive substances that can react with amino acids, peptides and proteins, thus altering the structural and functional properties of the cell. This enzyme has been implicated to the function in tissue browning (Coetzer et al., 2001; Gandia-Herrero et al., 2003), lignifications (López-Serrano et al., 2004, Gabaldón et al., 2005) and the defense mechanism against insects and plant pathogens (Mohammadi & Kazemi, 2002, Pinto et al., 2008). POD is a widely distributed plant enzyme with various physiological functions in plant cells, including the browning and discoloration in fruit and vegetables (Jiménez-Atiénzar et al., 2007), auxin metabolism (Schopher et al., 2002) and defense against numerous abiotic stresses (Bacardijeva et al., 1996; Dragišić Maksimović et al., 2007; Morina et al., 2008). Both phenolics and POD might be involved in the elimination of the reactive oxygen species (ROS) within the peroxide/phenols/ascorbate system during dehydration (Takahama & Oniki, 1997; Sgherri et al., 2003, 2004).

Desiccation tolerance is a common property of bryophytes and lichens, but very rare among angiosperms (Alpert & Oliver, 2002). Pteridophytes include almost 70 tolerant species, yet the mechanisms of their desiccation tolerance have only been investigated in a limited way (Oliver et al., 2000). The rustyback fern (*Asplenium ceterach* L.) belongs to the resurrection species and it is widespread in Western and Central Europe, including the Mediterranean region. The adult fern (sporophyte) is a perennial herbaceous rosette-like plant with leathery fronds, which grows in rock crevices and stone walls and may survive long dry periods between wet spells, passing quickly from anabiosis to full biological activity. It was recently shown that the fronds of the rustyback fern sporophyte contain an unusually large amount of phenolics (Živković, unpublished data) in comparison with other desiccation-tolerant plants (Sgherri et al., 2004; Farrant et al., 2007).

The results concerning dehydration induced changes in the enzyme activity and content of enzyme substrates and products are ambiguous and varied among plant species and conditions (Fazeli et al., 2007; Veljović-Jovanović et al., 2008). Furthermore, there is a striking lack of data in the literature concerning the desiccation tolerance in the rustyback fern (Schwab & Heber, 1984; Schwab et al., 1989; Živković, 2009). For the first time, the relation between the dehydration-induced changes of phenolics and phenolic oxidizing enzymes in *A. ceterach* has been investigated. In the present study we evaluated the comparative data on POD and PPO activities in the resurrection rustyback fern during short-term dehydration. The possible role of phenolics in the tolerance to dehydration has been discussed.

MATERIAL AND METHODS

Plant material

Mature sporophytes of rustyback fern (*Asplenium ceterach* L.) were collected during September 2008 in Herceg Novi (Montenegro) and grown in a greenhouse of the Institute for Biological Research "Siniša Stanković", Belgrade (Serbia).

Relative water content (RWC)

Discs (6 mm in diameter) were cut from fully expanded fronds and subjected to 24 h dehydration in an aqueous solution of PEG-8000 with different water potential: -1, -2, -3, and -4 MPa, respectively (calculated by software package according to Mitchell, 1983). The fresh (FW), turgid (TW) and dry weight (DW) of the discs was determined and the RWC was calculated using the equation: [RWC = (FW - DW) / (TW - DW) x 100]. TW was measured after disc incubation in deionized water for 24 h at 25°C. DW was determined after a 24 h lyophilization of the frond discs.

After treatment the plant material from several trials (twenty replicates each from three independent experiments) was thoroughly washed with distilled water and stored at -70°C until further use.

Determination of phenolic compounds

Lyophilized plant material was powdered in a mortar with liquid nitrogen and total phenolics were extracted with 80% methanol. After centrifugation at 10000x*g* for 10 min at 4°C the supernatant was collected and filtered (Econofilter, pore size 0.45 μm, Agilent Technologies, Germany) prior to analysis.

