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# Antioxidant responses of *in vitro* shoots of *Deschampsia antarctica* to polyethylene glycol treatment

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**Abstract:** To understand the adaptability to environmental stresses by *Deschampsia antarctica*, one of the two vascular plants growing in Antarctica, we analysed the activity of several antioxidant enzymes, including peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6)) and glutathion reductase (GR, EC 1.6.4.2), in shoots subjected to drought stress (PEG-8000, -0.3 MPa). Additionally, levels of total phenolic compounds, flavonoids and ascorbate, were determined. The content of malondialdehyde (MDA), chlorophyll and hydrogen peroxide did not change as a result of PEG-8000 treatment. In addition, treated plants showed higher enzymatic activity of CAT, POD and GR in shoots than control plants. In addition, a high capacity to scavenge free radicals was also detected in stressed plants. These results seem to indicate that in *D. antarctica* tolerance of drought stress is associated with enhanced activity of antioxidant enzymes and free radical scavenging capacity.

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**Key words:** Antarctic hairgrass, gramineae, osmotic stress, PEG-8000, reactive oxygen species (ROS)

## Introduction

Salinity, drought, and temperature extremes are among the major environmental constraints to plant productivity worldwide. These constraints will reduce productivity of major agricultural crops by more than 50% by the year 2050 (Wang *et al*. 2003). Both salinity and drought induce osmotic stress, altering cellular homeostasis and ion distribution in plants (Zhu 2001).

The best characterized biochemical response of plants to osmotic stress is the accumulation of compatible solutes, such as glycine-betaine, proline and soluble sugars (McCue & Hanson 1990). Compatible solutes mediate osmotic adjustment, protect subcellular structures and reduce oxidative damage caused by free radicals produced in response to osmotic stress (Crowe *et al*. 1993, Yeo 1998, Holmstrom *et al*. 2000, Chen & Murata 2002).

In plants, a wide range of abiotic stress factors can induce oxidative stress. Salinity, drought, and oxidative stress are accompanied by the formation of reactive oxygen species (ROS) molecules such as superoxide anion $O_2^-$, hydrogen peroxide $H_2O_2$, and hydroxyl ion $OH^-$, which damage membranes and macromolecules (Noctor & Foyer 1998, Asada 1999, Mittler 2002).

Different antioxidant defence systems have been developed in aerobic cells to counteract the damaging effects of ROS. Plants possess antioxidant defence systems comprised of enzymatic and non-enzymatic components, which normally maintain ROS balance within the cell. For instance, they may use a diverse array of enzymes such as superoxide dismutase (SOD), catalases (CAT) and peroxidases as well as low molecular mass antioxidants (glutathione, ascorbate phenolic compounds and lignans) to scavenge different types of ROS (Foyer *et al*. 1994, Pietta 2000).

*Deschampsia antarctica* Desv. (Poaceae) is the only grass endemic to the Antarctic, a habitat simultaneously affected by various environmental conditions such as extreme high UV-B radiation, low temperatures, high levels of salinity and low water availability (Alberdi *et al*. 2002). It has been reported that *D. antarctica* presents an efficient photosynthetic system, which results in a large accumulation of sugars (Zúñiga *et al*. 1996). However, the degree of tolerance to abiotic stress under natural conditions has been not described.

The aim of this research was to evaluate some enzymatic and non enzymatic responses of *in vitro* shoots of *D. antarctica* subjected to osmotic stress.

## Materials and methods

### *Plant material and* in vitro *growth conditions*

*Deschampia antarctica* plants were collected from King George Island, South Shetland Islands (62°14'S; 58°48'W), and then moved to the laboratory for *in vitro* culture at the Universidad de Santiago de Chile. Shoots were propagated vegetatively in soil: peat mixture (3:1) at 16°C ± 2°C in a growth chamber with photon flux density of 40 umol $m^{-2}$ $s^{-1}$ and 16/8 hr light/dark period. Crown tissue from axenic

plants was used to initiate cultures into culture vessels (250 ml) containing MS Propagation Medium (Murashige & Skoog 1962), (3% sucrose, 0.6% agar-agar, pH adjusted at 5.6 and autoclaved at 120°C for 20 min). Cultures were incubated at 14 ± 2°C under 16 hr photoperiod using a cold, white fluorescent light irradiance of 40 μmol $m^{-2}$ $s^{-1}$. Explants were sub-cultured to fresh medium at four week intervals.

