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Role of phenolics in the antioxidative status of the resurrection plant *Ramonda serbica* during dehydration and rehydration

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***Ramonda serbica* plants dehydrated for 14 days reached a relative water content of 4.2% and entered into anabiosis prior to being rehydrated for 48 h. Total ascorbate (AsA + DHA) and glutathione (GSH + GSSG) contents increased during dehydration and approached control values by the end of rehydration. Reduced ascorbate (AsA) and glutathione (GSH) were consumed during the first 13 days of dehydration when guaiacol-, syringaldazine- and phenolic peroxidases (EC 1.11.1.7) increased. At the end of dehydration AsA and GSH accumulated whereas peroxidases decreased to half the value of controls. In this period, plants of *R. serbica* face a phase of reduced metabolism and, thus, of reduced consumption of antioxidants. During rehydration, both AsA and GSH were utilized reaching, after 48 h, about 20 and 40% of their total pools, respectively; moreover peroxidases increased showing the recovery of metabolic activities. In the dehydration process total phenolic acids decreased, but accumulated after 5 h of rehydration and returned to control values at the end of rehydration. In *R. serbica* leaves, the most representative phenolic acids were protocatechuic, *p*-hydroxybenzoic and chlorogenic acids. Most concentrated phenolic acids, such as protocatechuic and chlorogenic acids, accumulated during the first period of rehydration when AsA decreased. These results suggest a role of ascorbate in inhibiting oxidation when phenolic peroxidases remain at low levels. As a consequence of this inhibition, ascorbate was oxidized and when most of it was consumed, oxidation of phenols resumed.**

Introduction

The ability of desiccation tolerant plants to revive from air-dried state includes both protective and repair adaptive mechanisms. Environmental fitness of resurrection plants, among which is *Ramonda serbica*, is performed by a compromise between small and slow biomass production, long life span and adaptive complexity response to harsh habitat conditions.

Ramonda serbica Panc. (*Gesneriaceae*) is a rare resurrection plant of the northern hemisphere, originating from the Balkan Peninsula as an endemic and relict species of the Tertiary period. It inhabits northern-positioned calcareous rocks of some gorges in Serbia, well-known as the refugia sites of the ancient flora and vegetation. There, it not only withstands high air and soil temperature, particularly during hot and sunny summers, but also the severe water shortage lasting more than 3 months (Stevanovic et al. 1997). From an ecological point of view, *R. serbica* is a perennial, herbaceous, shade-adapted species belonging to the group of homoiochlorophyllous poikilohydric plants which preserve more than 80% of the chlorophyll content during dehydration (Markovska et al. 1994, Drazic et al. 1999, Augusti et al. 2001).

A common feature of plants exposed to drought is their potential to increase the production of reactive oxygen species (ROS) until severe dehydration reduces metabolic activities and thus slows down production of ROS (Navari-Izzo et al. 1994, Sgherri et al. 1996). To defend themselves against ROS, which at biochemical

Abbreviations – AsA, ascorbate; D, dehydrated; C, control; DIECA, diethyldithiocarbamic acid; DHA, dehydroascorbate; GPOD, guaiacol peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; POD, peroxidase; R, rehydrated; ROS, reactive oxygen species; RWC, relative water content; SPOD, syringaldazine peroxidase.