Qualitative and quantitative analysis of phenolics was performed on a Hewlett-Packard HPLC system, model 1100 with DAD, using Hypersil BDS-C18 (5 μl), 125 x 2 mm I.D. The mobile phase contained 0.1 % phosphoric acid and acetonitrile (Acros Organics, Geel, Belgium). Phosphoric acid (A) and acetonitrile (B) were applied in elution gradient as follows: 7.5% B (0.00 min), 20% B (20.00 min), 25% B (25.00 min), 7.5% B (30.00 min). The flow rate was 0.500 ml min^{-1} and phenolics were detected at different wave-lengths (210, 266, 310 and 326 nm). Quantification was performed using a standard curve prepared with five different concentrations of standard mixtures containing coniferyl alcohol, Gallic, chlorogenic, caffeic, *p*-coumaric and ferulic acid (Sigma-Aldrich, Oakville, ON, Canada). Chromatogram analysis was performed by HP Chemstation chromatographic software (Palo Alto, CA, USA).

Protein extraction

Plant tissue was grinded in a mortar and pestle in liquid nitrogen to a fine powder, and extracted in 100 mM potassium-phosphate buffer (pH=6.5) supplemented with a 20 μl ml^{-1} protease inhibitor cocktail for plant tissue extracts (Sigma-Aldrich, USA) and 10% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15000x*g* for 20 min at 4°C. Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

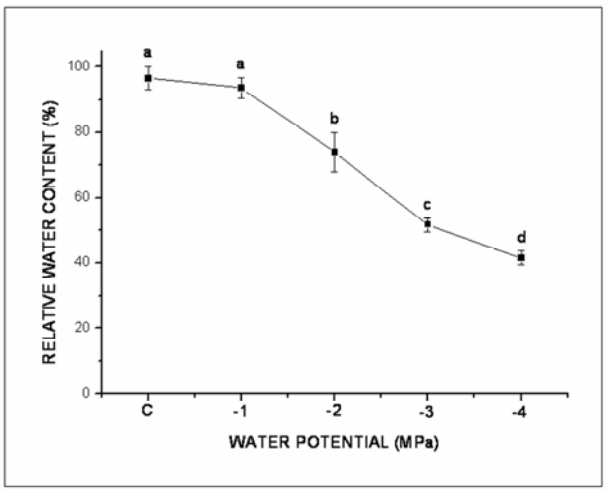

Fig. 1. Relative water content (RWC) in frond discs of *A. ceterach* subjected to short-term dehydration (24h). The water potential of the PEG solution ranged from Ψ= -1 to -4 MPa, respectively. Results are mean ± standard error from forty replicates of three different experiments. Means followed by different letters are significantly different at p≤0.05.

Electrophoresis and enzyme assays

Proteins were separated by native PAGE and isoelectrofocusing (IEF) to determine POD and PPO isoforms. Native electrophoresis was performed on 5% stacking and 10% separating gel, with a reservoir buffer containing 0.025 M Tris and 0.192 M Glycine (pH=8.3) at 120 V for 120 min. IEF was performed on 7.5% polyacrylamide gel with 3% ampholyte on a pH gradient from 3 to 9. The protein amounts applied to each well were 20 μg or 40 μg for POD or PPO analysis, respectively. To determine POD activity the gels were incubated in a 50 mM potassium phosphate buffer (pH=6.5) containing 10% 4-chloro-α-naphthol and 0.03% H_2O_2. PPO activity was visualized by staining the gels with 0.1% L-DOPA in a 100 mM potassium-phosphate buffer (pH=8.5). Relative band intensities were estimated using Image J, Version 1.32j (USA).

Spectrophotometric enzyme assays were carried out according to Jiménez-Atiénzar et al. (2007) with some modifications. The POD activity was measured using chlorogenic acid (CGA, A_{400} ε=0.01 $mM^{-1}cm^{-1}$)

and H_2O_2 as the hydrogen donors. The reaction mixture contained a 50 mM sodium acetate buffer (pH 5.0), 0.5 mM H_2O_2 and 2 mM CGA. The activity of PPO was determined using the substrate CGA by measuring the initial rate of the absorbance change at 410 nm. The assay medium contained 2 mM CGA in a 50 mM potassium phosphate buffer (pH 6.5). One unit (U) of PPO was defined as 0.001 absorbance change per minute in a 1 ml reaction volume.

Statistical analysis

Statistical analysis was performed using Stagraphics Centurion XV, Version 15.1.02 (StatPoint, Inc. 1982-2006, USA). The data were subjected to analysis of variance (ANOVA), using a multiple range test calculated at a confidence level of $p \leq 0.05$. Final graphical processing of data was performed using Origin 7.0 (OriginLab Corporation 1991-2002, USA).