Osmotic stress simulation was carried out over one year by culturing plants in MS media supplemented with PEG-8000 (-0.3 MPa). Sub-culturing of treated populations was carried out using the same timeframe as used for conventional propagation of shoots.

*Physiological parameters of damage*

Lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA) by thiobarbituric acid (TBA) assay (Ederli *et al.* 1997). 0.1 g of leaves were homogenized with 2 ml of TCA (1%) and centrifuged at 10.000 *g* for 5 min. 250 μl of the supernatant was mixed with 1 ml of TBA (0.5%) in TCA (20%). Mixtures were incubated in boiling water for 30 min, and then cooled to room temperature. Absorbance was determined at 532 nm and non-specific absorbance at 600 nm (Hodges *et al.* 1999). MDA content was determined using a molar extinction coefficient of 155 $mol^{-1}$ $cm^{-1}$.

Total chlorophyll content in leaves was analysed according to Lichtenthaler methodology (Lichtenthaler & Wellburn 1990). 0.1 g of leaves was homogenized with 2 ml of acetone 80%, maintaining the mortar on ice. Samples were centrifuged at 4°C for 10 min at 10.000 *g*. 50 μl of the supernatant was mixed with 1 ml of acetone 80% and absorbance was determined at 663.6 and 646.6 nm. Total chlorophyll content was calculated using the formula: Chl $a + b = 17.76A_{646.6} + 7.34A_{663.6}$.

Hydrogen peroxide content was determined using a reflectometric method by used Rqflex (Merck), and applying a sensitivity range between of 0.2–20 mg $l^{-1}$. Fresh tissue (0.1 g) was macerated with 2 ml of 50 mM sodium phosphate (pH 7.0), and immediately used for analysis.

*Non-enzymatic antioxidant response*

The extracts were prepared by taking 100 mg of tissue and extracting with 3 ml of 100% methanol. Extracts were sonicated at 50/60 Hz (Cole-Parmer, Model 8851) for 4 hr, and kept a 4°C for 96 hr in darkness prior to analysis.

The total phenolic content was determined using a modified Folin-Ciocalteu colorimetric method (Asami *et al.* 2003). Results were expressed as milligrams of gallic acid equivalents. The flavonoid content was determined using a modified colorimetric method as described by Liu *et al.* (2002). Results were expressed as milligrams of quercetine equivalents. Data are reported as means ± SD for at least three replicates.

Antioxidant activity of methanolic extracts was measured by the bleaching of 1,1-Difenil-2-Picril-Hidrazil (DPPH) cation radical. The DPPH solution is purple ($\lambda$ max = 517 nm), but loses its colour when the radical molecules are stabilized by antioxidants (Blois 1958). The measurement was carried out by determining the curve consumption DPPH in 180 sec, evaluating 50 μl extract with 950 μl of DPPH solution (Brand-Williams *et al.* 1995).

Ascorbate content was determined using a reflectometric method, Rqflex (Merck), and applying a sensitivity range of 25 to 450 mg $l^{-1}$. Fresh tissue (0.1 g) was macerated with 2 ml of 50 mM sodium phosphate (pH 7.0) and immediately used for analysis.

*Effects of PEG-8000 on the enzymatic antioxidant system*

To determine the activities of the antioxidant enzymes, 0.1 g of leaves were homogenized with 2 ml of potassium phosphate buffer 50 mM adjusted at pH 7.0 (extraction buffer). Samples were centrifuged at 4°C for 15 min at 11 000 *g* and the supernatant used for activity determinations. Protein content of samples was determined using a modified Bradford method (1 ml of Bradford reactive, 80 μl of 0.15 M NaCl and 20 μl of supernatants (Bradford 1976). Protein concentration was calculated using a calibration curve made with BSA (1 mg $ml^{-1}$).

APX (EC 1.11.1.11) activity was tested measuring the decomposition of ascorbate at 290 nm for 45 sec. The reaction mixture contained 1 ml of extraction buffer, 5 μl of 30% $H_2O_2$, 40 μl of 10 mM ascorbic acid and 20 μl of the supernatant. Enzyme activity was calculated using a molar extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$ (Zhao & Blumwald 1998).