levels cause drastic changes in carbohydrate and lipid metabolism (Navari-Izzo and Rascio 1999) and protein and chlorophyll degradation, plants are endowed with low-molecular mass antioxidants (i.e. ascorbate and glutathione) and protective enzymes, such as superoxide dismutase, peroxidases and glutathione reductase, which catalyse the elimination of activated oxygen forms in the cell. After dehydration, in the resurrection plant *Boea hygroscopica*, H_2O_2 production decreased whereas total ascorbate and glutathione levels became two and 50 times as high, respectively, when compared to the control (Sgherri et al. 1994a). In *R. serbica* drought induced changes in the protein : lipid ratio (Quartacci et al. 2002) and altered the structure of membranes, among them plasmamembranes, which are the first target of water deficit (Navari-Izzo and Rascio 1999). Among other functions, the polyphenolic structure also allows the scavenging of radical species (Rice-Evans et al. 1996). The antioxidant effect of flavonoids lies in their radical scavenging properties towards all types of oxidizing radicals, namely superoxide radical and singlet oxygen (Bors et al. 1996). Their action becomes particularly important during water depletion when superoxide radicals increase (Sgherri et al. 1996). Semi-quinones are both produced during the scavenging reaction and during auto-oxidation. The antioxidative properties of flavonoids is well established. In contrast, the antioxidative potential of simple polyphenols such as phenolic acids is less known (Caldwell 2001, Peterson 2001). Malterud et al. (1993) described similar functions for anthraquinones and anthrones. Inhibitory effects on lipid peroxidation may be considered the most general claim made in this area (Bors et al. 1996). One interpretation of the flavonoid antioxidative effect lies in the radical-scavenging properties of these compounds, both in model system and under in vitro conditions (Bors et al. 1990). Most papers affirm scavenging of superoxide anions, whereas other studies favour hydroxyl, peroxyl or alkoxyl radicals (Bors et al. 1996). Recently, it has also been suggested that peroxidases, in the presence of phenolics and ascorbate, could act as an efficient hydrogen peroxide scavenging system in plant vacuoles (Takahama and Oniki 1997, Sgherri et al. 2003). Vacuolar peroxidases can reduce hydrogen peroxide by using phenolics as primary electron donors. Phenoxyl radicals, generated upon oxidation, can be reduced by monodehydroascorbate radical which in its turn is generated by the phenoxyl-dependent oxidation of AsA (Takahama and Oniki 1997, Sgherri et al. 2003).

The aim of the present research was to evaluate in the resurrection plant *Ramonda serbica*, an attractive model-system plant due to the reversible anabiotic/biotic life style, the role of phenolic acids in the tolerance to desiccation. In addition, phenolic acid changes were related to the more studied antioxidants ascorbate and glutathione under conditions of dehydration and rehydration.

Materials and methods

Plant material

Ramonda serbica Panc. plants were collected in the south-east regions of Serbia in a gorge near the town of Nis. Plants were allowed to acclimate inside a greenhouse keeping them fully watered. Subsequently, plants from three independent experiments were subjected to dehydration by withholding irrigation for 14 days prior to rehydration for 48 h by spraying the plants with water to simulate rainfall and keeping the soil damp. For this study, mature and fully expanded leaves from the middle of the rosettes and comparable in size were selected. Leaves were harvested after 8 (D1), 9 (D2), 13 (D3) and 14 (D) days from the start of the experiment during dehydration and at 5 (R1) and 48 h (R2) during rehydration.

Relative water content (RWC) determination

At harvesting, leaves (from 10 replicates of three independent experiments) were cut and fresh, turgid and dry weights were determined in order to calculate the relative water content (RWC) according to Sgherri et al. (1994b). Turgid weights were measured after the leaves were left in distilled water for 24 h at 20°C in the dark. Dry weights were measured after the leaves were dried for 48 h at 80°C. The RWC were calculated as follows:

$$RWC = (fwt - dwt/twt - dwt) \times 100$$

Ascorbate and dehydroascorbate determinations

Fresh leaves (0.5 g) were homogenized in ice-cold 5% (w/v) trichloroacetic acid containing 4% (w/v) polyclar AT (BDH, Chemicals Ltd, Poole, England). After centrifugation at 12 000 *g* for 15 min, AsA and total ascorbate (AsA + DHA) were determined in the supernatant as reported previously (Sgherri and Navari-Izzo 1995). Total ascorbate was determined through the reduction of dehydroascorbate (DHA) to ascorbate by 1 m*M* DTT. A standard curve covering a 0–25 nmol AsA range was used. DHA levels were estimated on the basis of the difference between total ascorbate and AsA amounts.

Glutathione and oxidized glutathione determination

Fresh leaves (0.5 g) were homogenized in ice-cold 5% (w/v) trichloroacetic acid and centrifuged at 12 000 *g* for 15 min. The supernatant was used for total (GSH + GSSG) and GSSG determinations by the 5,5′-dithio-bis-nitro-benzoic acid-GSSG reductase recycling procedure as reported in Sgherri and Navari-Izzo (1995). GSSG was determined after GSH had been removed by derivatization with 2-vinylpyridine. Changes in absorbance of the reaction mixtures were detected at 412 nm at 25°C. GSH, expressed as GSSG equivalents, was obtained by the difference between total and oxidized glutathione contents. The total glutathione

contents were calculated from a standard curve (1–10 nmol of GSH equivalents).