RESULTS

The frond discs of rustyback fern were subjected to a PEG solution with different water potentials, according to Gibon et al. (2000). After 24 h of dehydration, in relation to the intensity of ***stress applied***, the mean relative water content (RWC) changed from 96.5% in fully hydrated specimens (control) to 41.5% at a water potential of - 4 MPa (Fig. 1).

HPLC analysis provided evidence that CGA is the main phenolic compound in the fronds of *A. ceterach* (Fig. 2A). All extracts tested showed one major peak with a retention time of 4.0 min. This peak accounted for more than 90% of the total peak area in the HPLC profile of rustyback fern extracts. During dehydration treatment the CGA content significantly decreased from 9.5 mg g^{-1} DW at a water potential of ψ=-1 MPa to 5.3 mg g^{-1} DW at a water potential of ψ=-4 MPa, showing an approximately 2-fold decrease compared to the control (Fig. 2B). Other phenolic acid - caffeic acids (CA), were found in a small amount, about 45-fold less than CGA (Fig. 2B insert). The content of CA also changed with dehydration reaching a 2-fold greater decrease as compared to the control during the dehydration process, with no significant differences between the single treatments.

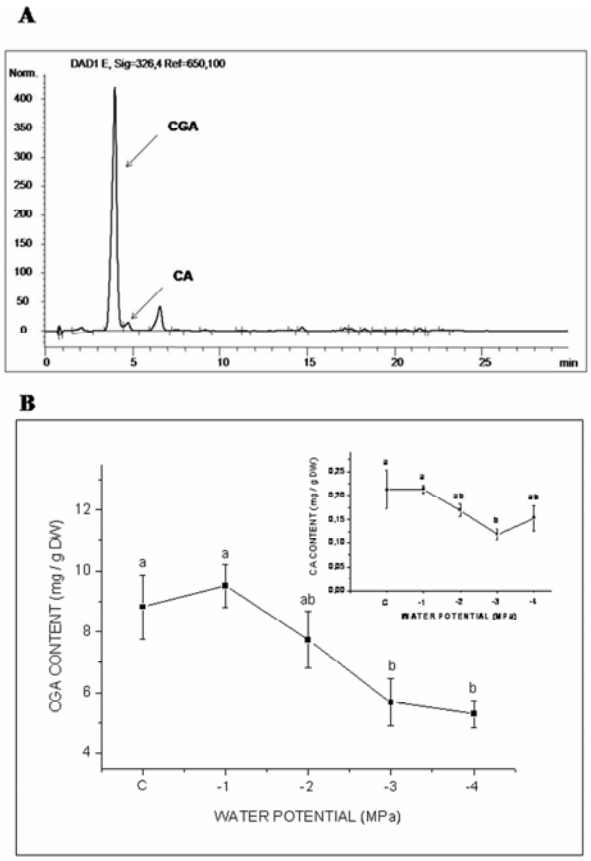

Fig. 2. HPLC analysis of methanol extracts of *A. ceterach* frond discs subjected to short-term dehydration. (A) HPLC chromatogram of methanol extracts. The peaks corresponding to chlorogenic acid (CGA) and caffeic acid (CA) are indicated with arrows. (B) The content of the most represented phenolic acid-CGA in methanolic extracts of frond discs. Inset shows changes in CA content during short-term dehydration. Values are means ± standard error from forty replicates of three different experiments. Letters represent significant difference at $p \leq 0.05$.

In order to characterize POD isoforms, IEF analysis was conducted. Bands were visualized using 4-chloro-α-naphthol and H_2O_2 as enzyme substrates. Separation of POD isoforms by their pI values revealed the presence of numerous anionic isoforms ranging from 4.4 to 5.8, and several distinct bands at pI 4.9, 5.3, 5.4 and 5.7 (Fig. 3A).

