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Dynamic responses of photosynthesis and the antioxidant system during a drought and rehydration cycle in peanut plants

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Abstract. Drought stress is one of the most important environmental factors that adversely affect the productivity and quality of crops. Most studies focus on elucidating plant responses to this stress but the reversibility of these effects is less known. The aim of this work was to evaluate whether drought-stressed peanut (*Arachis hypogaea* L.) plants were capable of recovering their metabolism upon rehydration, with a focus on their antioxidant system. Peanut plants in the flowering phase (30 days after sowing) were exposed to drought stress by withholding irrigation during 14 days and subsequent rehydration during 3 days. Under these conditions, physiological status indicators, reactive oxygen species production and antioxidant system activity were evaluated. Under drought stress, the stomatal conductance, photosynthetic quantum yield and $^{13}C:{}^{12}C$ ratio of the peanut plants were negatively affected, and also they accumulated reactive oxygen species. The antioxidant system of peanut plants showed increases in superoxide dismutase-, ascorbate peroxidase- and glutathione reductase-specific activities, as well as the total ascorbate content. All of these responses were reversed upon rehydration at 3 days. The efficient and dynamic regulation of variables related to photosynthesis and the antioxidant system during a drought and rehydration cycle in peanut plants was demonstrated. It is suggested that the activation of the antioxidant system could mediate the signalling of drought stress responses that enable the plant to survive and recover completely within 3 days of rehydration.

Additional keywords: ascorbate, *Arachis hypogaea* L., reactive oxygen species, water stress.

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Introduction

Abiotic stresses are the primary causes of crop loss worldwide, with drought stress being particularly relevant in semiarid regions. At the molecular and cellular levels, ABA is the first signal molecule that elicits responses to cope with drought stress (Xia *et al*. 2015). One of these responses is the trigger of reactive oxygen species (ROS) production. Superoxide anion (O_2) and H_2O_2 may act as signals to develop adaptive responses (Foyer and Noctor 2015). However, they also can be the source of other damaging ROS, such as lipid-derived radicals and the·OH radical, in the presence of trace amounts of Fe, Cu or other transition metals, through the Fenton reaction (Halliwell and Gutteridge 1999). ROS negatively affects plant development by causing oxidative damage of lipids and proteins (Sassi *et al*. 2008; Zabalza *et al*. 2008).

Plants possess an efficient defence system comprising nonenzymatic and enzymatic antioxidants to scavenge overproduced ROS, thereby avoiding their deleterious effects. Ascorbate (ASC) and glutathione (GSH) are potent nonenzymatic antioxidants within plant cells, so that cells that participate in the ASC–GSH cycle are capable of detoxifying H_2O_2. The antioxidant enzymes include superoxide dismutase (SOD), which reacts with superoxide radicals and converts them to O_2 and H_2O_2; catalase (CAT) and peroxidases (POD), which detoxifies H_2O_2; and ascorbate peroxidase (APX) and glutathione reductase (GR), which operate in the ASC–GSH cycle. In addition, GST catalyses the *S*-conjugation of reactive compounds with GSH (Foyer and Noctor 2009). Antioxidant enzyme activities are broadly used in studies dealing with plant stress responses; additionally, plant stress tolerance may be improved by the enhancement of *in vivo* levels of antioxidant enzymes (Cruz de Carvalho 2008; Gill and Tuteja 2010).

Peanut (*Arachis hypogaea* L.) is an economically important legume crop throughout the world. All landraces of peanuts are probably derived from one or a few plants. This has constrained the advances in genetics that are necessary for efficient modern breeding and has led to a very limited diversity in some traits of agricultural interest (Moretzsohn *et al*. 2005). The limited variability of the cultivated tetraploid peanut has been explained as the result of a genetic bottleneck resulting from a single domestication event ~3500 years ago (Simpson *et al*. 2002; Kottapalli *et al*. 2007). Other reasons are the nature

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of self-pollinating species, its origin from one or few events of hybridisation in a relatively recent evolutionary time and the application of rigorous selective breeding schemes (Pozzi *et al.* 2014). In Argentina, the peanut production area intermittently suffers water deficit periods almost every year, leading to important losses (Collino *et al.* 2001). Studies focussed on peanut antioxidant activity and expression have revealed that plants exposed to water stress by adding polyethylene glycol showed increased APX or GR and CAT activity, depending on the cultivar (Celikkol Akcay *et al.* 2010). Under these conditions, molecular studies have shown that 52 genes are induced, two of which correspond to sequences coding for antioxidant enzymes (APX and GST) (Luo *et al.* 2005*a*). These authors also identified the induction of genes related to the antioxidant system in leaves (GST and CuZn-SOD) and immature pods (GST, CAT, CuZn-SOD and APX) on drought-stressed peanut (Luo *et al.* 2005*b*). These articles contribute to our understanding of peanut's responses to drought stress but the antioxidant enzyme activity has not been elucidated during the rehydration period. In natural environments, drought stress is usually intermittent and hence the rehydration process is an important phenomenon to be considered. Moreover, biotechnological approaches for improving peanut tolerance to drought stress are scarce (Bhatnagar-Mathur *et al.* 2007). Therefore, this research is expected to provide information towards understanding the stress and rehydration response model of this legume. Besides this, it hopes to identify the genes that are able to be used in biotechnological approaches aimed towards generating plants capable of growing under adverse environmental conditions.

