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Senescence- and drought-related changes in peroxidase and superoxide dismutase isoforms in leaves of *Ramonda serbica*

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Abstract

***Ramonda* sp. (Gesneriaceae) is an endemic and relic plant in a very small group of poikilohydric angiosperms that are able to survive in an almost completely dehydrated state. Senescence- and drought-related changes in the activity of peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (EC 1.11.1.11), and superoxide dismutase (SOD; EC 1.15.1.1) were determined in leaves of different age and relative water content. The results indicate that different POD isoforms were stimulated during senescence and dehydration. Two of the soluble POD isoforms were anionic with pI 4.5, and two were cationic with pI 9.3 and 9.0. The activity of ascorbate peroxidase remained unchanged either by drought or senescence. For the first time, SOD isoforms have now been determined in this resurrection plant. Several SOD isoforms, all of the Mn type, were found to be anionic with pI 4 and a few others had pI from 5 to 6, while one band of FeSOD with a lower molecular weight was neutral. Rehydration brought about a remarkable decrease over the first hour in the activity of all the antioxidant enzymes examined but activity recovered 1 d after rehydration. The results confirmed that dehydration and senescence caused disturbance in the redox homeostasis of *Ramonda* leaves, while inducing different POD isoforms. A physiological role of peroxidase reaction with hydroxycinnamic acids in conservation and protection of cellular constituents of desiccated *Ramonda* leaves is suggested.**

Key words: Desiccation, peroxidase, *Ramonda*, senescence, superoxide dismutase.

Introduction

Water deficit, like other abiotic and biotic stresses in plants, may disturb the redox homeostasis and may even lead to oxidative stress under severe conditions (Winston, 1990; Price and Hendry, 1991; Quartacci and Navari-Izzo, 1992; Vertucci and Farrant, 1995; Navari-Izzo *et al.*, 1995). Peroxidase (POD) and superoxide dismutase (SOD) constitute the first line of defence against reactive oxygen species (ROS), and changes in their activity and amounts have been identified as an indicator of a redox status change under drought conditions in homoiohydric plants (Moran *et al.*, 1994; Schwanz and Polle, 2001). Under prolonged harsh environmental conditions, most plants that are homoiohydric or desiccation intolerant get irreversibly damaged, which leads to early senescence and sudden death. Less than 1% of flowering plants belong to a group known as 'resurrection' plants (Porembski and Barthlott, 2001), which are able to survive by maintaining metabolic functions in an almost completely dehydrated state, and then to recover their activity readily upon rehydration (Gaff, 1989). The capacity to recover, based on an alteration in gene expression that becomes evident upon rehydration, is especially strong and extremely rapid in poikilohydric desiccation-tolerant plants. Investigation of this unique adaptation mechanism to water deficit, developed through dehydration and recovery of cell

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constituents after a state of anabiosis, may give new insights into the process of water stress in plants or even help improve drought resistance in crops. Over the years it has also become clear that free-radical scavenging systems are important components in the mechanisms of drought and desiccation tolerance (Navari-Izzo *et al.*, 1997; Ingram and Bartels, 1996; Noctor *et al.*, 2002). *Ramonda serbica* Panc. (Gesneriaceae) belongs to a very small group of homoiochlorophyllous and poikilohydric angiosperms of the northern hemisphere that includes five or six species (families Gesneriaceae and Cyperacea). *Ramonda* sp. is an endemic and relic plant of the tertiary period originating from the Balkan peninsula, and is a resurrection, perennial, herbaceous, and shade-adapted species. Studies of the metabolism of *Ramonda* sp. plants under dehydration and subsequent rehydration (Augusti *et al.*, 2001; Quartacci *et al.*, 2002) have indicated a role of oxidative processes in impairment of photosynthesis and plasma membrane structures. Despite many studies of the distribution and activity of isoforms of antioxidative enzymes in other plants (Polle *et al.*, 1994; Bernards *et al.*, 1999; Schwanz and Polle, 2001), no analyses have so far been made of POD and SOD isoform profiles in *Ramonda* sp. plants. Accumulation of ROS during senescence (Leshem, 1981; Thomson *et al.*, 1987; Mahalingam and Fedoroff, 2003) has been shown to accompany programmed cell death, implicating the impairment of cellular antioxidant defence (Kar and Feierabend, 1984; Jiménez *et al.*, 1998). Although changes in the activities of ascorbate peroxidase (APX), POD, SOD, and catalase, during senescence of homoiohydric plants have been reported (Pastori and del Río, 1994; del Río *et al.*, 1998; Prochazkova *et al.*, 2001; Kukavica and Veljovic-Jovanovic, 2004), there is no clear evidence as yet of a physiological role of these enzymes in senescence. A correlation between desiccation- and senescence-induced changes in antioxidative metabolism has been reported, showing that some desiccation-tolerant plants may lose tolerance with ageing (Gaff and Giess, 1986; Gaff, 1989). For the first time, senescence-related changes in the antioxidative metabolism in one of the resurrection plants have been investigated. The objective of this study was to analyse peroxidase and SOD changes in the resurrection plant *Ramonda serbica*, an attractive model-system plant, in senescence and, upon dehydration and rehydration, to evaluate similarities in antioxidative responses induced under these conditions. In accordance with the widespread classification of peroxidases, the superfamily is subdivided into three classes based on sequence alignments and biological origin (Welinder, 1992). Two classes of peroxidase, i.e. ascorbate peroxidase (EC 1.11.1.11) that belongs to class I and glycosylated unspecific peroxidase (POD; EC 1.11.1.7) belonging to class III, were analysed. It remains to be clarified in future studies whether there is a unique mechanism underlying antioxidative defence, or are there some basic properties conferring such 'broad spectrum' tolerance to oxidative stress.

Materials and methods

Plant material

Specimens of the desiccation-tolerant plant *Ramonda serbica* Pan. & Petrov. were collected from their natural habitat in a gorge near the city of Niš in south-eastern Serbia. Plants were harvested together with the attached layers of soil. After collection, the plants were acclimated for 4 weeks under full watering until the beginning of the experiments. For the senescence study, leaves of different ages, comparable in size, were collected from four plants according to their position in the rosette: the young leaves which were also the smallest in size were taken from the central part, the mature and fully expanded ones from the middle, and the senescent yellowish ones from the peripheral part of the rosette (Fig. 1). After taking samples for the senescence study, the plants were subjected to drought and relative water content (RWC) was measured in leaves sampled from the same plants (Fig. 2). The plants were dehydrated for 2 weeks by withholding water, and were kept under room temperature and ambient photoperiod. After this period, rehydration was induced by spraying the plants every 2 h with water to simulate rainfall and to keep the soil damp. The samples were collected during dehydration and over the first 3 d of rehydration.