Determination of phenols

Phenolic acids were determined as reported previously (Sgherri et al. 2003). Phenolic acids were extracted from fresh leaves (2 g) for 1 h with 50% methanol containing 1% HCl under continuous stirring at room temperature. After centrifugation at 12 000 g for 15 min, the supernatant was collected and the extraction was repeated three times on the pellet. Methanolic extracts were collected, vacuum dried and re-suspended in 80% methanol. Before analysis, samples were passed through a Sartorius filter (Minisart 0.45 μm) to remove any suspended material.

A measure of total phenolics was obtained by recording A_{280} before and after addition of Polyclar AT (BDH, Chemicals Ltd, Poole, England) to the extract (1 : 10 w/v) as reported by Sgherri et al. (2003). The calculations were performed using the absorbance, obtained by the difference between the two readings, and by using a calibration curve for total phenolics prepared with gallic acid as standard.

Qualitative and quantitative analysis was performed by reverse phase HPLC (Talcott and Howard 1999). Twenty microlitres were injected into a Waters model 515 HPLC system fitted with a 3.9×20 mm Sentry Nova-Pak C_{18} precolumn and a 3.9×150 mm Nova-Pak C_{18} column (Waters, Milford, MA). Detection of phenolic acids was at 280 nm using a Waters 2487 dual λ UV-visible detector. Mobile phase A contained 98% of water and 2% acetic acid, and mobile phase B contained 68% water, 30% acetonitrile and 2% acetic acid. A linear gradient from 0 to 30% of mobile phase B for 30 min at 1 ml min^{-1} was used. The system precolumn-column was washed with 100% mobile phase B and equilibrated with 100% mobile phase A before the next sample injection. Identity of phenolic acids was confirmed by co-chromatrography on HPLC with authentic standards (Sigma Chemical Co., St. Louis, MO), and quantification was performed using a standard curve in the range 0.2–2 μg of a standard mixture as reported previously (Sgherri et al. 2003). Chromatogram analysis was performed by the software MILLENNIUM 32 (Waters).

Peroxidase (POD, EC 1.11.1.7) analysis

Leaves were ground in an ice-cold mortar with sand and 0.1 M Tris/HCl, pH 7.8, containing 1 mM EDTA and 10 mM DIECA at 4°C. The homogenate was filtered through Miracloth (Calbiochem, LaJolla, CA) and centrifuged at 20 000 g for 15 min at 4°C. The protein content (Lowry et al. 1951) and the enzyme assay were measured in the supernatant at 25°C. POD activities were measured spectrophotometrically using 3 mM guaiacol and 5 mM syringaldazine as substrates. POD activity with guaiacol (GPOD) as hydrogen donor was determined following Bergmeyer et al. (1974). One unit of guaiacol peroxidase was defined as the amount of enzyme oxidizing 1 μmol guaiacol min^{-1}. POD activity with syringaldazine (SPOD) as hydrogen donor was determined according to Imberty et al. (1985). One unit of syringaldazine peroxidase was defined as the amount of enzyme oxidizing 1 μmol syringaldazine min^{-1}.

Phenolic peroxidase activities

Sample extractions were the same as for POD analysis. Phenolic peroxidase activity was determined in the supernatant according to Hadzi-Taskovic Sukalovic et al. (2003). The assay mixture contained the extract and 0.1 mM chlorogenic acid in 50 mM phosphate buffer pH 6. Phenolic peroxidase activity was determined by measuring the initial rate of the decrease in absorbance at 324 nm at 30°C after the addition of 2.5 mM H_2O_2. Specific activity was determined by using an extinction coefficient of 20 200 M^{-1} cm^{-1} and measuring the protein contents according to Lowry et al. (1951).

Statistical analysis

Results are the means from three replicates of three independent experiments. The significance of differences between mean values was determined by one-way ANOVA. Comparisons among means was performed using Newman–Keuls test. Reported means followed by different letters are significantly different at $P \leq 0.01$.

Results

The mean relative water content (RWC) decreased from 98% in fully hydrated leaves of the control plants (C) to 4.2% in the dried ones. Following the desiccation process, water loss increased gradually, reaching the RWC of 78.2% (D1), 34.1% (D2), 31.5% (D3) and 4.2% (D4). However, upon rehydration, the RWC restored rapidly to 31.7% (R1) and 86.9% (R2) after 5 and 48 h, respectively (Fig. 1).