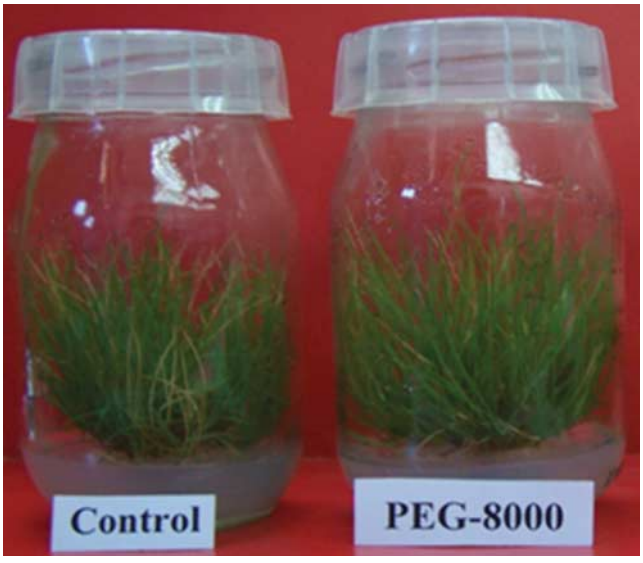


**Fig. 1.** Phenotype of *in vitro* shoots of *D. antarctica* in control media or in PEG-8000 (-0.3 MPa) induced osmotic stress media. Shoots were kept for 12 months under those conditions.

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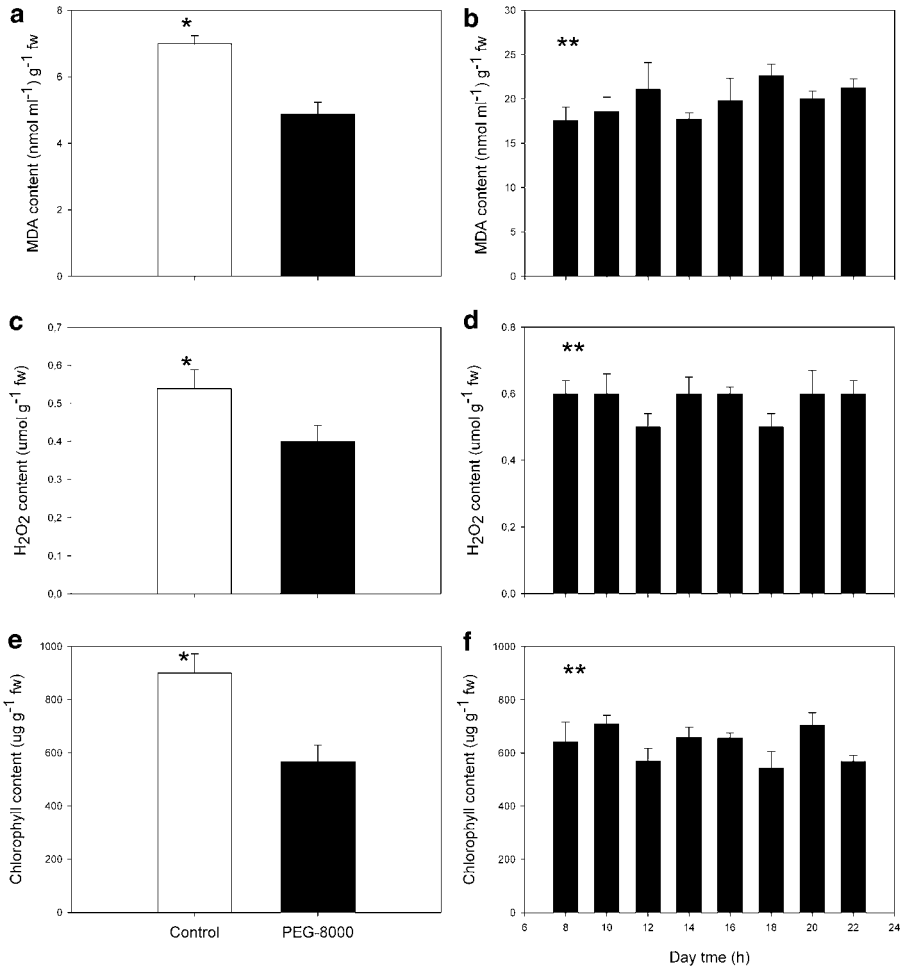


**Fig. 2.** Content of **a, b.** Malondialdehyde, **c, d.** hydrogen peroxide, and **e, f.** total chlorophyll on *in vitro* and *in situ* shoots of *D. antarcti*ca. **a., c. & e.** Control plants and PEG-8000 treated plants, and **b., d. & f.** *in situ* growing plants. Each value corresponds to the mean of six replicates ± standard error ($g^{-1}$ f. wt). Bars with * are significantly different ($P < 0.05$), ** = no significant difference.