Relative water content

Measurements of RWC were taken at regular intervals (every other or third day during dehydration, and at 6 h intervals during the first

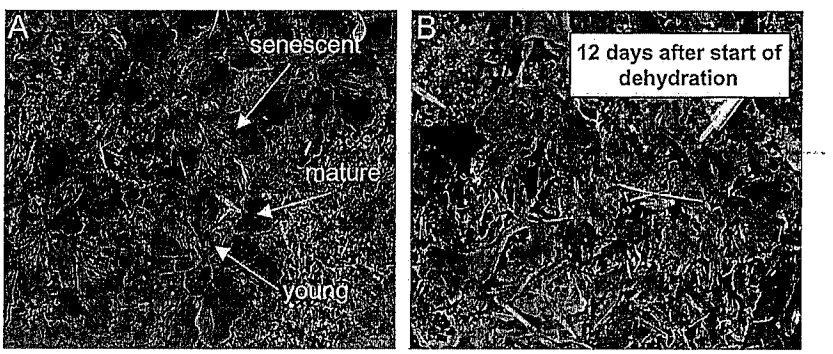

Fig. 1. Effect of dehydratation and rehydration on the phenotype of *R. serbica*: (A) plants with leaves (96% RWC) of different ages identified; (B) plant after 12 d of dehydration.

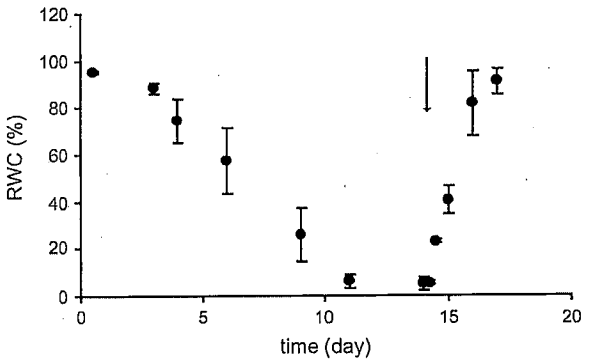

Fig. 2. Relative water content (RWC) in leaves of *R. serbica* subjected to dehydration and rehydration. Results are means ±standard error of four to six separate samples. After 2 weeks of water deficit, plants were sprayed with water to start rehydration (indicated by an arrow).

day of rehydration, and on the second and third days of rehydration) according to a reported procedure (Sgherri *et al.*, 1994*a*). Mature and fully expanded leaves from the middle part of the rosette, comparable in size, were selected for the analyses. RWC of leaves was calculated according to formula: 100×[(fresh weight–dry weight)/(saturated weight–dry weight)].

Protein extraction and measurements of POD activity

For enzyme analysis, leaves taken from the same plant as the one used for RWC determination were weighed and frozen in liquid N_2. Plant material was crushed into powder in a mortar containing liquid N_2 and extracted in 100 mM K-phosphate buffer (pH 6.5), 10 mM ascorbic acid, 2 mM PMSF (phenylmethylsulphonyl fluoride), and 2 mM EDTA with the addition of 5% (w/v) insoluble polyvinylpyrrolidine. The homogenate was centrifuged at 10 000 *g* for 15 min at 4 °C. To determine peroxidase activity, pyrogallol (A_{430}; ε=2.7 mM^{-1} cm^{-1}), guaiacol (A_{470}; ε=2.6 mM^{-1} cm^{-1}), caffeic acid (A_{450}), and ferulic acid (A_{356}) were used as hydrogen donors. In the case of hydroxycinnamates, the rate of peroxidase reaction was measured as an absorbance increase. The K_m values were calculated from Hanes plots for all substrates. The reaction mixture consisted of an aliquot of extract diluted 300-fold and 3.3 mM H_2O_2 in 100 mM K-phosphate buffer (pH 6.5), with 30 mM pyrogallol or a different concentration of reducing substrates. To measure APX activity, absorbance decrease at 290 nm (ε=2.8 mM^{-1} cm^{-1}) was monitored in a reaction mixture consisting of 0.3 mM ascorbate, 0.1 mM H_2O_2, and 50 µl extract in 50 mM K-phosphate (pH 7.2). Estimation of specific enzyme activity was done on a protein basis. Protein content was measured according to Bradford (1976).

Electrophoresis

SDS-PAGE was performed on 12% running gel. Samples (15 µg of proteins) were diluted in loading buffer to final concentrations of 62.5 mM TRIS-HCl, 0.1% (w/v) SDS, 10% (w/v) glycerol, 100 mM DTT, and 0.002% (w/v) bromophenol blue. After heat treatment for 30 min at 100 °C, samples were loaded onto the gels. Protein bands were visualized after staining with CBB (0.1% CBB, 50% methanol, 10% acetic acid). Molecular mass standards (Bio-Rad) were used. Proteins were separated by native PAGE and isoelectrofocusing (IEF) to determine POD and SOD isoforms. Native electrophoresis was performed on 5% stacking and 10% running gel, with a reservoir buffer consisting of 0.025 M TRIS and 0.192 M Gly (pH 8.3), at 24 mA for 120 min. IEF was carried out in 7.5% polyacrylamide gel with 3% ampholyte on a pH gradient of 3–9. Markers for IEF of the pI range 3.6–9.3 were purchased from Sigma (IEF-M1A). The amount of total protein applied to each well was 50 µg for native electrophoresis and 25 µg for IEF. To determine POD activity, the gel was incubated with 10% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM K-phosphate buffer (pH 6.5). Determination of SOD activity on the gels was performed according to Beauchamp and Fridovich (1971). After incubation in reaction mixture (0.1 M EDTA, 0.098 mM nitroblue tetrazolium, 0.03 mM riboflavin, and 2 mM TEMED in K-phosphate buffer, pH 7.8) for 30 min in the dark, the gel was washed in distilled water and illuminated. Different SOD isoenzymes (CuZn, Mn, and FeSOD) were identified by preincubation with either 5 mM KCN or 5 mM H_2O_2 before staining. Relative band intensities were estimated, measuring density with Total Lab.