After 9 days of dehydration (D2), the total ascorbate pool (AsA + DHA) increased, maintained the same value till 13 days of dehydration and increased further after 14 days of dehydration (D4), being about five-fold higher than that of the fully irrigated control plants (Fig. 2). Upon rehydration (R1) the total ascorbate had largely decreased at 5 h , regaining the control value at 48 h after re-watering (R2). As for the total ascorbate pool, the percentage of AsA after 8 days of dehydration (D1) remained at the same level as in the control leaves. Thereafter, in moderately desiccated plants (D2), AsA started to decrease, approaching in rather dry leaves (D3) 40% of total ascorbate. However, in plants in the state of anabiosis (D4), AsA% increased by 33% in comparison with the plants dehydrated for 13 days (D3). Furthermore, upon rehydration, the AsA percentage decreased again reaching 18% of total ascorbate in fully rehydrated leaves (Fig. 2).

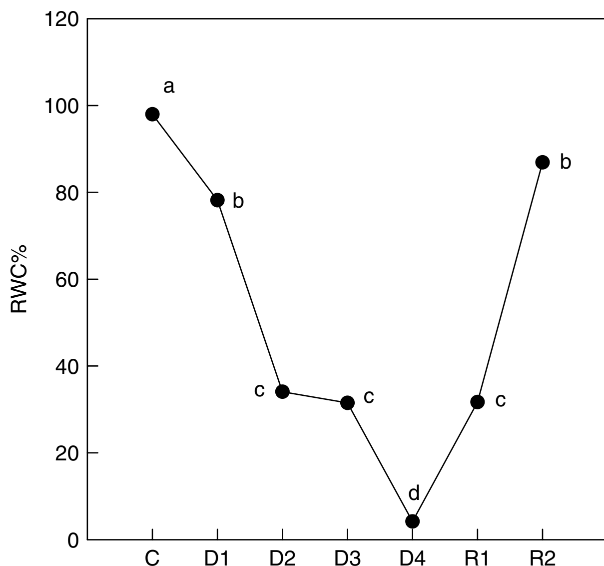

Fig. 1. Relative water content (RWC) of *R. serbica* plants subjected to dehydration and rehydration. C, control; D1, dehydrated for 8 days; D2, dehydrated for 9 days; D3, dehydrated for 13 days; D4, dehydrated for 14 days; R1, rehydrated for 5 h; R2, rehydrated for 48 h.

Total glutathione showed a similar pattern of change as that observed for total ascorbate. It increased in the course of gradual dehydration and accumulated in dried leaves (D4), as it was for ascorbate, being about four-fold higher than in control plants. Upon rehydration total glutathione was restored to the control level. However, GSH percentage decreased both during dehydration and rehydration with the exception of the D4 sample when GSH increased by 21% in comparison with D3 (Fig. 3).

During dehydration, total phenolic acids decreased from 2.7 in the leaves of fully hydrated plants (C) to 0.98 mg g^{-1} DW in the more desiccated leaves (D4). From D2 to D4 the value of total phenolic acids remained constant. In R1 they reached a value four-fold higher than in D4, and after complete rehydration (R2) they regained the control level (Fig. 4). Main representative phenolic acids of *R. serbica* leaves were protocatechuic, *p*-hydroxybenzoic and chlorogenic acids. All of them decreased during dehydration, but, both protocatechuic and chlorogenic acids reached values six-fold higher in R1 in comparison with D4, whereas *p*-hydroxybenzoic acid continuously increased during