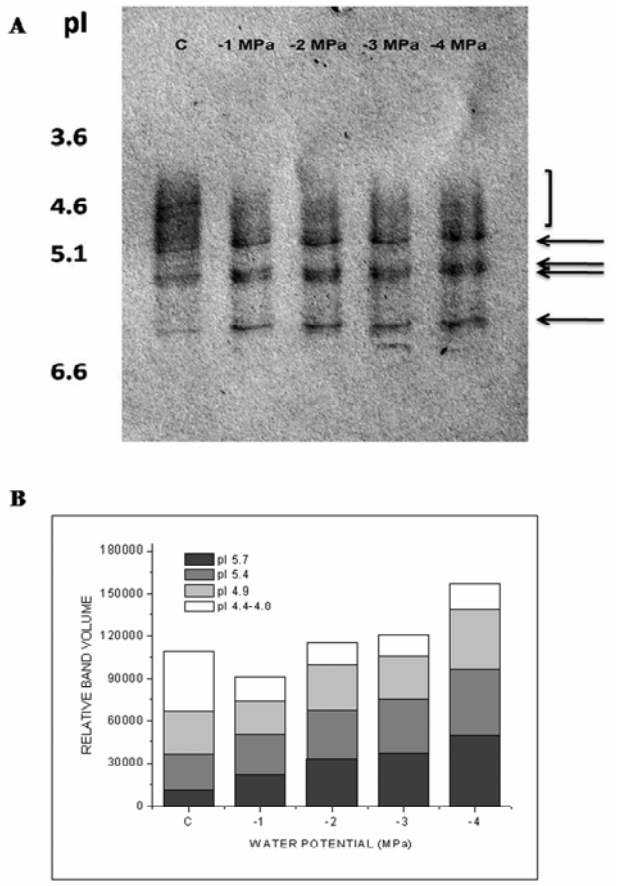

Fig. 3. (**A**) Isoenzymatic pattern of soluble POD in *A. ceterach* frond discs during dehydration. IEF was performed in a pH gradient of 3-9. Arrows indicate POD isoforms with different pI values. An amount of 20 μg of total proteins was applied to each well. (**B**) Relative band volume of POD isoforms.

However, cationic isoforms of POD was not identified in the fronds of the rustyback fern after short-term dehydration. The activity of pI 4.9, 5.3, 5.4 and 5.7 isoforms increased during all treatments, while the group of isoforms ranging between pI 4.4 and 4.8 showed higher activity under control conditions (Fig. 3B). Isoelectrofocusing showed that the higher total POD activity was a result of the accumulation of existing isoforms during dehydration, rather than the induction of new forms of the enzyme.

POD activity was further analyzed spectrophotometrically using CGA, a major natural phenol found in *A. ceterach*. A significant increase in activity was observed in all dehydration treatments, showing a 1.9-fold rise at the water potential ψ=-3 MPa compared to the control (Fig. 4).

PPO isoform characterization was performed by IEF using L-DOPA as a substrate for band visualization. Two PPO isoforms were detected: an anionic form with pI 6.3 and a cationic form with pI of ≈ 9.0 (Fig. 5A). While the number of PPO isoforms remained unchanged in treatments compared to the control, IEF analysis revealed a significant increase in the PPO activity of both isoforms after short-term dehydration (Fig. 5B). Total PPO activity was 8- to 12-fold higher in the treatments compared to the control.

CGA was used as a substrate for the spectrophotometric measurement of PPO activity. An increase in enzyme activity was detected during short-term dehydration at water potentials lower than -1 MPa, reaching the highest level at -2 MPa (Fig. 6).

DISCUSSION

Desiccation tolerance in the active stages of the life cycle in plants is both rare and widespread (Alpert, 2000). Among vascular plants a small group of angiosperms known as poikilohydric or resurrection plants can tolerate extreme dehydration (Gaff, 1987), while desiccation-tolerant gymnosperms are not known at all. On the other hand, many lichens, bryophytes and ferns can survive in a dried state. According to Oliver and colleagues (2000) approximately 60 to70 species of pteridophyte belong to resurrection plants. Proctor and Tuba (2002) mention 21 genera of DT ferns, including *Asplenium*. *Asplenium ceterach* belongs to a group of poikilohydric ferns and could sustain different rates of desiccation, recovering uninjured from complete dryness (Schwab et al., 1989). It always grows under suitably humid conditions (especially high air humidity) in its habitat, and becomes quiescent when water is unavailable.