Statistical analysis

Data were analysed with Mann–Whitney *U* test. Comparison tests were used to determine levels that differed significantly (P <0.05).

Results

Changes in soluble proteins and peroxidase

Throughout the dehydration–rehydration cycle, leaves of the fully hydrated control plants showed no significant differences in RWC or enzyme activities. For this reason the data relative to control plants represent the mean value of the control plants at each harvest time. The mean RWC decreased from 97% in fully hydrated leaves of control plants to 7% in the dried ones (Fig. 1). The rate of water loss was quite low over the first 3 d, RWC decreasing only by about 3% each day. Soon the rate of water loss increased so that, after the next few days of withholding water, the RWC was lowered to 58–60%, and in the following 5 d it decreased further down to 7% (Fig. 2). After 12 h of rehydration, which took place after 4 d, the leaves quickly regained RWC and recovered to control values after 70 h. Compared with mature turgid leaves (referred to as controls), the protein level in senescent turgid *Ramonda* leaves was found to be 20% lower, and in dehydrated mature leaves 40% and 20% lower (at 50% and 20% RWC, respectively) than in controls (Table 1). However, comparison of the electrophoretic patterns of denatured proteins from heat- and SDS-treated leaf extracts of senescent and dehydrated leaves revealed significant differences in the contents of peptides (Fig. 3). In senescent leaves all bands had rather low intensity and some of them were completely missing in comparison with young and mature leaves, while the bands of dehydrated leaves were more distinct and some were intensified. It was noticed that by measuring band density the intensity of the band with M_r 60 increased by 38–47%, the M_r 31 band increased by 69–87% and the one with M_r 14 increased by 80–86% in dehydrated leaves compared with mature turgid leaves. New bands with M_r 33 and 25 appeared upon dehydration (Fig. 3). To measure class III POD activity, pyrogallol as an electron donor, guaiacol and two hydroxycinnamic acids, i.e. caffeic and ferulic acids, were used. The highest specific activity was measured for guaiacol, and the lowest

Table 1. *Ascorbate peroxidase and POD activity in young, mature, senescent, and mature leaves with different RWC*

In the same leaves soluble protein content was measured and expressed on the basis of fresh and dry mass of leaves. The asterisk indicates significant differences ($P \leq 0.05$).

	APX (mmol min^{-1} mg^{-1} protein)	POD (mmol min^{-1} mg^{-1} protein)	Soluble proteins	
			(mg g^{-1} FW)	(mg g^{-1} DW)
Young	1.2±0.3*	9.5±2.3	5.5±0.2	20.4±0.9
Mature	3.2±0.6	12.5±0.8*	6.1±0.1	32.6±2.2
Senescent	3.6±0.8	18.6±4.5*	2.8±0.5*	26.2±2.0
50% RWC	3.7±0.7	21.4±0.5	6.0±0.2	19.6±1.7*
20% RWC	2.9±0.5	12.5±0.8	18±1.0*	26.7±4.4

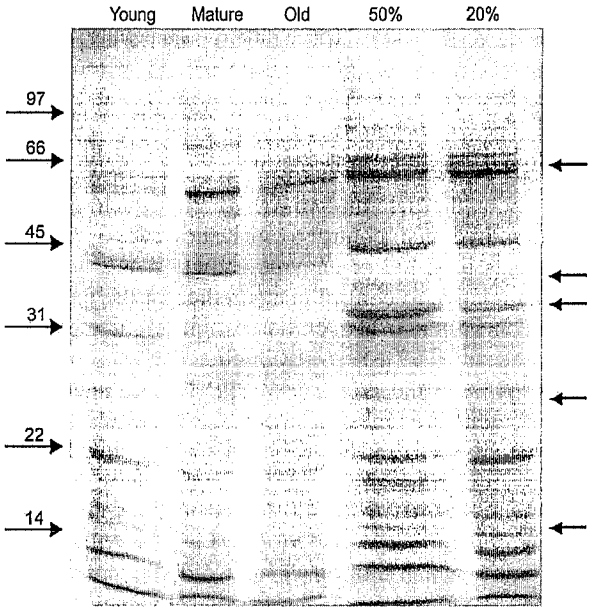

Fig. 3. Peptide profiles of soluble proteins extracted from young, mature, and senescent leaves and those of dried leaves at 50% and 20% RWC. Arrows indicate intensified and new peptide bands in extracts of leaves subjected to drought. An amount of 15 μg of total proteins was applied to each well.

for ferulic acid (Fig. 4). Calculated K_m (mM) values obtained from Hanes plots were 0.4 for caffeic acid, 1.9 for ferulic acid, 5.6 for guaiacol, and 5.4 for pyrogallol. Increase in the activity of POD, with pyrogallol as electron donor, was significant both in the senescent and dehydrated leaves (50% RWC) compared with mature and control leaves (Table 1). In leaves with 20% RWC, the specific POD activity was similar to the activity measured in control leaves. APX activity in young leaves measured half the activity in mature leaves and did not change during senescence or drought (Table 1). Separation of POD isoforms based on molecular weights showed differences in POD profiles between mature, young, and old leaves (Fig. 5). A form of high mobility that was present in young leaves disappeared in mature and old leaves, while a form of low mobility appeared only in old leaves. Dehydration of plants down to 50% and 20% RWC induced two additional isoforms with higher mobility than in mature leaves. Separation of POD isoforms by their pI values revealed two anionic isoforms with similar pI (4.5) and two cationic ones with pI of about 9.3–9.0 (Fig. 6). During ageing, anionic isoforms increased by 180% in mature and 280% in senescent leaves compared with young leaves, while drought had no influence on the amount of anionic isoforms. On the other hand, the intensity of cationic isoforms was 4-fold higher in the leaves of 50% RWC, and two new isoforms with pI 8.4 and 8.6 appeared. In the leaves with 20% RWC, two cationic isoforms (pI 9.3–9.0) were 3-fold intensified compared with turgid leaves. The anionic isoforms remained unchanged in leaves of different RWC (Fig. 6). Over the 2 week period of withholding water, leaves were gradually dehydrated from 95% to about 7% RWC (Fig. 2). Leaves with similar RWCs were grouped and the enzyme activity plotted against the RWC of the corresponding plant (Fig. 7). Specific POD activity increased significantly (from 8.7 μmol mg^{-1} protein min^{-1} in turgid leaves to 17.1 μmol mg^{-1} protein min^{-1} in the leaves of 57% RWC), and decreased gradually as dehydration progressed (Fig. 7B). POD activity varied greatly in the desiccated leaves due to different ages. APX activity was measured in fresh extracts of *Ramonda* leaves as a 50% decrease in the activity was detected after 2 h of storage on ice (data not shown). When specific APX activity was plotted against the RWC of leaves, it showed no significant changes even in desiccated leaves (Fig. 7A).