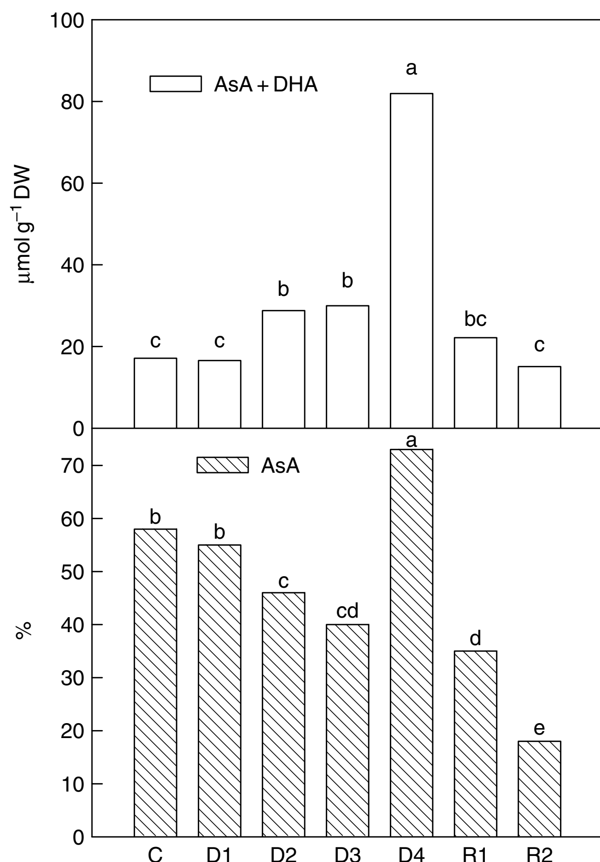

Fig. 2. Ascorbate contents of *R. serbica* plants subjected to dehydration and rehydration. AsA, ascorbate; DHA, dehydroascorbate. For other details see Fig. 1.

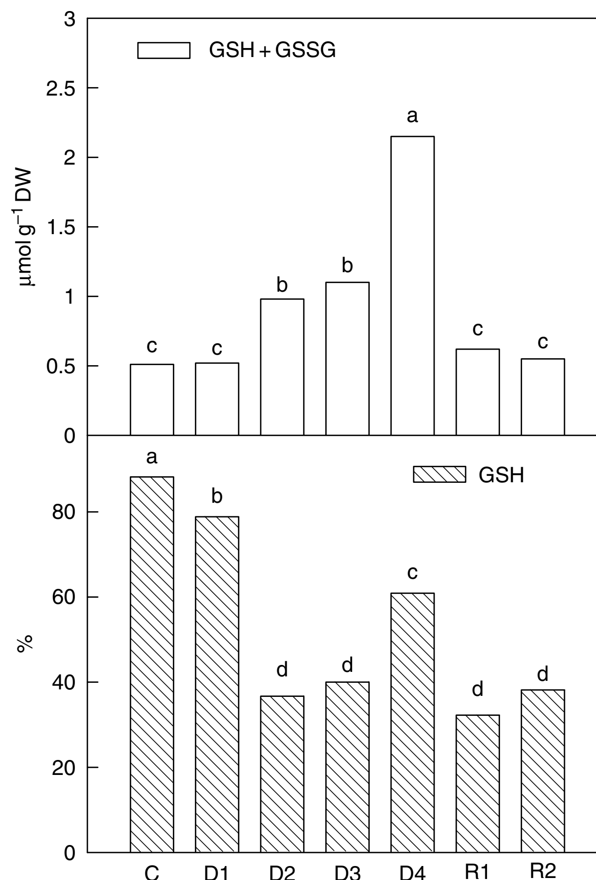

Fig. 3. Glutathione contents of *R. serbica* plants subjected to dehydration and rehydration. GSH, reduced glutathione; GSSG, oxidized glutathione. For other details see Fig. 1.

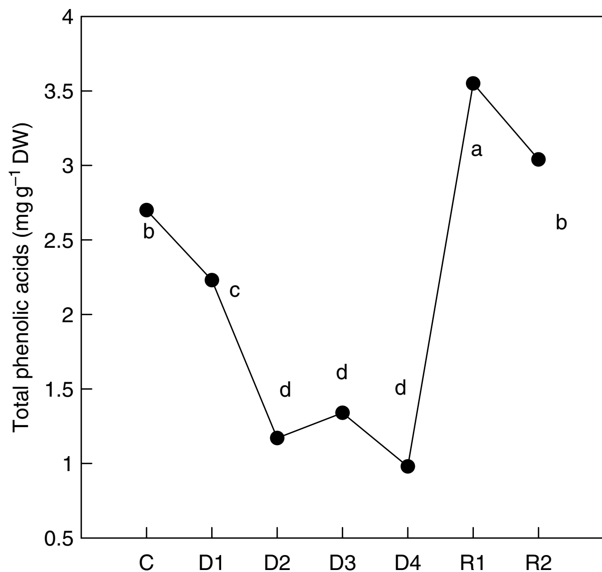

Fig. 4. Total phenolic acids of *R. serbica* plants subjected to dehydration and rehydration. For other details see Fig. 1.

rehydration reaching in R2 a value similar to that of the control sample (Fig. 5). Other phenolic acids identified in small amounts (about 100-fold less than the main phenolic acids) were gallic, vanillic, caffeic, siringic, *p*-coumaric and ferulic acid. As observed with the main representative phenolic acids, the least representative ones also decreased during dehydration. Most of the phenolic acids were restored to the control values after rehydration (Fig. 6).