In our study, the frond discs of the rustyback fern were dehydrated for 24 h at several different

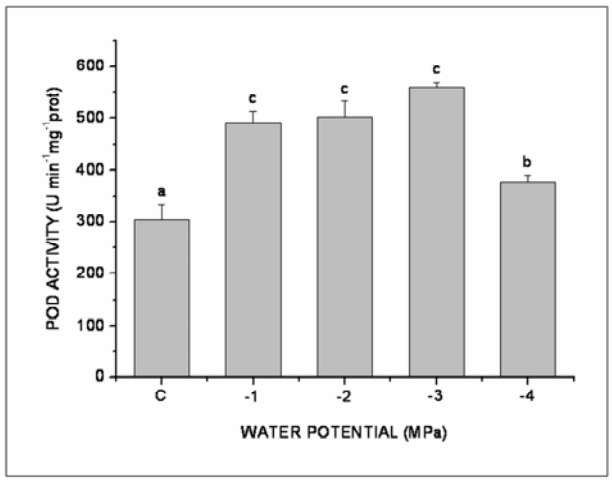

Fig. 4. Activity of soluble POD in *A. ceterach* frond discs during short-term dehydration. CGA was used as an electron donor, and absorbance change was measured at 400 nm. Results are mean ± standard error from forty replicates of three different experiments. Means followed by different letters are significantly different at p≤0.05.

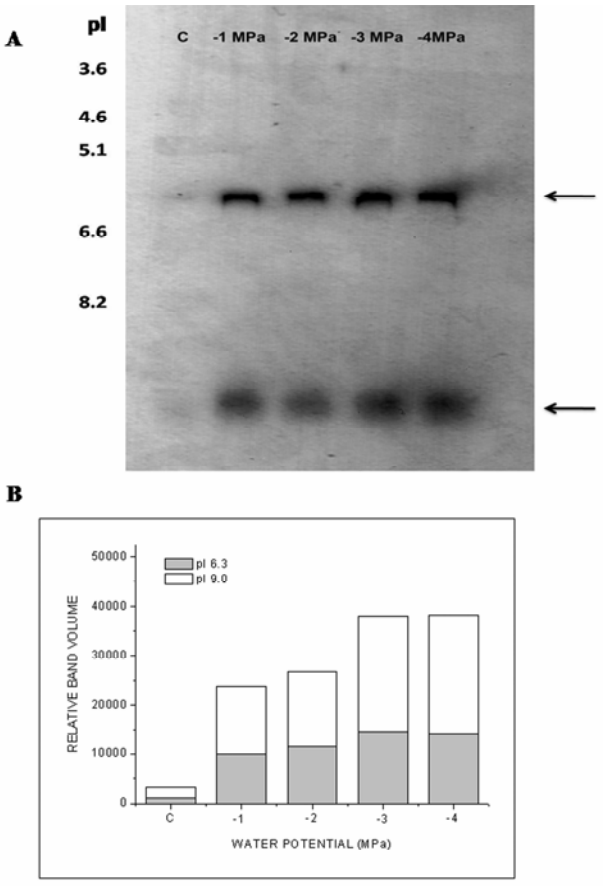

Fig. 5. (**A**) PPO isoenzyme pattern in *A. ceterach* frond discs during short-term dehydration. Isoelectrofocusing was done in a pH gradient of 3-9. An amount of 40 μg of total proteins was applied to each well. PPO isoforms with different pI values are indicated with arrows. (**B**) Relative band volume of PPO isoforms.

water potentials. During exposure to dehydration the water content in the fronds of *A. ceterach* decreased gradually, reaching almost 40% (Fig. 1), which suggests that short term dehydration induces a relatively moderate water loss in plant tissue (partial desiccation), even at ψ=-4 MPa. Previous studies on electrolyte leakage in the rustyback fern during short-term dehydration indicated that relatively moderate water stress results in cellular reaccommodation to water loss (Stevanović et al., 1997). It may be explained as plasma membrane stability during the initial phase of water loss in the fronds of *A. ceterach*. Similar results have been obtained in other fern species, i.e. slowly drying fronds of *Polypodium virginianum* (Bewley et al., 1993) and desiccation-tolerant fronds of *Mohria caffrorum* (Farrant et al., 2009).