Previously, we have demonstrated that drought stress negatively affected peanut growth and that plants showed ABA accumulation and oxidative damage, which were completely reversed upon rehydration for 3 days (Furlan *et al.* 2012). The aim of this work was to evaluate whether drought-stressed peanut plants were capable of recovering their metabolism upon rehydration, with a focus on antioxidant system. The hypothesis was that physiological parameters relating to photosynthetic activity and the antioxidant system are sensitive to stress conditions and that these have sufficient functional plasticity to reverse upon rehydration. Moreover, the resilience of plants is an important aspect to be considered in order to improve productivity in natural environments experiencing intermittent drought.

Materials and methods

Plant material and treatments

Seeds of peanut (*Arachis hypogaea* L.) cv. Granoleico (Criadero El Carmen, General Cabrera, Córdoba, Argentina) were surface-sterilised (Vincent 1970) and pregerminated in Petri dishes for 96 h. Pregerminated seeds were transferred to pots that had a diameter of 8 cm and a height of 12 cm. Each pot was filled with 200 g of sterile volcanic sand. Plants were grown in a controlled growth chamber (light intensity: 200 µmol m^{-2} s^{-1}; 16-h day 8-h night cycle; 28°C; relative humidity, 50%). The strain *Bradyrhizobium* sp. SEMIA6144, which is able to infect peanut plants, was provided by MIRCEN (Porto Alegre, Brazil). Seven days after sowing, plants were inoculated with 4 mL of yeast extract–mannitol culture containing 10^8 CFU (colony forming units) mL^{-1}. In order to keep the soil water at 13%, which was determined to correspond to field capacity of volcanic sand via the pressure-plate method (Burk 1996), plants were irrigated twice a week alternately with distilled water and Hoagland nutrient solution without N (Hoagland and Arnon 1950). Thirty days after sowing plants at the beginning of the flowering growth stage (R1) (Boote 1982) were separated at random into three experimental groups: (a) the control, where plants were kept under normal irrigation conditions (soil water content at 13%); (b) drought stress, where the irrigation was suspended during 14 days and (c) rehydrated, where plants subjected to drought stress were reirrigated during 3 days. The water potential of plants subjected to different treatments was measured in the second expanded leaf from the top of the main stem of each plant collected between 1000 and 1200 hours using a pressure bomb (Model 10, Bio-Control, Buenos Aires, Argentina) (Scholander *et al.* 1964). The plant water potentials (Ψ_w) for the different treatments were: control Ψ_w, –0.22 MPa; drought stress Ψ_w, –0.8 MPa; rehydrated Ψ_w, –0.34 MPa. Leaves were harvested into liquid N and stored at −80°C until use.

Physiological status indicators

At the end of the water-deficit stress treatment (14 days without irrigation) and the rehydration treatment (14 days without irrigation followed by 3 days of reirrigation), stomatal conductance was measured on fully expanded second nodal leaves at midday (1000–1300 hours) using a portable porometer (SC-1, Decagon Devices Inc., Pullman, WA, USA) by the steady-state method as indicated in the operator's manual (version 11). Using another pair of leaves under the same conditions as above, the effective quantum yield of PSII (ΦPSII) was measured using the Mini-PAM Fluorometer (Walz, Germany) set at a light intensity of 230 µmol quanta m^{-2} s^{-1} under each treatment as indicated.

The δ^{13} C analysis was carried out at the Archeometry Department, University of Cape Town, South Africa. Fully expanded leaves from the second node were oven-dried and milled in a Wiley mill using a 0.5-mm mesh (Arthur H Thomas, Philadelphia, PA, USA). Between 2.1 mg and 2.2 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd, Okehampton, Devon, UK) on a Sartorius Cubis® Ultramicro balance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 Series 2 CHN analyser (Fisons Instruments SpA, Milan, Italy). The C isotopic ratio of a sample was expressed as:

$$\delta^{13}C = \left(\frac{R(\text{sample})}{R(\text{standard})} - 1\right) \times 1000,$$

where, $\delta^{13}C$ is the isotope ratio in δ units relative to a standard based upon CO_2 derived from limestone from the Pee Dee formation in South Carolina, and R(sample) and R(standard) are the absolute isotope ratios of the sample and standard, respectively (Mortimer *et al.* 2008, 2009). The $\delta^{13}C$ values thus calculated are expressed in parts per thousand.

In situ *detection of ROS*

In order to detect O_2^- production, fully expanded leaves from the second node were detached and vacuum-infiltrated with 10 mM

sodium citrate buffer (pH 6) containing 1 mM nitroblue tetrazolium (NBT) and were incubated for 8 h at 25°C under light. They were then boiled in 80% (v/v) ethanol for 10 min and photographed using an Axiophot microscope (Carl Zeiss, Göttingen, Germany) (Frahry and Schopfer 2001).