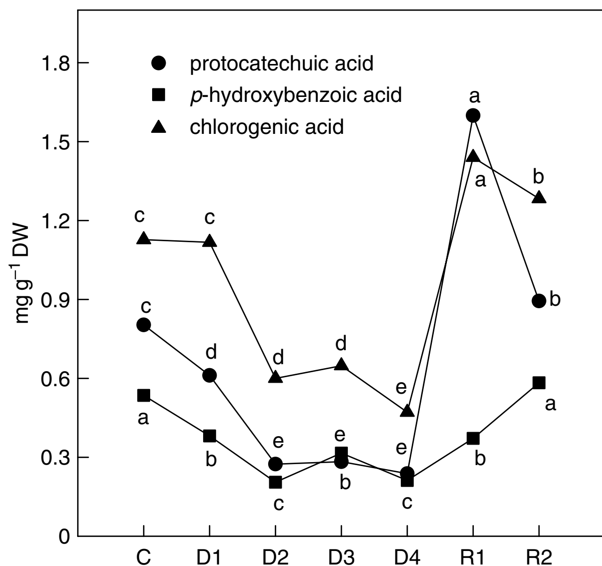

Fig. 5. Most representative phenolic acids of *R. serbica* plants subjected to dehydration and rehydration. For other details see Fig. 1.

Both SPOD and GPOD showed the same pattern during the dehydration–rehydration cycle of *R. serbica* (Fig. 7). During dehydration SPOD and GPOD activities increased and in D3 reached values that were two- and three-fold higher, respectively, than in the control. In D4 PODs dramatically decreased but they recovered their activity and exceeded the control value during rehydration (Fig. 7). Specific phenolic peroxidases also increased during dehydration but, at the end of dehydration (D4), they decreased to approximately half the value of the control. During the first period of rehydration (R1) specific PODs remained at low levels whereas, later they doubled and reached the control value (Fig. 8).

Discussion

During the first 13 days of dehydration (D1–D3) *R. serbica* experienced high levels of active oxygen species generated within its tissues, as is a common feature of plants under environmental stress conditions (Sgherri et al. 1996). Free radical damage may be avoided by production of antioxidative substances during the periods of severe water and temperature stresses (Sgherri et al. 1994a, Navari-Izzo et al. 1997, Takahama and

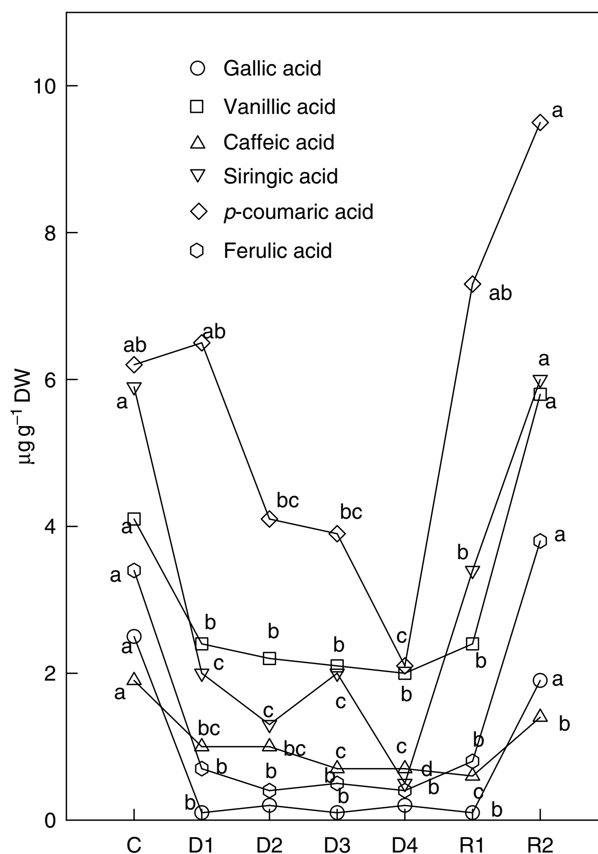

Fig. 6. Least representative phenolic acids of *R. serbica* plants subjected to dehydration and rehydration. For other details see Fig. 1.