A transient decrease in the activity of both APX and POD was followed by an increase on the first day of rehydration (Fig. 8). Whereas POD activity decreased again and did not rise to control values even after 70 h, APX reached an activity similar to control leaves after 10 h (Fig. 8).

Changes in SOD isoforms during dehydration and rehydration

Both senescence and dehydration brought about a slight increase in the level of SOD, which is particularly evident on the IEF gel (Fig. 10). Using specific inhibitors, KCN and H_2O_2, several SOD isoforms were identified in *Ramonda*

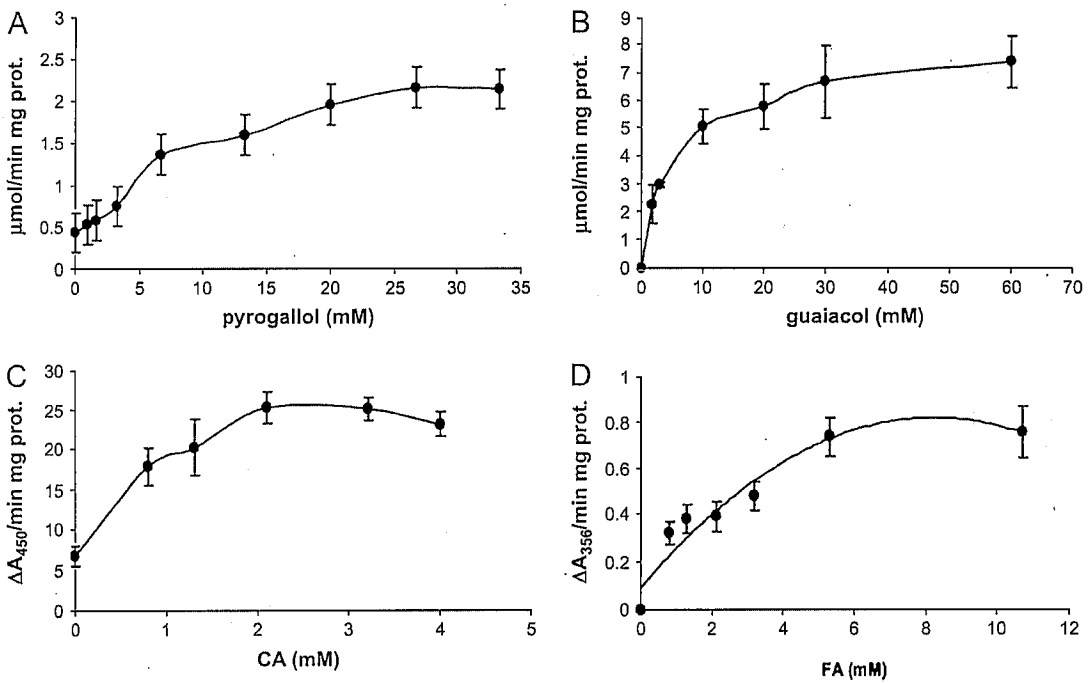

Fig. 4. Concentration dependence of the specific rate of peroxidase reaction. Reducing substrates were pyrogallol (A), guaiacol (B), caffeic acid (C), and ferulic acid (D) and the reaction was started by adding hydrogen peroxide (1 mM). The reaction was performed at 30 °C in 100 mM K-phosphate buffer (pH 6.5) for pyrogallol and guaiacol and at pH 5 for ferulic and caffeic acids. Values are means (±standard deviation) for three or four leaves. K_m values were calculated for each substrate after linearizing average values using Hanes plots.

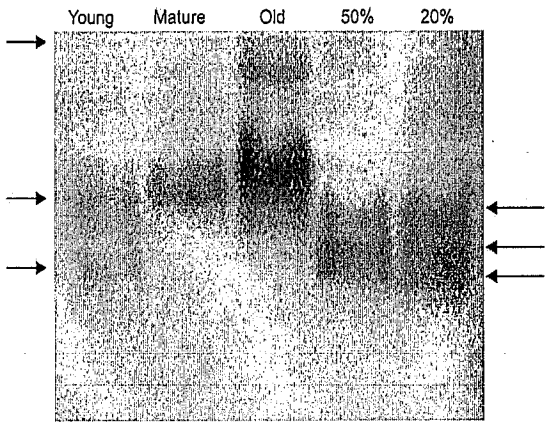

Fig. 5. Native-PAGE electrophoresis of soluble proteins stained for POD activity in *R. serbica* leaves of different age and RWC. Arrows indicate different POD isoforms. An amount of 50 µg of total proteins was applied to each well.

leaves, five of them being MnSOD and one of higher mobility FeSOD (Fig. 9). However, it was not possible to identify CuZnSOD in *Ramonda* leaves. IEF revealed numerous MnSOD anionic isoforms with pI from 6 to 5 and one with pI 4, while one band of FeSOD of smaller molecular weight was neutral. Interestingly, a high level of SOD activity was found in dried *Ramonda* leaves. SOD was inactivated during the initial hour of rehydration (Fig. 11). The recovery of SOD activity in *Ramonda* occurred within 24 h of rehydration.