For the detection of H_2O_2, fully expanded leaves from the second node were detached and incubated with 1 mg mL^{-1} diaminobencidine-HCl (pH 3.8) for 16 h in darkness at room temperature. Afterwards, they were exposed to light for 15 min, boiled in 96% (v/v) ethanol as described for O_2^- and visualised using an Axiophot microscope (Zilli *et al*. 2009).

Antioxidant enzyme activities

The SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) enzyme extracts were derived from fully expanded leaves from the second node extracted in a medium consisting of 50 mM potassium phosphate (pH 7.8), 0.5 mM EDTA, insoluble polyvinylpyrrolidone and 0.5% (v/v) Triton X-100 (Sigma-Aldrich). The supernatants were obtained after centrifugation at 10 000*g* for 12 min and were used to determine enzymatic activities. The total protein content was assessed according to Bradford (1976) using BSA as a standard. References on the methodologies used to quantify the specific activity of each antioxidant enzyme and its units are listed in Table 1.

SOD isozymes were individualised and identified on 11% polyacrylamide native gels by incubation with specific inhibitors (5 mM potassium cyanide-KCN or 10 mM H_2O_2 for 1 h) and subsequent staining for SOD activity (Rao *et al*. 1995). Activity bands resistant to KCN but inhibited by H_2O_2 were assigned to Fe-SOD isozymes and those resistant to both inhibitors to Mn-SOD isozymes. SOD activity was determined according to Beauchamp and Fridovich (1973) in a O_2^--generating solution containing NBT in the presence of riboflavin. One mL of the reaction mixture (0.54 µM EDTA, 75 µM NBT, 777 µM methionine and 50 mM phosphate buffer at pH 7.8), 4 µM riboflavin and 100 µg protein extract was placed under fluorescent light for 15 min to allow the O_2^- production. The absorbance was determined spectrophotometrically at 560 nm (Spectronic® Genesys 2, Thermo Scientific, Madison, WI, USA) and SOD-specific activity was expressed as units mg^{-1} protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the reduction of NBT.

CAT activity was measured using the method described by Aebi (1984). The assay mixture in 1 mL contained 25 µg protein extract, 50 mM phosphate buffer at pH 7.5 and 5 mM H_2O_2. The reaction was measured by the H_2O_2 decomposition at 240 nm. One unit of CAT is defined as the quantity of enzyme needed to degrade 1 µmol H_2O_2 min^{-1}.

APX (EC 1.11.1.11), GR (EC 1.6.4.2), GPX (EC 1.11.1.9) and GST (2.5.1.18) were extracted in a medium containing 100 mM potassium phosphate at pH 7.4 and 1 mM EDTA. The extraction buffer for APX was supplemented with 5 mM ASC, which allows the measurement the total activity, since APXs from organelles are inactive in extraction media without ASC (Dalton *et al*. 1986; Amako *et al*. 1994). Protein concentration was measured as described above.

APX activity was assayed as described by Nakano and Asada (1987). The reaction was developed using 12.5 µL of protein extract, 50 mM phosphate buffer at pH 7.0, 0.5 mM ASC, 0.1 mM EDTA and 0.1 mM H_2O_2 in a total volume of 1 mL. The decomposition of H_2O_2 was followed at 290 nm. One unit of APX is defined as the quantity needed to degrade 1 mmol H_2O_2 min^{-1}.

GR activity was determined as described by Schaedle and Bassham (1977). One mL of the assay mixture contained 50 µL protein extract, 0.5 mM oxidised glutathione (GSSG) and reaction buffer (50 mM Tris-HCl, 0.15 mM NADPH and 3 mM $MgCl_2$ at pH 7.5). A reaction without GSSG was performed in order to determine the nonspecific consumption of NADPH. The reduction of GSSG by GR was followed by NADPH oxidation at 340 nm. One unit of GR was defined as the quantity of enzyme needed to produce 1 µmol $NADP^+$ min^{-1}.

GPX activity was determined as described by Flohé and Gunzler (1984). One mL of the assay mixture contained 50 µL of protein extract, 0.1 M phosphate buffer at pH 7.4, 0.24 U GR and 10 mM GSH. Next, 1.5 mM NADPH and 1.5 mM H_2O_2 were added. The reaction was measured following NADPH oxidation at 340 nm. One unit of GPX was defined as the quantity of enzyme needed to produce 1 µmol $NADP^+$ min^{-1}.

GST activity was determined by measuring the conjugation of GSH with 1-chloro-2 4-dinitrobenzene (CDNB) as described by Habig *et al*. (1974). One mL of the assay mixture contained 125 µL 0.25 mM CDNB, 400 µL 4 mM GSH, 200 µg of protein extract and a phosphate buffer (0.1 M, pH 7). The reaction was measured following the absorbance at 340 nm for 5 min. The extinction coefficient of CDNB (9.6 mM^{-1} cm^{-1}) was used to calculate GST-specific activity. One unit of GST produced 1 µmol CDNB-GSH conjugate min^{-1}.

Total and reduced ASC contents

Total (reduced ASC + dehydroascorbate (DHA)) and ASC content were measured based on the method of Law *et al*.