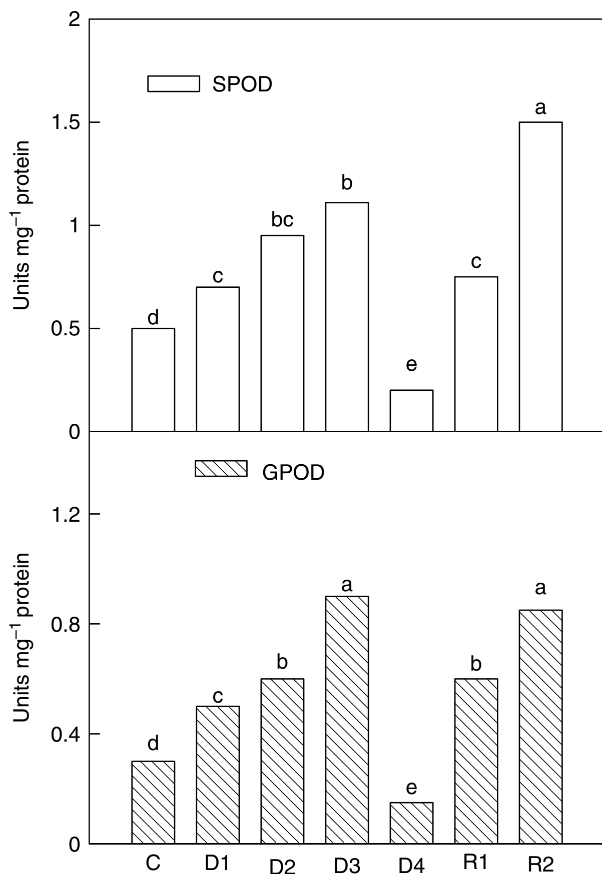

Fig. 7. Syringaldazine (SPOD) and guaiacol (GPOD) peroxidases in *R. serbica* plants subjected to dehydration and rehydration. For other details see Fig. 1.

Oniki 1997, Zancani and Nagy 2000, Caldwell 2001). In these conditions the detoxification processes have to be activated, which was evident in dehydrated leaves of *R. serbica* (D1, D2 and D3) because of the oxidation of AsA and GSH and the increase in DHA and GSSG (Figs 2 and 3). The decrease in total phenolic acids during dehydration suggests that these substances might also play an important role against oxidation (Figs 4–8) as substrates of peroxidases (Hadzi-Taskovic Sukalovic et al. 2003, Sgherri et al. 2003). However, in the state of anabiosis (D4), the very slow, almost suspended metabolism of this plant accompanied by a reduced production of superoxide radicals and hydrogen peroxide (Navari-Izzo et al. 1994, Sgherri et al. 1994a; Navari-Izzo 2002, personal communication) was followed by an accumulation of both total ascorbate and glutathione (Figs 2 and 3). Glutathione and ascorbate are relevant in metabolizing hydrogen peroxide in the ascorbate/glutathione cycle, as it has been previously seen for *Boea hygroscopica* (Sgherri et al. 1994a, Navari-Izzo et al. 1997). The lower oxidation of these low-molecular-mass antioxidants during anabiosis (D4) actually effected the increase of their reduced forms (Figs 2 and 3). Their accumulation, probably also due to a new synthesis, during the last phase of dehydration (D4) may constitute a reserve which allows *R. serbica* plants to tolerate oxidative damage during dehydration and even more during rehydration, when cellular injury caused by dehydration has to be repaired (Sgherri et al. 1994a, b).

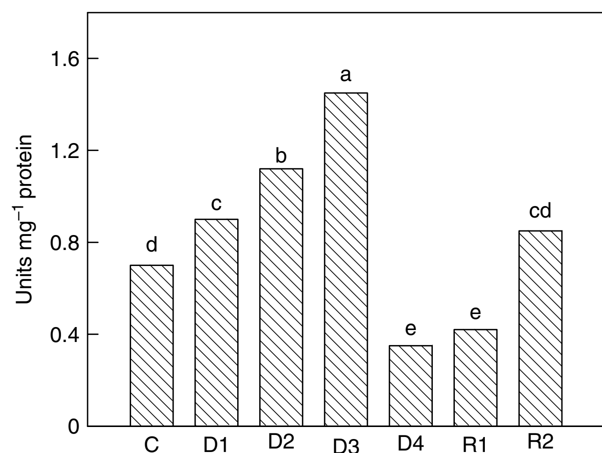

Fig. 8. Phenolic peroxidases in *R. serbica* plants subjected to dehydration and rehydration. For other details see Fig. 1.