Dehydration resulting from water deficit could directly lead to the enhanced generation of reactive oxygen species (ROS). Although oxidative stress is a primary component of desiccation-induced cellular injuries, plants possess very efficient antioxidant systems that control the level of ROS and protect cells under stress conditions. Plant tissue contains a number of enzymes that scavenge ROS (superoxide dismutase, catalase, peroxidases and glutathione peroxidase) or participate in the detoxification of lipid peroxidation products (glutathione S-transferases, phospholipid-hydroperoxide glutathione peroxidase and ascorbate peroxidase), and a network of low molecular mass oxidants (ascorbate, glutathione, tocopherols and phenolic compounds) (Blokhina et al., 2003). It is known that phenolic acids are synthesized in plants as a response to various stresses and that they are stored primarily in the vacuole or in the apoplast, playing either a signaling or direct role in defense. Direct defense includes

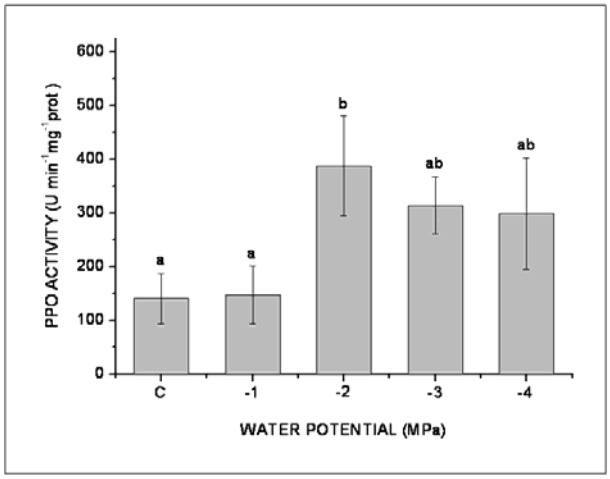

Fig. 6. PPO activity in *A. ceterach* frond discs during dehydration. CGA was used as substrate, and the initial rate of the absorbance change was measured at 410 nm. Values are means ± standard error from forty replicates of three different experiments. Letters represent significant difference at p≤0.05.

protection from injurious UV radiation, deterrence of grazing animals and feeding insects (because of their astringent, toxic nature), resistance to pathogens etc. (Beckman, 2000). Furthermore, reduced forms of phytophenolics are powerful antioxidants equivalent to ascorbate (Sakihama et al., 2002). Although the antioxidant activity of fern phenolics has been intensively studied (Garcia et al., 2006; Chang et al., 2007; Ding et al., 2008), there are only a few data relating to the composition and antioxidant role of phenolics in *A. ceterach.*

Đurđević and coworkers (2007) concluded that the fronds and rhizome of the rustyback fern contain much larger amount of phenolic compounds than *Asplenium trichomanes* L. and *Asplenium adiantum-nigrum* L. Furthermore, the content of phenolics in *A. ceterach* fronds was unusually elevated in comparison to other resurrection plants (Sgherri et al., 2004; Moore et al., 2005). CGA, an ester of caffeic and quinic acid, is one of the most common phenylpropanoid metabolites in vascular plants (Mølgaard & Ravn, 1988) and the main phenolic acid in *A. ceterach* fronds (Fig. 2A). During dehydration the CGA content declined approximately 2-fold in the fronds that were exposed to the water potential ≤ -3 MPa (Fig. 2B). These dehydration-related changes of CGA in the fronds of *A. ceterach* imply a possible role of this phenolic acid in anti-oxidative defense and adaptation to water deficit in the rustyback fern. Furthermore, the contribution of a less abundant phenolic acid, i.e. caffeic acid, in *A. ceterach* oxidative protection should not be omitted. A wide range of biotic and abiotic stresses could effect a change in the level of CGA in a variety of plant tissues (Dixon & Paiva, 1995). The elevated levels and polyhydroxy nature of this phenolic acid suggest that it might be of great importance in the free radical scavenging processes during dehydration and at the start of rehydration, when oxidative stress increases due to the recovery of the metabolism in plants (Sgherri et al., 1994a, b). Also, the oxidation products of phenolic compounds appear to be involved in the defense of plants against phytopathogens, including fungi, bacteria and viruses (Friedman, 1997).

Considering the decline in the amount of CGA and the less abundant phenolic acid - CA during short-term dehydration, our research focused on the enzymes involved in their oxidative catabolism, namely POD and PPO. CGA is considered to be the endogenous reductant for class III PODs, which use phenolics as preferential electron donors (Kukavica & Veljović-Jovanović, 2004; Takahama et al., 1999). Class III POD activity in *A. ceterach* was induced during dehydration (Figs. 3 and 4) and mostly correlated with the decline in phenolic acid abundance. Similar results have been reported for the resurrection plant *Ramonda serbica* Panc. Since the activities of POD increased during dehydration, the authors hypothesized that the oxidation process was responsible for the decline in phenolic acids content (Sgherri et al., 2004; Veljović-Jovanović et al., 2006).