Discussion

The production of ROS such as the superoxide radical increases in senescence (del Río *et al.*, 1998) and under drought stress (Navari-Izzo and Rascio, 1999; Alscher *et al.*, 2002), leading to disturbance in the redox homeostasis and also playing a key role in redox signalling in leaves (Foyer and Noctor, 2003). Increases in POD and MnSOD activity in dehydrated and senescent *Ramonda* leaves (Figs 5, 7, 9; Table 1) can therefore be considered part of an antioxidative defence under such conditions. An increase in total peroxidase activity in *Ramonda* leaves during dehydration has been already reported (Sgherri *et al.*, 2004). In the present experiment, this increase did not continue with decreasing RWC, but POD activity decreased to the values obtained in turgid mature leaves (Fig. 7). The enzymes examined were not inactivated in desiccated leaves but they stayed intact and conserved, being transiently inactivated only upon rewatering. The different result in dried leaves could lie in the higher (7%) RWC reached in the present experiment in comparison with the experiment of the previous authors, where the RWC attained was 4.2%. In addition, IEF analysis reveals

that different isoforms were being induced in senescence and drought (Fig. 6). Dehydration induced cationic and POD isoforms with lower molecular weight, while anionic isoforms with pI 4.5 were induced in senescent leaves. One of the challenges associated with studying the peroxidases remains the determination of the physiological role of different isoforms. All efforts to assign a specific function to any particular peroxidase isoform (Goldberg *et al.*, 1983; Polle *et al.*, 1994; Bernands *et al.*, 1999) is complicated by the broad spectrum of substrates and the ability of class III peroxidases (glycosylated unspecific peroxidases) to compensate the absence of a specific isoform with another. The present analysis of substrate specificity of *Ramonda* POD showed a similar affinity for phenols, i.e. guaiacol and pyrogallol (K_m 5.6 and 5.9 mM), and higher affinity for caffeic and ferulic acids (0.4 mM and 1.9 mM, respectively). In *Ramonda serbica* the content of phenolic acids was unusually elevated in comparison with other plants (Booker and Milller, 1998), having a high level of chlorogenic, protocatechuic, and *p*-hydroxybenzoic acids (Sgherri *et al.*, 2004). The decrease in total phenolic acids during dehydration suggests their important role in antioxidative defence. A protective role of the peroxidase/phenolic/ascorbate cycle (Takahama and Oniki, 1997; Takahama, 2004) operating in the vacuole and apoplast of *Vicia faba* against oxidative stress (Takahama and Oniki, 1997) may be assumed as a detoxification system of H_2O_2 in *Ramonda* subjected to dehydration.

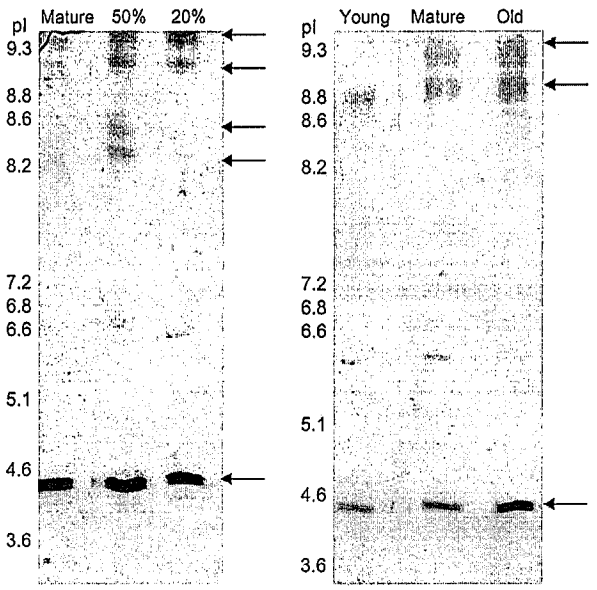

Fig. 6. Changes in POD pattern in *R. serbica* during dehydration (A) and ageing (B). Isoelectrofocusing was done in a pH gradient of 3–9. Arrows indicate POD isoforms with different pI values. An amount of 30 μg of total proteins was applied to each well.

While class III peroxidases, which use phenolics as preferential electron donors, participate in polymerization reactions in the cell wall, the metabolism of indolacetic acid or ethylene (De Gara, 2004), APX (class I peroxidase) uses ascorbate as the preferential electron donor and operates as a H_2O_2 scavenger within the cell. Although a decrease in the ascorbate content is often considered to be an indicator of senescence in many annual plants (Takahama *et al.*, 1999; Dertinger *et al.*, 2003) APX activity was shown not to change in senescent leaves, but rather to remain the same throughout the period of drought (Fig. 7; Table 1). However, contrasting results related to ascorbate and glutathione levels and the redox state need further investigation of ascorbate metabolism in this unique plant (Augusti *et al.*, 2001; Sgherri *et al.*, 2004). A simultaneous decrease in the levels of phenolics reported by Sgherri *et al.* (2004) for *Ramonda* leaves during dehydration and increase in POD activity suggests the role of the peroxidase/phenolics/ascorbate system in scavenging ROS and releasing oxidative stress during dehydration. An increase in the content of phenolic acids over the first hour of rewatering, in the initial hour of rehydration (Sgherri *et al.*, 2004) may result from an intensified biosynthesis and decreased peroxidase activity (Fig. 8B). Rehydration also brought about a transient decrease in the activity of the other two antioxidant enzymes, SOD (Fig. 11) and APX (Fig. 8A), and an

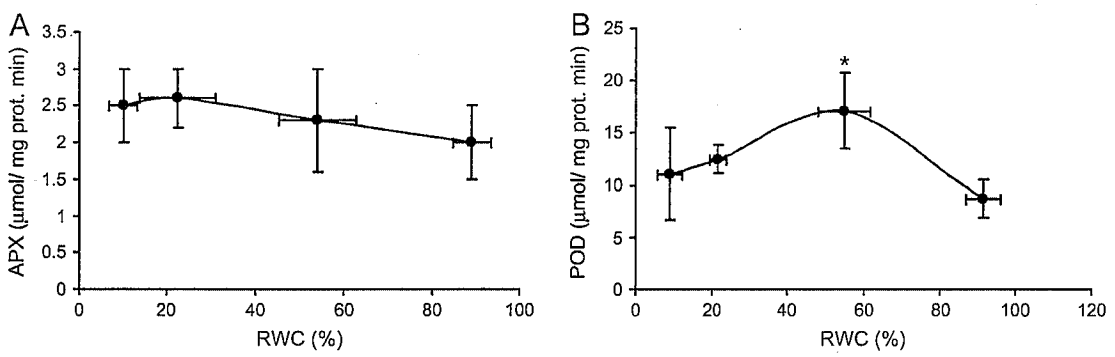

Fig. 7. Ascorbate peroxidase (A) and peroxidase activity (B) in mature leaves of *R. serbica* of different RWC obtained during a 2 week period of drought. Activity was detected by the protein levels in leaf extracts. Values are means (±standard deviation) for seven to ten leaves. The asterisk indicates significant differences ($P \leq 0.05$).