Table 1. Units used for measuring enzyme levels in peanut under drought stress and rehydration
SOD, Superoxide dismutase; CDNB, 1-chloro-2 4-dinitrobenzene; GSH, glutathione

Enzyme	Reference	Units
SOD	Beauchamp and Fridovich (1973)	U SOD mg^{-1} protein
Catalase	Aebi (1984)	mmol H_2O_2 min^{-1} mg^{-1} protein
Ascorbate peroxidase	Nakano and Asada (1987)	µmol ascorbate min^{-1} mg^{-1} protein
Glutathione reductase	Schaedle and Bassham (1977)	µmol $NADP^+$ min^{-1} mg^{-1} protein
Glutathione peroxidase	Flohé and Gunzler (1984)	µmol $NADP^+$ min^{-1} mg^{-1} protein
GST	Habig *et al*. (1974)	µmol CDNB–GSH conjugate min^{-1} mg^{-1} protein

(1983) with modifications. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ASC in an acidic solution. Fe^{2+} then forms complexes with bipyridyl, giving a pink colour that absorbs at 525 nm. Total ASC was determined through a reduction of DHA to ASC by dithiothreitol. Leaves (0.1 g) were homogenised in liquid N with 10% (w/v) trichloroacetic acid (1 : 10) and centrifuged at 10 000*g* for 20 min. Half a sample of supernatant was assayed for total ASC content and the other half was assayed for ASC only. DHA concentration was then deduced from the difference. A 100-µL sample of the supernatant was added to an equal volume of 150 mM sodium phosphate buffer (pH 7.4) and 2 mM DTT. After vortex-mixing, the sample was allowed to stand at room temperature in darkness for 10 min. This mixture was added to the assay reaction, 1 mL of which contained 0.025% (w/v) *N*-ethylmaleimide, 8.8% (v/v) ortophosphoric acid, 2% (w/v) trichloroacetic acid, 0.8% (w/v) 2–2'-bypiridil in 70% (v/v) ethanol and 0.3% (p/v) $FeCl_3$. *N*-ethylmaleimide was omitted for ASC determination and this volume of DTT was replaced with distilled water when added to the. After vortex-mixing, samples were incubated at 37°C for 60 min, the reaction was stopped on ice and the absorbance at 525 nm was recorded. A standard curve in the range of 0–50 nmol of ASC was used for calibration.

Total and oxidised GSH contents

Leaves (0.1 g) were homogenised in liquid N with 5% (w/v) 5-sulfosalicylic acid (1 : 10) and centrifuged at 10 000*g* at 4°C 10 min to remove cell debris. The supernatants were used for measuring the GSH content by following the method of Anderson (1985) using baker's yeast GSH reductase. The supernatant (25 µl) was added to 0.174 mg mL^{-1} NADPH, 0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) and distilled water to complete 1 mL. This mixture was incubated at 30°C for 15 min. Next, 5 µL of GR (266 units mL^{-1}) was added. The reaction was followed at 412 nm and the total GSH content was calculated from a standard curve using GSH (0–30 µmol). In order to measure GSSG, the supernatant was incubated with 2 µL of 2-vinylpyridine followed by 5 µL of triethanolamine (TEA), the latter being placed on the side of the tube above the level of the liquid. The addition of 2-vinylpyridine and TEA ensured that GSSG was the only form of GSH that could react with the 5,5′-dithio-bis(2-nitrobenzoic acid) reagent. The solution was vortex-mixed for 30 s and left at room temperature for 60 min. Afterwards, 100 µL of the resulting solution was assayed as described above. Calibration curves were carried out using GSSG samples (0–10 µmol) treated exactly as above. The intracellular GSH content was expressed as µmol GSH mg^{-1} protein.

Statistical analysis

Experiments were conducted in a completely randomised design and were repeated three times. The data were analysed using ANOVA and Duncan's test at $P \leq 0.05$. Prior to the test of significance, the normality and homogeneity of variance were verified using the modified Shapiro–Wilk and Levene tests, respectively. If the homogeneity of variance was not given, data were transformed using an appropriate function.

Results

Physiological status of peanut

Stomatal conductance was reduced ~35% in plants exposed to drought stress for 14 days; however, after 3 days of rehydration plants increased these values and did not show a statistical difference from well-watered plants (Fig. 1). The quantum yield of PSII determined by chl a fluorescence analysis dropped by 50% in drought-stressed plants. The photosynthetic quantum yield of rehydrated plants increased, reaching values that did not differ statistically from the control (well-watered) plants (Fig. 2). The $\delta^{13}C$ of drought-stressed plants was more positive compared with that of the control plants, which indicated enrichment in ^{13}C. However, upon rehydration, the $\delta^{13}C$ value was restored to normal and was comparable to the well-watered control plants (Fig. 3).