CAT (EC 1.11.1.6) activity was tested by measuring the decomposition of hydrogen peroxide at 240 nm for 45 sec. The reaction mixture contained 1 ml of extraction buffer, 3 µl of 30% $H_2O_2$ and 20 µl of the supernatant. Enzyme activity was calculated using a molar extinction coefficient of 39.4 $mM^{-1}$ $cm^{-1}$ (Pinhero *et al*. 1997).

POX (EC 1.11.1.7) activity was tested measuring the appearance of tetraguaiacol at 470 nm for 45 sec. The reaction mixture contained 1 ml of extraction buffer, 5 µl of 30% $H_2O_2$, 5 µl of guaiacol and 10 µl of the supernatant. Enzyme activity was calculated using a molar extinction coefficient of 26.6 $mM^{-1}$ $cm^{-1}$ (Pinhero *et al*. 1997).

The activity of GR (EC 1.6.4.2) was determined by measuring the oxidation of NADPH at 340 nm for 3 min. The reaction mixture contained 1 ml of extraction buffer, 2 mM $Na_2EDTA$, 0.15 mM NADPH, 0.5 mM GSSG and 100 µl extract. Enzyme activity was calculated using a molar extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ (Schaedle & Bassham 1977).

*Statistical analysis*

All studies were performed in triplicate and results were analysed using the *t*-test with a confidence interval of 95%. Analyses were made using the software Statgraphics Plus 5.1 software (Manugistics Inc, Rockville, MD, USA).

## Results

*Deschampsia antarctica* shoots were successfully introduced into *in vitro* cultivation by use of PM and a proper sub-culturing rate was set up every 21–30 days. Within five months, shoot populations reached approximately 200 individuals in a general healthy looking state. The population was then split into control shoots kept under PM, and treated shoots, kept and sub-cultured in treatment medium (TM). During one year of cultivation in TM, shoots showed no visual sign of damage by browning, chlorosis, leaf deformation or stunting, and their sub-culturing time was similar to control shoots (Fig. 1).

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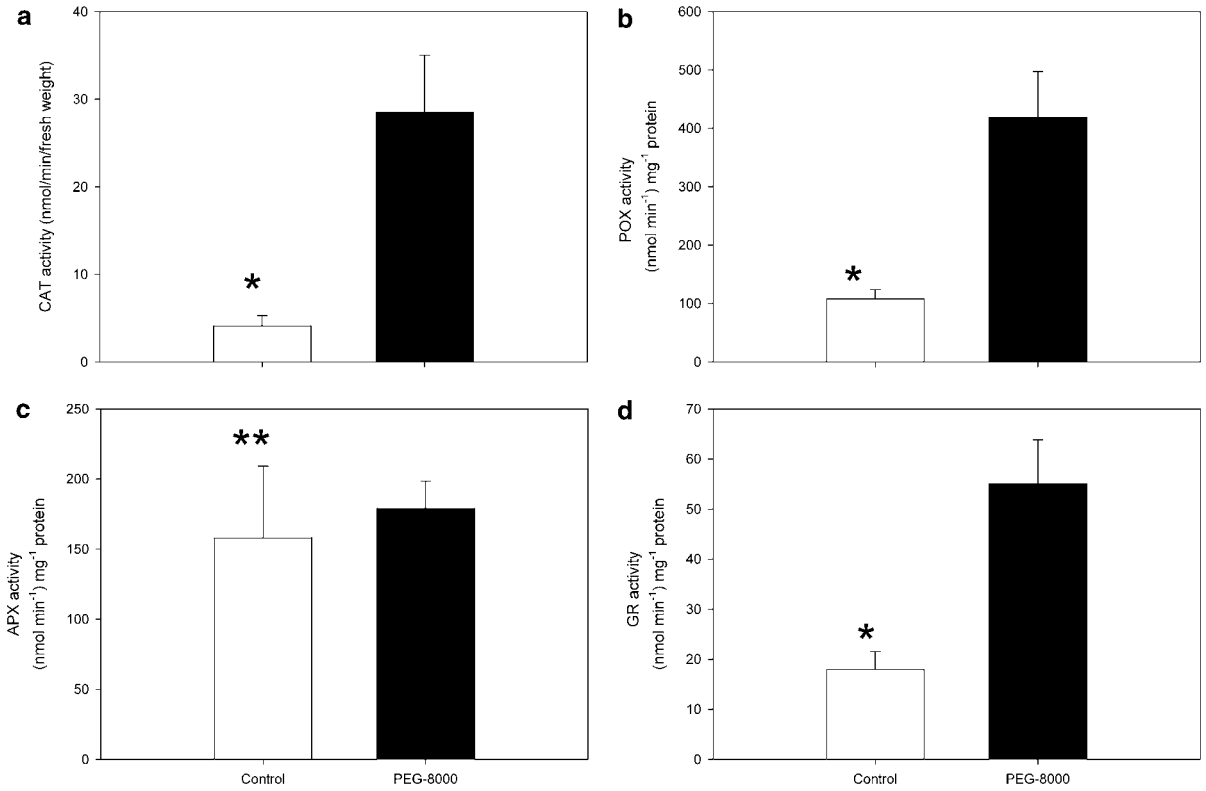