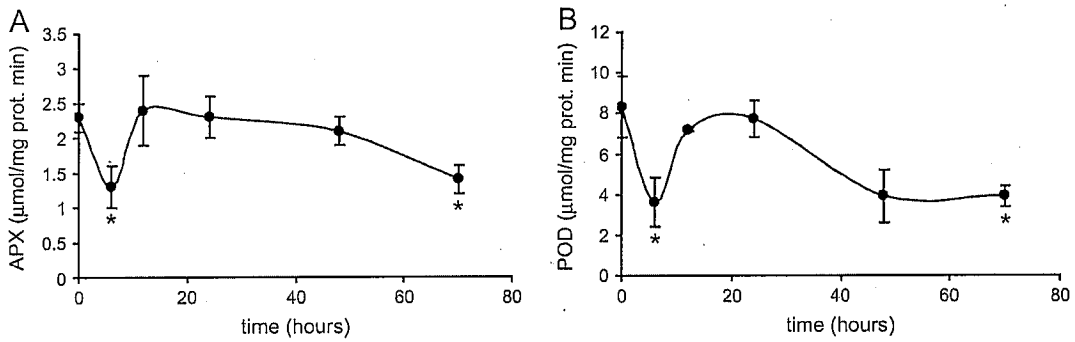

Fig. 8. Changes in ascorbate peroxidase (A) and peroxidase specific activity (B) during rehydration. Values are means (±standard deviation) for four leaves. The asterisk indicates significant differences ($P \leq 0.05$).

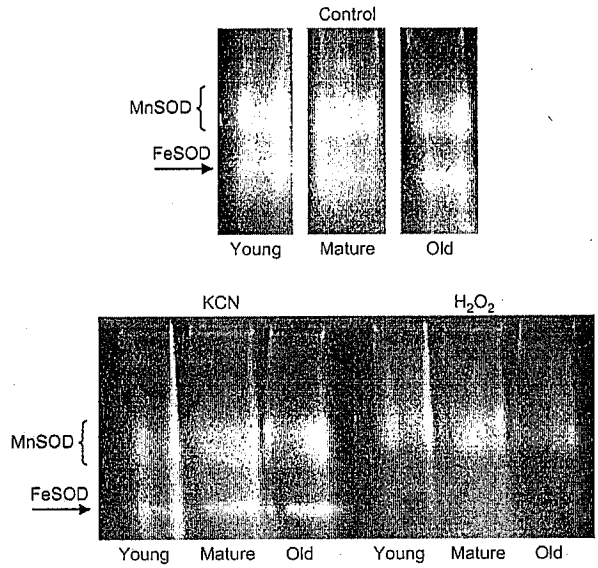

Fig. 9. Native-PAGE electrophoresis of soluble proteins stained for SOD activity in *R. serbica* leaves of different age and RWC. The effect of 5 mM KCN and 5 mM H_2O_2 on SOD activity was used to determine the presence of CuZnSOD, MnSOD, and FeSOD isoforms. Arrows indicate different SOD isoforms identified in the leaf extract of *R. serbica*. An amount of 50 μg of total proteins was applied to each well.

increase to higher activity in the following days, suggesting their role in alleviating oxidative stress during rehydration. Results confirm that the most dramatic period regarding cellular oxidative injury is when plant recovery begins (Sgherri *et al.*, 1994*a*, *b*; Navari-Izzo and Rascio, 1999; Augusti *et al.*, 2001). The leaf of *Ramonda* has several SOD isoforms, most of them being MnSODs and one of them FeSOD. FeSOD is an ancient form of SOD that is localized in the chloroplasts of several evolutionarily old plants (Salin and Bridges, 1981). Senescence was found to induce more intense bands of MnSOD. The induction of MnSODs, extracellular and mitochondrial, previously found in senescent pea (Jimenez *et al.*, 1998), ginkgo, and birch leaves (Kukavica and Veljovic-Jovanovic, 2004), has been proposed to be part of a protective mechanism that accompanies an intensified respiration process. Combined activities of these two enzymes, the H_2O_2-generating SOD and H_2O_2-metabolizing POD, contribute to increasing drought resistance and delaying the senescence process. The present data on the contents of soluble proteins in *Ramonda* leaves showed that senescence and drought both induced a similar protein decrease expressed on a dry weight basis (Table 1). Senescence in perennial plants is accompanied by protein degradation as a consequence of intensified catabolic oxidative reactions and activation of specific proteases (Thomson *et al.*, 1987). Contrasting results were reported on the protein levels in dehydrated leaves of resurrection plants, these being five to six times as high as in control leaves (Sgherri *et al.*, 1994*a*), or reduced to 30% of the control value (Sgherri *et al.*, 1994*b*). In dehydrated leaves, agreeing with some previous findings on other resurrection plants (Sgherri *et al.*, 1994*a*), the SDS-PAGE analysis of soluble proteins in *Ramonda* leaves at 50% and 20% RWC showed two new polypeptide bands of around 25 and 35 kDa, while bands with 60 kDa and two below 15 kDa were intensified. Decrease in the amount of peptides in senescent leaves (Fig. 3) was probably due to a reported increase in amino acid contents in senescent leaves (Soudry *et al.*, 2005). The existence of a close link between oxidative modification of proteins and their proteolysis has been suggested (Davies *et al.*, 1987). On the other hand, the amino acid decrease in *Ramonda* leaves (Živković *et al.*, 2004) may implicate a *de novo* protein synthesis (Fig. 3). Conservation of enzymes in desiccated leaves is a unique process unrelated to ageing, as obtained in senescent leaves, and it requires further study. Group 1 late embryogenesis-abundant proteins are a subset of hydrophilins that are postulated to play important roles in protecting plant macromolecules from damage during desiccation (Bartels and Salamini, 2001).

In conclusion, the present results confirm that dehydration, like senescence, causes disturbance in the redox homeostasis of the resurrection plant *Ramonda* by a preferential induction of class III peroxidase. Oscillatory changes in peroxidase activity (class III), accompanied by

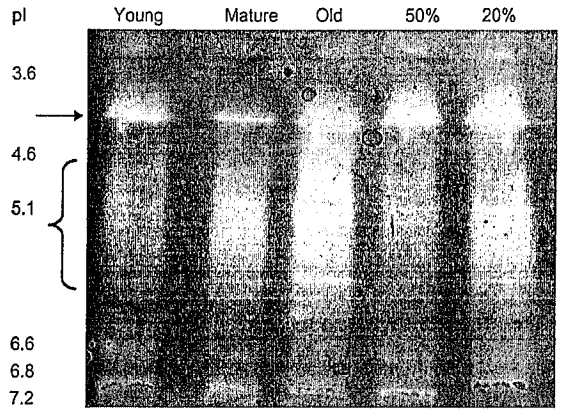

Fig. 10. IEF pattern of SOD isoforms isolated from *R. serbica* leaves of different age and RWC. An amount of 30 μg of total proteins was applied to each well.