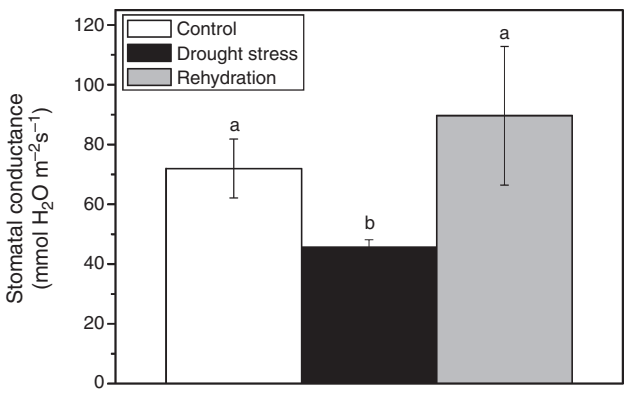

Fig. 1. Stomatal conductance in peanut leaves exposed to drought stress and rehydration. Values are means ± s.e. ($n = 9$). Different letters in each column indicate significant differences at $P < 0.05$ according to Duncan's test.

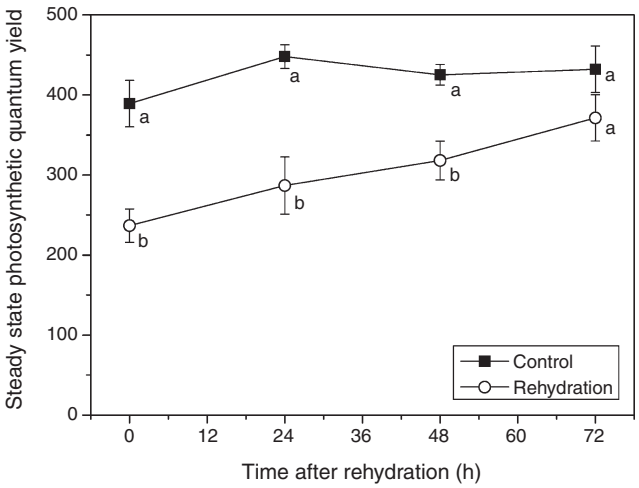

Fig. 2. Photosynthetic quantum yield in peanut leaves exposed to drought stress and rehydration. Values are means ± s.e. ($n = 9$). Different letters at each time-point indicate significant differences at $P < 0.05$ according to Duncan's test.

ROS production

Superoxide production was visualised by the formation of a blue precipitant (formazan) resulting from the reaction of NBT with O_2^-. Control and rehydrated leaves showed fewer O_2^- formazan deposits than those subjected to drought conditions (Fig. 4*a–c*). H_2O_2 production was detected through diaminobencidine staining, which relies on the presence of tissues peroxidases. H_2O_2 detection in control and rehydrated leaves was lower than in those exposed to drought stress (Fig. 4*d–f*).

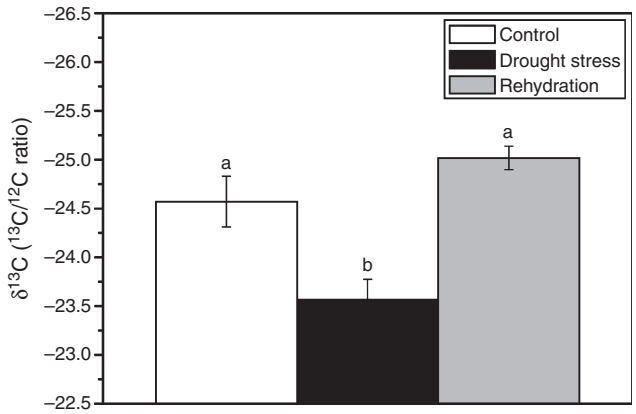

Fig. 3. δ^{13} C in peanut leaves exposed to drought stress and rehydration. Values are means ± s.e. ($n = 5$). Different letters indicate significant differences at $P < 0.05$ according to Duncan's test.

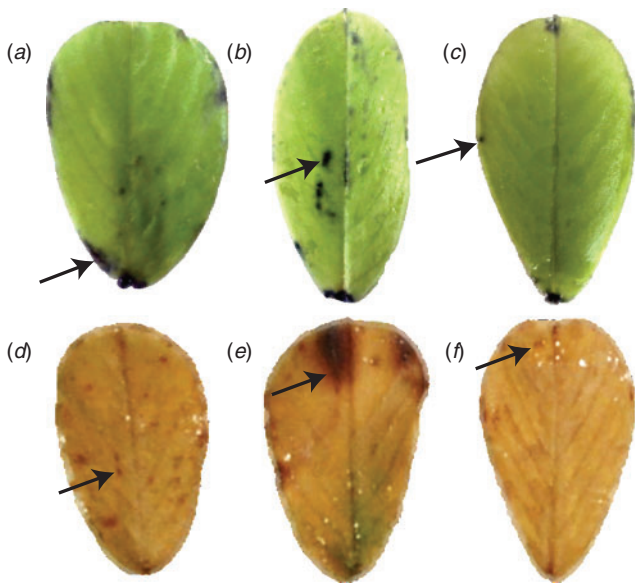

Fig. 4. Histochemical staining of O_2^- and H_2O_2 in peanut leaves. (*a*) Control, (*b*) drought-stressed and (*c*) rehydrated (3 days) leaves, after treatment with nitroblue tetrazolium. Arrows indicate formazan precipitate. (*d*) Control, (*e*) drought-stressed (*f*) and rehydrated leaves after treatment with diaminobencidine (DAB). Precipitate resulting from the H_2O_2–DAB reaction is indicated by arrows. The images presented are representative of several analysed leaves ($n = 6$). Scale bars = 1 mm.