**Fig. 3.** Effect of PEG-8000 induced osmotic stress on the activities of the antioxidant enzymes **a.** catalase (CAT), **b.** guaiacol peroxidase (POX), **c.** ascorbate peroxidase (APX), and **d.** glutation reductase (GR) in *D. antarctica* shoots. Each value represents the mean of six replicates ± standard error. Bars with * are significantly different ($P < 0.05$), ** = no significant difference.

*Effect of PEG-8000 - physiological parameters of damage*

Despite the fact that PEG-8000 did not affect any visual phenotypic characteristic in the shoots, its effect on levels of MDA, chlorophyll and hydrogen peroxide was compared with those in samples of plants growing under field conditions in the Antarctic. Figure 2 shows that MDA content was lower in treated than in control plants. It is important to notice that the levels of MDA in shoots growing in medium without PEG-8000, are significantly lower than levels found in the samples obtained from the Antarctic zone ($P < 0.05$) (Fig. 2a). In these plants, TBARS levels were about four-fold higher than *in vitro*, but remained unchanged during the day. Antarctic conditions did not induce signs of oxidative stress in plants of *D. antarctica*. In addition, the levels of hydrogen peroxide

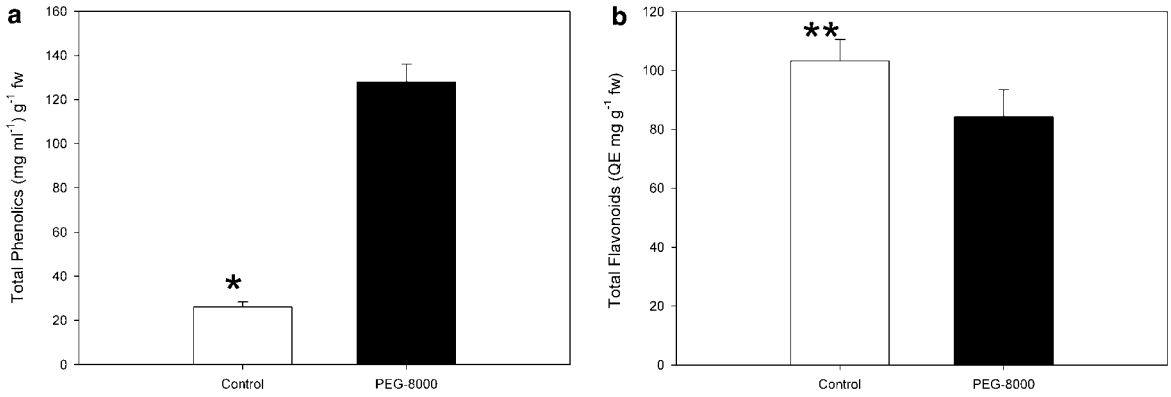


**Fig. 4.** Effect of PEG-8000 induced osmotic stress in the content of **a.** total phenolic compounds, and **b.** flavonoids in *D. antarctica* shoots. Each value represents the mean of six replicates ± standard error. (QE = quercetine equivalent). Bars with * are significantly different ($P < 0.05$), ** = no significant difference.