In *R. serbica* the contents of phenolic acids were unusually elevated in comparison with other higher plants (Booker and Miller 1998, Beckman 2000, Peterson 2001, Sgherri et al. 2003). Total amounts were about three-fold higher than in *Raphanus sativus* where all phenolic acids analysed increased with copper treatment (Sgherri et al. 2003). Beckman (2000) pointed out that phenolic acids are synthesized by plants in response to physical injury, infection or other stresses and that they are often stored primarily in the apoplast or in the vacuole, strategically playing either a signalling or direct role in defence. Phenolic acid contents in *R. serbica* could be the result of the balance between their oxidation and synthesis. During the first phase of rehydration (R1), we could hypothesize an increase in synthesis of phenolic acids since specific phenolic peroxidase activity remained at low levels (Fig. 8) and phenolic acid contents increased (Figs 4–6). The recovery of the activities of non-specific PODs (Fig. 7), together with data regarding decreases in ascorbate and glutathione amounts (Figs 2 and 3), points out that for *R. serbica* the first phase of rehydration (R1), when plant recovery started, was the most dramatic for exhibiting cellular injuries (Sgherri et al. 1994a, Navari-Izzo and Rascio 1999, Augusti et al. 2001). Indeed upon rehydration, in resurrection plants *Boea hygroscopica* and *Sporobolus stapfianus,* hydrogen peroxide levels increased in comparison with the dehydrated and control samples (Sgherri et al. 1994a,b) and in *R. serbica* passed from 47 (fully dehydrated) to 133 (fully rehydrated) $\mu mol\,g^{-1}$ DW (Navari-Izzo 2002, personal communication). In contrast, during dehydration (D1–D3) and in R2 we could hypothesize that oxidation of phenolic acids prevailed since their

contents decreased and activities of specific phenolic POD and non-specific POD increased (Figs 4–8). In leaves of a multiple-stress-tolerant cv. of wheat both SPOD and GPOD increased following cadmium treatment but when stress intensity was more severe (i.e. in the roots at the highest Cd concentration) these antioxidative enzymes were inhibited (Milone et al. 2003).

Chlorogenic acid, as seen previously in *R. sativus* (Sgherri et al. 2003), represents one of the major forms of phenolic acid in *R. serbica* (Fig. 5). Moreover, in *R. serbica* chlorogenic acid concentration was about 10-fold higher than in *R. sativus*. The elevated levels and polyhydroxy nature of this compound suggest that it might have a role in the scavenging of free radicals of oxygen during dehydration and above all during the first phase of rehydration (Fig. 5) when hydrogen peroxide and oxidative stress increased (Sgherri et al. 1994a, b). According to Rice-Evans et al. (1996) chlorogenic acid appears to be more active as an antioxidant than the hydroxy-derivatives of benzoic acid, such as *p*-hydroxybenzoic, vanillic and siringic acid. Since the main representative phenolic acids, namely protocatechuic, *p*-hydroxybenzoic and chlorogenic acid, accumulated after 5 h of rehydration (R1), when the reduced form of ascorbate decreased (Fig. 2), it could be argued that AsA may also play a role in inhibiting the oxidation of phenolic acids in R1 (Fig. 5). This would be consistent with the model proposed previously that describes a role of phenolic compounds in the peroxide/phenolics/ascorbate antioxidant system (Sgherri et al. 2003). This model would predict that, as a result of the inhibition of the oxidation of phenolics by AsA, it is only when the majority of AsA has been consumed that oxidation of phenols (Fig. 8) can resume (R2). Moreover, this model may be also applied to the least representative phenolic acids which also increased during rehydration and, in the case of *p*-coumaric acid the increase resulted in levels that at the end of the rehydration phase exceeded control values (Fig. 6).

In conclusion, the pattern of changes of phenolic acids during the dehydration/rehydration cycle (Figs 4 and 5) and the accumulation of ascorbate during anabiosis as well as its further decrease immediately upon rehydration (Fig. 2) suggest, as already highlighted in *R. sativus* (Sgherri et al. 2003), that a peroxide/phenolics/AsA system can also function in *R. serbica* as a detoxification system of hydrogen peroxide (Takahama and Oniki 1997, Takahama et al. 1999, Zancani and Nagy 2000). Keeping these in mind, we might also conclude that the relationship between the more studied principal antioxidative mechanisms and phenol metabolism could increase the plant resistance to water deficits. Furthermore, it will be interesting to determine the strategic location of the phenolic storing compounds, through investigations that are now in progress.

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