Antioxidant system activity

Six SOD isozymes were detected according to their mobility and different inhibition by H_2O_2 or KCN (data not shown). They were classified as Mn-SOD I, Mn-SOD II, Fe-SOD, CuZn-SOD I, CuZn-SOD II and CuZn-SOD III (Fig. 5). Peanut plants exposed to drought stress increased antioxidant enzyme activity of SOD, APX and GR in leaves. SOD and APX activities increased by ~30% under drought stress conditions, whereas GR doubled its activity. All increases were reversed upon rehydration. The specific activity of CAT, GPX and GST showed no differences between treatments (Fig. 6). Total ASC content increased by ~40% during drought and this variation was explained by increases in ASC and DHA contents. After rehydration, plants showed similar values to control plants. Total GSH and GSH leaf contents did not indicate differences among treatments, whereas GSSG content decreased by ~50% in drought-stressed and rehydrated plants (Table 2).

Discussion

Drought stress is an environmental constraint affecting plant growth and crop production, and has attracted an increasing level of interest because of the economic losses produced. After exposure to drought, plants increase ROS production, with ROS acting as damaging and signalling molecules to counteract the stressful conditions. Plants suffering drought stress decrease their photosynthetic rate because of low CO_2 availability in response to stomatal closure, as regulated by ABA accumulation (Mittler and Blumwald 2015). The effects of drought stress on peanut stomatal conductance are consistent with previous results that indicated that peanut plants accumulate ABA in response to drought stress. Besides this, after rehydration, both parameters showed the same pattern, suggesting a tight and coordinated functional relationship (Furlan *et al*. 2012). In a similar manner, the quantum yield of PSII was lower during drought and increased after rehydration. Moreover, the increase in δ^{13}C (higher $^{13}C : {}^{12}C$ ratio = less negative) in the drought-stressed

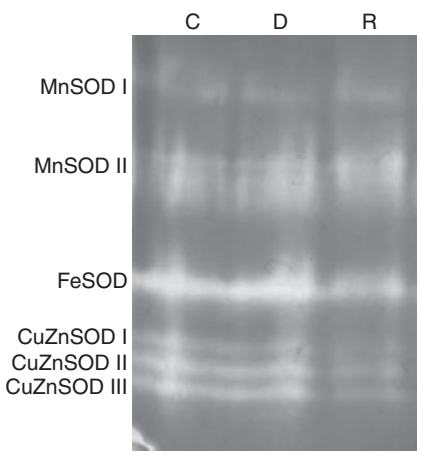

Fig. 5. Superoxide dismutase (SOD) isozyme activity in peanut leaves following drought stress and rehydration in nondenaturing polyacrylamide gels. C, control; D, drought stress; R, rehydration. All lanes were loaded with 25 µg protein.