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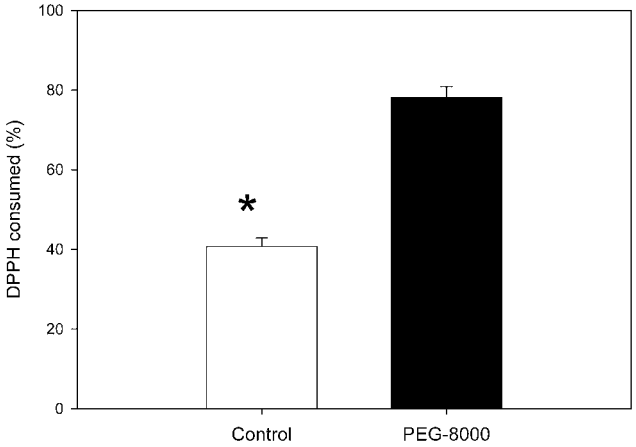


**Fig. 5.** Effect of PEG-8000 induced osmotic stress in the antioxidant capacity of leaf extracts of *in vitro* shoots of *D. antarctica*. Antioxidant capacity was expressed as percentage of the free radical $DPPH^+$ consumed in 180 sec of reaction. Each value represents the mean of six replicates ± standard error. * is significantly different ($P < 0.05$).

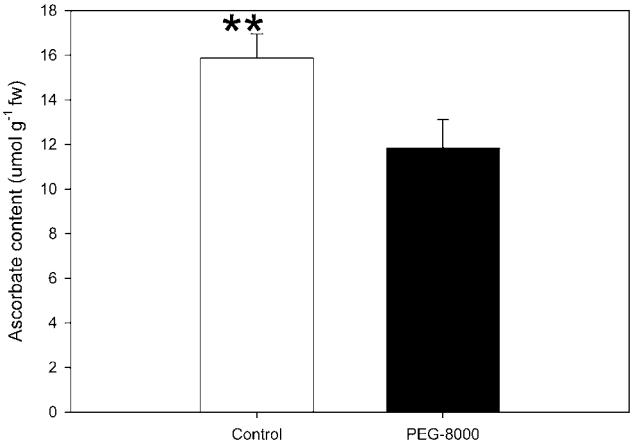


**Fig. 6.** Effect of PEG-8000 induced osmotic stress in the ASC content in shoots of *D. antarctica*. Each value represents the mean of six replicates ± standard error. ($\mu mol\ g^{-1}$ f. wt). * is significantly different ($P < 0.05$).

and total chlorophyll in plants treated with PEG-8000 decreased significantly compared to control plants ($P < 0.05$) (Fig. 2b & c). These results suggest that *in vitro* shoots of *D. antarctica* are capable of controlling the oxidative damage induced by PEG-8000.

### *Effect of PEG-8000 on antioxidant enzyme activities*

ROS can be controlled by efficient enzymes (Jiang & Zhang 2002). For this reason we evaluated the effect of PEG-8000 on the activity of antioxidant enzymes such as CAT, APX, GR and POX on *in vitro* shoots of *D. antarctica* (Fig. 3). The activity of CAT in plants grown in the presence of PEG increases significantly as compared to control plants (seven-fold) ($P < 0.05$) (Fig. 3a).

PEG-induced activities of POX in *D. antarctica* were four-fold higher than controls ($P < 0.05$) (Fig. 3b). APX activity did not show significant changes ($P < 0.05$) (Fig. 3c) in PEG treated plants. Additionally, the activity of GR found in shoots of plants treated with PEG-8000 showed a three-fold increase compared to control shoots ($P < 0.05$). These results suggest that the null effect observed in plants growing in PEG-8000 could be due, at least in part, to the induction of the activity of these antioxidant enzymes (CAT, GR and POX).

### *Effect of PEG-8000 on non-enzymatic antioxidant system*

In order to determine whether the non-enzymatic antioxidant system is affected by PEG-8000, concentrations of phenolic compounds and flavonoids were measured (Fig. 4). The content of phenolic compounds was significantly increased in plants growing in media with PEG-8000 (five-fold) with respect control shoots ($P < 0.05$) (Fig. 4a). In addition, the content of flavonoids, did not change significantly between shoots from both media ($P > 0.05$) (Fig. 4b).