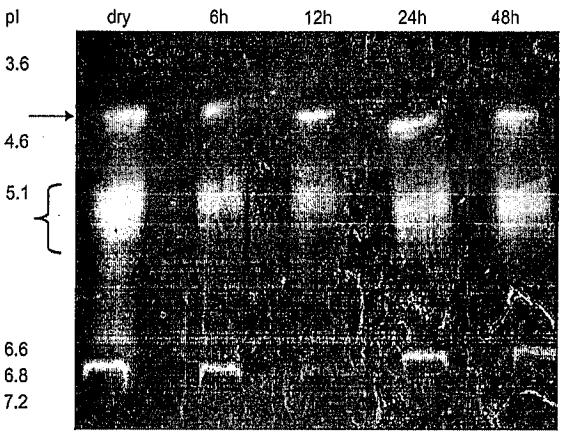

Fig. 11. SOD activity on IEF PAGE in *R. serbica* leaves of different RWC. Rehydration lasted 70 h and the hours indicate time after the beginning of watering, when samples were taken. An amount of 30 μg of total proteins was applied to each well.

transient SOD and APX inactivation obtained during rehydration, confirm that plants experience a severe oxidative stress in the first few hours of restoration of the original membrane structure within cells (Navari-Izzo and Rascio, 1999; Quartacci *et al.*, 2002). The isoperoxidase and peptide profile of dehydrated leaves and senescent leaves, compared with mature turgid leaves, supports an opinion that senescence and dehydration develop different mechanisms of protein protection when metabolism is slowed down. It is proposed that different POD isoforms induced under dehydration or senescence could have different physiological roles, anionic in lignifying cell wall, induced in senescence, and cationic in protecting cellular constituents in drought, oxidizing phenolics which are abundant in *Ramonda* leaves. The higher affinity of POD for hydroxycinnamic acids, compared with simple phenols (Fig. 4), and the unusually high level of chlorogenic and *p*-dihydroxybenzoic acids measured in *Ramonda* leaves (Sgherri *et al.*, 2004) imply that such a peroxidase reaction occurs *in situ*. Decreases in phenolic acids and reduced ascorbate in dehydrated leaves (Sgherri *et al.*, 2004), accompanied by an increase in soluble cationic isoperoxidase activity (Fig. 6), suggest that the enzymatic oxidation of hydroxycinnamic acids coupled to ascorbate oxidation by phenolic radicals (Takahama and Oniki, 1997) provides an efficient system for scavenging H_2O_2. In the absence of ascorbate in desiccated leaves, when transport from cytosol to apoplast and vacuole is impaired due to water loss and membrane disruption (Quartacci *et al.*, 2002), *o*-quinones, oxidation products of chlorogenic and *p*-dihydroxybenzoic acids, can be either substrates for peroxidase in the presence of excess amounts of H_2O_2 (Zapata *et al.*, 1992) or can have antimicrobial activity in the apoplast (Beckman and Siedow, 1985). Some of the oxidation products, *o*-diphenols can inhibit proteolysis, thus helping conservation of proteins in the anabiotic state of *Ramonda* leaves (Sullivan and Hatfield, 2006).

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References

Alscher RG, Erturk N, Heath LS. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany* **53,** 1331–1341.

Augusti A, Scartazza A, Navari-Izzo F, Sgherri CLM, Stevanovic B, Brugnoli E. 2001. Photosystem II photochemical efficiency, zeaxanthin and antioxidant contents in the poikilohydric *Ramonda serbica* during dehydration and rehydration. *Photosynthesis Research* **67,** 79–88.

Bartels D, Salamini F. 2001. Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*: a contribution to the study of drought tolerance at the molecular level. *Plant Physiology* **127,** 1346–1353.

Beauchamp C, Fridovich I. 1971. Superoxide dismutase: improved assay and assay applicable to acrylamide gels. *Analytical Biochemistry* **44,** 276–287.

Beckman JS, Siedow JN. 1985. Bactericidal agents generated by the peroxidase-catalyzed oxidation of *para*-hydroqinines. *Journal of Biological Chemistry* **260,** 14604–14609.

Bernards M, Fleming W, Lewellyn D, Prifer R, Yang X, Sabatino A, Plourude G. 1999. Biochemical characterization of the suberization-associated anionic peroxidase of potato. *Plant Physiology* **121,** 135–145.

Booker F, Miller J. 1998. Phenylpropanoid metabolism and phenol composition of soybean (*Glycine max* (L.) Merr.) leaves following exposure to ozone. *Journal of Experimental Botany* **49,** 1191–1202.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Davies KJA, Delsignore ME, Lin SW. 1987. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *Journal of Biological Chemistry* **262,** 9902–9907.

De Gara L. 2004. Class III peroxidases and ascorbate matabolism in plants. *Phytochemistry Reviews* **3,** 195–205.

del Río LA, Pastori GM, Palma JM, Sandalio LM, Sevilla F, Corpas FJ, Jimenez A, Lopez-Huertas E, Hernandez JA. 1998. The activated oxygen role of peroxisomes in senescence. *Plant Physiology* **116,** 1195–1200.

Dertinger U, Schaz U, Schulze ED. 2003. Age-dependence of the antioxidative system in tobacco with enhanced glutathione reductase activity or senescence-induced production of cytokinins. *Physiologia Plantarum* **119,** 19–29.

Gaff DF. 1989. Responses of desiccation tolerant 'resurrection' plants to water stress. In: Kreeb KH, Richter H, Hinckley TM, eds. *Structural and functional responses to environmental stress: water shortage.* The Hague: SPB Academic Publishers, 255–268.

Gaff DF, Geiss W. 1986. Drought resistance in water plants in rock pools of southern Africa. *Dinteria* **18,** 17–37.

Goldberg R, Catesson AM, Czaninski Y. 1983. Some properties of syringaldazine oxidase, a peroxidase specifically involved in the lignification process. *Zeitschrift für Pflanzenphysiologie* **110,** 259–266.

Foyer CH, Noctor G. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum* **119,** 355–364.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Molecular Biology* **47,** 377–403.