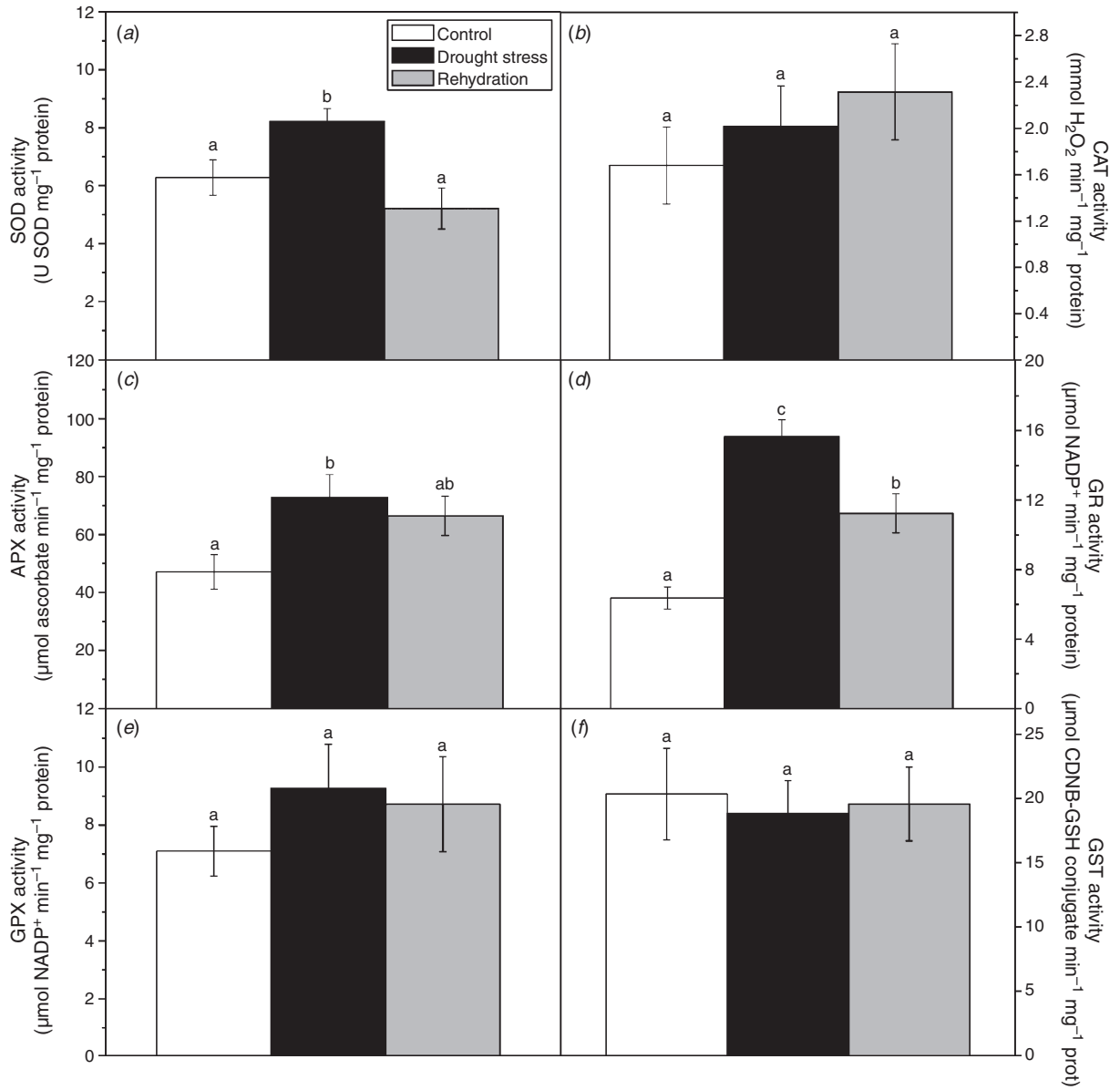

Fig. 6. Effect of drought stress and rehydration on antioxidant enzyme activities in peanut leaves. Values are means ± s.e. ($n = 6$). Different letters in each column indicate significant differences at $P < 0.05$ according to Duncan's test. (*a*) Superoxide dismutase (SOD); (*b*) catalase (CAT); (*c*) ascorbate peroxidase (APX); (*d*) glutathione reductase (GR); (*e*), glutathione peroxidase (GPX); (*f*) glutathione-S-transferase (GST).

peanut plants was another indicator revealing that plants were experiencing water stress. The altered $^{13}C:^{12}C$ ratio may be explained by the stomatal closure under drought, which would result in an increase of ^{13}C fixation, leading to less ^{13}C discrimination (Kume *et al*. 2003; Bellaloui 2011). However, the partial stomatal closure was restored to normal and was comparable to the well-watered control plants upon rehydration. The results suggest that impaired photosynthetic activity may be restored upon rehydration. These findings are supported by earlier studies that revealed a strong relationship between net photosynthesis and stomatal conductance, which regulate transpiration in peanut (Nautiyal *et al*. 1995).

It is known that ABA triggers H_2O_2 production in plants subjected to drought stress conditions (Cho *et al*. 2009). In peanut, this mechanism was demonstrated as a first response to drought stress (within 12 h), which was then exacerbated by ABA-independent mechanisms (Furlan *et al*. 2013). As expected, in the present study, evidence of H_2O_2 and O_2^- production was demonstrated. This ROS production can be related to the onset of oxidative stress, which was confirmed by prior studies that showed an accumulation of peroxidised lipids (estimated as malondialdehyde (MDA)) and oxidised proteins (estimated as carbonyl groups) (Furlan *et al*. 2012). The relationship between ROS and oxidative damage becomes clear if we consider the

Table 2. Antioxidant metabolite contents of peanut leaves exposed to drought stress and rehydration

Values are means ± s.e. ($n = 6$). Different letters in each row indicate significant differences at $P < 0.05$ according to Duncan's test. ASC, ascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidised glutathione

Antioxidant metabolites	Control (μmol g DW^{-1})	Drought stress (μmol g DW^{-1})	Rehydration (μmol g DW^{-1})
Total ASC	12.03 ± 1.62a	18.78 ± 2.33b	10.47 ± 0.60a
ASC	7.50 ± 1.36ab	10.55 ± 1.26b	6.52 ± 0.91a
DHA	4.53 ± 0.96a	8.22 ± 0.92b	3.94 ± 1.14a
Total GSH	4.66 ± 0.85a	4.36 ± 0.59a	3.96 ± 0.53a
GSH	4.50 ± 0.87a	4.27 ± 0.58a	3.91 ± 0.52a
GSSG	0.15 ± 0.03b	0.08 ± 0.01a	0.05 ± 0.01a

formation of highly oxidising ROS, such as the hydroxyl radical, which, in turn, depends on trace amounts of metal ions in the presence of H_2O_2 (Stadtman 1992; Halliwell and Gutteridge 1999).