PODs consist of families of isoenzymes that perform different physiological functions in plant cells (Gaspar et al., 1985) and comprise both basic and acidic isoforms. Acidic isoforms are located in the cell wall free spaces, while basic forms with pI > 9.0 are detected in the vacuoles (Escribano et al., 2002).

Taking this into consideration, POD is thought to be involved in the oxidation of the cell wall and vacuolar phenolics, thus participating in the strengthening of the cell wall and the turnover and degradation of phenolic compounds (Jiménez-Atiénzar et al., 2007). Our results revealed the presence of a number of acidic POD isoforms with pI in a range from 4.4 to 5.8 in *A. ceterach* fronds, but none of the basic isoforms. Interestingly, short term dehydration did not induce new POD isoforms, but promoted the accumulation of pI 5.7, 5.4 and 4.9 isoforms. Isoforms in the range of 4.4 - 4.9 were more abundant in the control samples. On the basis of these results it can be assumed that the anionic POD isoforms could participate in protecting cellular constituents by oxidizing the phenolics in fern fronds. It has been proposed that PODs could act as an efficient H_2O_2 scavenging system in plant vacuoles in the presence of phenolics and reduced ascorbate; phenolics are oxidized to phenoxyl radicals which can be reduced by ascorbate (Zancani & Nagy, 2000, Sgherri et al. 2003). This cycle can occur in both the apoplast and in the vacuole, where phenolics are particularly concentrated (Santiago et al., 2000).

On the other hand, in the resurrection angiosperm *Ramonda serbica* dehydration induced cationic and POD isoforms with a lower molecular weight, while anionic isoforms with pI 4.5 were induced in senescent leaves (Veljović-Jovanović et al., 2006). It has been proposed that the different POD isoforms induced under dehydration and senescence could have different physiological roles where anionic ones are responsible for cell wall lignifications.

While POD is mainly localized in the epidermal and vascular cells of leaves and stems (Ros Barceló et al., 2003), in green leaves a considerable part of PPO activity is localized in the chloroplasts. The isoforms of PPO were reported mostly within the range of 35-70 kDa in plants (Yoruk & Marshall, 2003). Using the IEF technique a variety of mainly anionic PPO isoforms has been described. When PPO was purified from the chloroplasts of broad bean leaves, all four detected isoforms were with pI values ranging from 4.9 to 5.9 (Hoyle, 1977). Marri et al. (2003) determined in potato one diffuse single band with a pI of 6.5, and peach PPO showed an acidic isoform pattern (Jiménez-Atiénzar et al., 2007). When *A. ceterach* frond proteins were subjected to native IEF, a basic isoform of pI ≈ 9 was detected besides the prominent anionic form with pI value 5.5. An *in gel* assay with L-DOPA revealed a significant increase in the activity of both PPO forms during dehydration (Fig. 5). An increase in total PPO activity was also detected by spectrophotometric assay when CGA was used as the enzyme substrate (Fig. 6). Similar results were obtained in the resurrection species *Ramonda serbica* during prolonged dehydration, where the total PPO activity rose 4-fold, mainly due to the anionic isoform with pI 4.8 (Veljović-Jovanović et al., 2008). The authors concluded that the oxidation of CGA by molecular oxygen catalyzed by PPO is the preferred reaction in the leaf extract of resurrection angiosperm *R. serbica* during desiccation. We could speculate that the increase in PPO activity and the possible accumulation of the oxidative products of phenolic compounds represent part of the general defense strategy of *A. ceterach* against different biotic and abiotic stresses, including water stress.

In conclusion, the presented results indicate that short-term dehydration in the fronds of *A. ceterach* may induce changes in phenolic acid metabolism by increasing the POD and PPO activity. To our knowledge this is the first attempt to analyze POD and PPO isoform profiles in the rustyback fern. Changes in the enzyme activity and content of substrates during dehydration may play an important role in the adaptation of the rustyback fern to water deficit, and increase the overall plant resistance to stress conditions. Examination of the activity and possible role of these enzymes during long-term dehydration and the rehydration process will be a course of our further work.

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