Jiménez A, Hernández JA, Ros Barceló A, Sandalio LM, del Río LA, Sevilla F. 1998. Mitochondrial and peroxisomal ascorbate peroxidase of pea leaves. *Physiologia Plantarum* **104,** 687–692.

Kar M, Feierabend J. 1984. Metabolism of activated oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. *Planta* **160,** 385–391.

Kukavica B, Veljovic-Jovanovic S. 2004. Senescence-related changes in the antioxidant status of ginkgo and birch leaves during autumn yellowing. *Physiologia Plantarum* **22,** 321–327.

Leshem YY. 1981. Oxy free radicals and plant senescence. What's new? *Plant Physiology* **12,** 1–4.

Mahalingam R, Fedoroff N. 2003. Stress response, cell death and signalling: the many faces of reactive oxygen species. *Physiologia Plantarum* **119,** 56–68.

Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P. 1994. Drought induces oxidative stress in pea plants. *Planta* **194,** 346–352.

Navari-Izzo F, Meneguzzo S, Loggini B, Vazzana C, Sgherri CLM. 1997. The role of glutathione system during dehydration of *Boea hygroscopica. Physiologia Plantarum* **99,** 23–30.

Navari-Izzo F, Rascio N. 1999. Plant response to water-deficit conditions. In: Pessarakli M, ed. *Handbook of plant and crop stress.* New York, NY: Marcel Dekker, 231–270.

Navari-Izzo F, Ricci F, Vazzana C, Quartacci MF. 1995. Unusual composition of thylakoid membranes of the resurrection plant *Boea hygroscopica*: changes in lipids upon dehydration and rehydration. *Physiologia Plantarum* **94,** 135–142.

Noctor G, Veljovic-Jovanovic S, Driscoll S, Novitskaya L, Foyer CH. 2002. Drought and oxidative load in wheat leaves: a predominant role for photorespiration? *Annals of Botany* **89,** 841–850.

Pastori GM, del Río LA. 1994. Activated oxygen species and superoxide dismutase activity in peroxisomes from senescent pea leaves. *Proceedings of the Royal Society of Edinburgh* **102B,** 505–509.

Polle A, Otter T, Seifert F. 1994. Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L.). *Plant Physiology* **106,** 53–60.

Porembski S, Barthlott W. 2001. Genetic and gneissic outcrops (Inselbergs) as centers for diversity of desiccation-tolerant vascular plants. *Plant Ecology* **151,** 19–28.

Price AH, Hendry GAF. 1991. Iron-catalysed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. *Plant, Cell and Environment* **14,** 477–484.

Prochazkova D, Sairam RK, Srivastava GC, Singh DV. 2001. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Science* **161,** 765–771.

Quartacci MF, Glisić O, Stevanović B, Navari-Izzo F. 2002. Plasma membrane lipids in the resurrection plant *Ramonda serbica* following dehydration and rehydration. *Journal of Experimental Botany* **53,** 1–8.

Quartacci MF, Navari-Izzo F. 1992. Water stress and free radical mediated changes in sunflower seedlings. *Journal of Plant Physiology* **139,** 621–625.

Salin ML, Bridges SM. 1981. Absence of the iron-containing superoxide dismutase in mitochondria from mustard (*Brassica campestris*). *Biochemical Journal* **195,** 229–233.

Schwanz P, Polle A. 2001. Differential stress responses of antioxidative systems to drought in pedunculate oak (*Quercus robur*) and maritime pine (*Pinus pinaster*) grown under high CO_2 concentrations. *Journal of Experimental Botany* **52,** 133–143.

Sgherri CLM, Loggini B, Bochicchio A, Navari-Izzo F. 1994*a*. Antioxidant system in *Boea hygroscopica*: changes in response to desiccation and rehydration. *Phytochemistry* **37,** 377–381.

Sgherri CLM, Loggini B, Puliga S, Navari-Izzo F. 1994*b*. Antioxidant system in *Sporobolus stapfianus*: changes in response to desiccation and rehydration. *Phytochemistry* **35,** 561–565.

Sgherri C, Stevanovic B, Navari-Izzo F. 2004. Role of phenolic acid during dehydration and rehydration of *Ramonda serbica. Physiologia Plantarum* **122,** 478–485.

Soudry E, Ulitzur S, Gepstein S. 2005. Accumulation and remobilization of amino acids during senescence of detached and attached leaves: in planta analysis of tryptophan levels by recombinant luminescent bacteria. *Journal of Experimental Botany* **56,** 695–702.

Sullivan ML, Hatfield RD. 2006. Polyphenol oxidase and *o*-diphenols inhibit post-harvest proteolysis in red clover and alfalfa. *Crop Physiology and Metabolism* **46,** 662–670.

Takahama U. 2004. Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase: physiological significance of the oxidation reactions. *Phytochemistry Reviews* **3,** 207–219.

Takahama U, Hirotsu M, Oniki T. 1999. Age-dependent change in levels of ascorbic acid and chlorogenic acid and activities of peroxidase and superoxide dismutase in the apoplast of tobacco leaves: mechanism of oxidation of chlorogenic acid in the apoplast. *Plant Cell Physiology* **40,** 716–724.

Takahama U, Oniki T. 1997. A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. *Physiologia Plantarum* **101,** 845–852.

Thomson JE, Ledge RL, Barber RF. 1987. The role of free radicals in senescence and wounding. *New Phytologist* **105,** 312–344.

Vertucci CW, Farrant JM. 1995. Acquisition and loss of desiccation tolerance. In: Kigel J, Galili G, eds. *Seed development and germination.* New York, NY: Marcel Dekker, 237–271.

Welinder KG. 1992. Superfamily of plant, fungal and bacterial peroxidases. *Current Opinion in Structure Biology* **2,** 388–393.

Winston GW. 1990. Physiochemical basis for free radical formation in cells: production and defences. In: Alscher RG, Cumming JR, eds. *Stress responses in plants: adaptation and acclimation mechanisms.* New York, NY: Wiley-Liss, 57–86.

Zapata JM, Calderon AA, Munoz R, Ros Barcelo A. 1992. Oxidation of hydroquinone by both cellular and extracellular grapevine peroxidase fractions. *Biochimie* **74,** 143–148.

Živković T, Quartacci M, Stevanović B, Marinone F, Navari-Izzo F. 2005. Low-molecular weight substances in the poikilohydric plant *Ramonda serbica* during dehydration and rehydration. *Plant Science* **168,** 105–111.