A close relationship between oxidative stress and ABA in ROS-dependent drought responses was reported by Noctor *et al*. (2014). The authors described that 70% of the drought-associated genes induced by oxidative stress were also induced by ABA. These percentages were higher than the overall proportion of the drought-induced genes that were induced by ABA (46%). However, the antioxidant system was induced by ABA application in *Stylosanthes guianensis* (Aubl.) Sw. (Zhou *et al*. 2005), *Arabidopsis thaliana* (L.) Heynh. (Bright *et al*. 2006), maize (*Zea mays* L.) (Zhang *et al*. 2007) and *Cynodon dactylon* (L.) Pers. (Lu *et al*. 2009). In our experimental system, ABA accumulation (Furlan *et al*. 2012) may explain the overall increase in antioxidant enzyme activity.

The antioxidant system's response to drought stress and rehydration in plants depends on the species, cultivar, vegetal tissue, and stress duration or intensity (Cruz de Carvalho 2008). In this regard, pea (*Pisum sativum* L.), maize, wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) plants increased their antioxidant activity under drought stress conditions (Mittler and Zilinskas 1994; Jiang and Zhang 2002; Luna *et al*. 2004; Guo *et al*. 2006). In contrast, some authors found that the antioxidant system was nonresponding or decreasing in stressful conditions (Iturbe-Ormaetxe *et al*. 1998; Lascano *et al*. 2001; Guo *et al*. 2006). The variability of responses in different species makes the study of antioxidant activity essential and allows us to generate knowledge that may be used in plant engineering to improve crops. Few studies have focussed on the involvement of antioxidative defence mechanisms in drought tolerance in peanuts (Celikkol Akcay *et al*. 2010) and to date, most studies have focussed on the response of peanut seedlings. Celikkol Akcay *et al*. (2010) compared the antioxidant responses to drought between 16-day-old seedlings of a drought-resistant peanut cultivar (Florispan) and a drought-sensitive cultivar (Gazipasa). They found significant levels of proline accumulation in the drought-resistant cultivar, whereas proline accumulation did not appear to form part of the protection mechanism in the drought-sensitive cultivar. A comparison of enzyme activities indicated that alongside proline accumulation, the activities of the enzymes CAT and APX were important mechanisms for maintenance of drought tolerance in peanut plants. It is noteworthy that testing drought tolerance in field trials is difficult, even if controlled irrigation is available, because of the unpredictable variability of weather, soil, rain and pests or disease (Deikman *et al*. 2012). Furthermore, the current investigation reports on findings from younger plants than would normally be expected to grow to full maturity in agricultural practices. Nonetheless, our findings are about drought stress responses at a specific developmental stage of peanuts and would be applicable to agricultural plants at this same stage. Since these responses are likely to be underpinned by genetic regulation, it can be expected that peanut's responses would be similar at further developmental stages. In peanut, an induction in SOD activity contributing to H_2O_2 accumulation was found and the three known isoforms of SOD (CuZn-SOD, Mn-SOD and Fe-SOD) were detected. The SOD enzyme system is strictly regulated in every plant species at the gene expression level, which is affected by both environmental and developmental stimuli (Bowler *et al*. 1994). Although an induction in the overall activity was seen in the present work, no differences among the isozyme patterns were found. The ASC–GSH cycle-related enzymes involved in H_2O_2 removal (APX and GR) and the total ASC content were increased, indicating that the antioxidant system actively detoxified ROS during the stress period. The increase in APX and GR activity is more common than the decreased or unchanged activity (Cruz de Carvalho 2008). In peanut the results presented are in accordance with previous reports. This work revealed that APX and GR overexpression may be used in peanut biotechnology; this is an approach developed successfully in other plant species such as *Nicotiana tabacum* L., tomato (*Solanum lycopersicum* L.) and wheat (Gill and Tuteja 2010). Costs in biomass production due the overexpression of an enzyme are important issues to consider and cannot be discarded. However, if present, these costs can be compensated by an increase in stress tolerance that will ultimately lead to an increase in biomass production in plants exposed to environmental stresses (Li *et al*. 2009; Xu *et al*. 2014).

In peanut, the content of the thiol GSH did not show changes in response to drought stress and rehydration. However, the content of the oxidised form was reduced, although the effect may not have biological significance, since it contributed to only 1–3% of the total GSH. This feature has been reported in leaves experiencing drought, where the lack of accumulation of GSSG under drought contrasted with some other stresses, in which GSH oxidation was evident (Noctor *et al*. 2014). A possible explanation for such a response may be that changes in thiol status were likely to be involved in redox signalling pathways. Consequently, the activity of antioxidant enzymes related to thiol metabolism may have an indirect role in signalling (Noctor *et al*. 2014). In summary, peanut's responses to a drought and rehydration cycle showed the capacity of the plant to recover after 3 days of rehydration in terms of photosynthesis and antioxidant system activity. The marked increase in ASC–GSH cycle activity during the drought stress period did not appear to be sufficient to protect plants from oxidative stress but may play a positive role in signalling responses to this abiotic stress. In this sense, effective ROS signalling may require increased flux through antioxidative

components, notably by thiol-dependent enzymes. The established framework opens the path for future work to detail the role of the ASC–GSH cycle plant signalling responses to drought stress and possibly to use the generated information in plant biotechnology.

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