Changes in the content of total phenolic compounds in shoots of control and stressed *D. antarctica* plants are presented in Fig. 4. The results show that the total content of phenolic compounds in methanolic extracts of PEG-8000 treated plants are higher than in control ones ($P > 0.05$), suggesting that the increased levels of phenolic compounds could have a function related to ROS detoxification. Methanolic extracts showed antiradical activity against the DPPH radical (Fig. 5).

Other molecules that showed changed levels under osmotic stress were ascorbate and glutathione. Figure 6 shows that the level of ascorbate content in plants subjected to PEG-8000, were significantly lower than levels determined in control shoots ($P < 0.05$). These differences could be attributed to severe changes in steady-state of this molecule, which could be caused by its over consumption in addition to a relatively low rate of regeneration.

## Discussion

In this study, *D. antarctica* was shown to be tolerant to PEG-induced osmotic stress. It is already known that free radicals generated under stress conditions induce peroxidation of lipid membranes (Jain *et al.* 2001). In the present work, MDA levels and $H_2O_2$ did not increase as a result of PEG-8000 treatment. In addition, MDA and $H_2O_2$ remained constant during a daily cycle in plants growing under Antarctic conditions. These results suggest that *D. antarctica* has efficient mechanisms to control the generation of ROS.

To resist oxidative damage, plants possess antioxidant defence systems that include metabolites such as ascorbate and glutathione (GSH), present in tissues at mM concentrations. Another part of the ROS scavenging

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system depends on enzymes, such as SODs, peroxidases and catalases (Noctor & Foyer 1998, Asada 1999). These enzymes catalyze redox reactions, many of which rely on electrons supplied by reductants of low molecular weight, (Noctor & Foyer 1998). In this work we observed enhanced activities of CAT, POX and GR and high antioxidant activity of methanolic extract in plants treated with PEG-8000. It has been suggested that constitutive levels of CAT and POX were always higher in species tolerant to osmotic stress (Sairam *et al*. 1998).

Catalases convert $H_2O_2$ to water and $O_2$ (Smirnoff 1993). These enzymes have extremely high catalytic rates, but low substrate affinities, since the reaction requires the simultaneous access of two $H_2O_2$ molecules at the active site. An alternative mode of $H_2O_2$ detoxification is via peroxidases, which are present in most organelles and show higher affinity for $H_2O_2$ than CAT. However, peroxidases require a reductant, since they reduce $H_2O_2$ to $H_2O$. In plant cells, the most important reducing substrate for $H_2O_2$ detoxification is ascorbate. This may explain the decreased content of this molecule in plants treated with PEG-8000. Ascorbate is a powerful antioxidant, which reacts directly with $O_2^-$ and $HO^-$ radicals. It has an important role in preserving the activities of enzymes that contain prosthetic groups with transition metal ions. Apoplastic ascorbate is also considered crucial in scavenging ROS, particularly those arising from exposure to atmospheric pollutants such as ozone. Ascorbate may also be involved in the regulation of the cell cycle (Kerk & Feldman 1995). Arrest of cell cycle during oxidative stress prevents replication of damaged DNA, and appears to correlate with a decreased ratio of ascorbate to DHA (Sánchez-Fernández *et al*. 1997).

GR also plays a key role in oxidative stress by converting oxidized glutathione, GSSG to GSH (Fadzilla *et al*. 1997). Increased GR activity in corn plants has been reported to be closely related with drought tolerance (Pastori & Trippi 1993). In our experiments GR activity increased in *D. antarctica* under osmotic stress. It has been shown that $O_2^-$ and $H_2O_2$ generated during water stress might be responsible for the induction of GR (Baisak *et al*. 1994). The increase in GR in *D. antarctica* plants treated with PEG-8000, might have resulted in a higher pool of GSH, which could be used in ascorbate generation. GR could play a key role in protection against oxidative stress (Gamble & Burke 1984).

By analysing the antioxidant activity of shoot extracts, it was demonstrated that methanolic extracts had the capacity to scavenge DPPH free radicals (Fig. 4). A positive correlation was demonstrated between the overall content of phenolic compounds in the extracts and their antioxidant capacity (Fig. 5). The methanolic extract is being characterized to identify the molecules with antioxidant activity.

In conclusion, based on the data obtained from MDA, $H_2O_2$ and chlorophyll content, it is clear that *D. antarctica* is tolerant to osmotic stress. It is possible that this osmotic stress tolerance is associated with its ability to induced activity of CAT, POX and GR resulting in lower $H_2O_2$ production and lipid peroxidation.

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