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Antioxidant enzyme activities and gene expression patterns in peanut nodules during a drought and rehydration cycle

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Abstract. Drought stress is one of the most important environmental factors that affect plant growth and limit biomass production. Most studies focus on drought stress development but the reversibility of the effects receives less attention. Therefore, the present work aims to explore the biological nitrogen fixation (BNF) of the symbiotic association between peanut (*Arachis hypogaea* L.) and *Bradyrhizobium* sp. during a drought–recovery cycle with a focus on the response of enzyme activity and gene expression of the antioxidant system. Peanuts exposed to drought stress had impaired BNF, as indicated by lower nitrogenase activity, and decreased leghaemoglobin content; the latter was reversed to control values upon rehydration. Previous results demonstrated that reactive oxygen species ($O_2^{\cdot -}$ and H_2O_2) were accumulated as a consequence of drought stress, suggesting that nodules experience oxidative stress. In addition, marker transcripts responsive to drought, abscisic acid and H_2O_2 were upregulated. Increased transcript levels of glutathione reductase were associated with an increased enzyme activity but superoxide dismutase and glutathione S-transferase activities were unchanged, despite upregulated gene transcription. In contrast, increased activity of ascorbate peroxidase (APX) was unrelated with changes in cytosolic APX transcript levels suggesting isogene specificity. In conclusion, the work exemplarily demonstrates the efficient and dynamic regulation of antioxidant enzymes and marker compounds during drought cycling, which is likely to be a prerequisite for functional optimisation of nodule metabolism.

Additional keywords: antioxidant system, *Arachis hypogaea*, biological nitrogen fixation, oxidative stress, reactive oxygen species.

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Introduction

Legume nodules are unique symbiotic organs that develop on roots and, in a few species, also on stems after infection with rhizobia. Biological nitrogen fixation (BNF) contributes significantly to the biological nitrogen cycle and nitrogen input into bio- and agrosystems (Reid *et al*. 2011). Several processes in nitrogen-fixing systems generate reactive oxygen species (ROS), making antioxidant defence indispensable for survival. ROS include the superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), which are produced concomitant with the high respiration rates and the reduced state of electron transport carriers required to support N_2 fixation and are also produced by autoxidation of the oxygenated leghaemoglobin (Lb) form, and the oxidation of several proteins with strong reducing potential (e.g. nitrogenase, ferredoxin, oxygenases and hydrogenase) (Becana *et al*. 2010). Furthermore, ROS, lipid-derived radicals and $^{\cdot}OH$ radical can be formed in the presence of trace amounts of Fe, Cu and other transition metals through the Fenton reaction (Halliwell and Gutteridge 1999).

Those oxidative challenges must be overcome by efficient antioxidant and repair systems formed by compounds and enzymes that contribute to coping with oxidative stress. Antioxidant compounds, namely ascorbate (ASC) and glutathione (GSH) participate in the ASC–GSH cycle and are capable of detoxifying H_2O_2 by a series of reactions. Outside the chloroplast, the ASC–GSH cycle ultimately relies on the reducing power of NADPH. In the enzymatic ROS-scavenging pathways, ascorbate peroxidase (APX) and glutathione reductase (GR) are responsible for operating the ASC–GSH cycle for H_2O_2 removal. Superoxide dismutase (SOD) converts $O_2^{\cdot -}$ to H_2O_2 and O_2; APX, catalase (CAT), peroxiredoxins and glutathione peroxidases (GPX) are involved in the reduction of H_2O_2 to H_2O (Foyer and Noctor 2009). GST catalyses the *S*-conjugation of reactive compounds with GSH, and also fulfils many different functions in plants. GST is a large family with 54 and 83 members in *Arabidopsis thaliana* (L.) Heynh and *Populus trichocarpa* (Torr. & Gray), respectively (Mittler *et al*. 2004; Foyer and Noctor 2009; Jacquot *et al*. 2013). The responses and dynamics of

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antioxidant enzyme activities or transcripts are commonly used to study plant stress responses (Becana *et al*. 2010). Whereas enzymatic activities reflect changes in overall capacity averaged over different compartments and isoforms, transcript analysis allows the easy distinction of gene-specific responses. As a consequence, activity and transcript regulation often fail to provide a consistent view, since they measure different levels of response (Bian and Jiang 2009). In recent years, such approaches were often accompanied by the analysis of regulatory marker transcripts, which provided additional information on the activation of the specific plant signalling pathways that control biochemical and transcriptional responses (Oelze *et al*. 2012). Thus, drought-, ABA- and H_2O_2-responsive marker transcripts are also interesting targets in nodules for assessing their metabolic and signalling state.

Drought stress has profound effects on crop productivity that are partly similar to those of freezing, salinity and high temperature, which usually have a water stress component. At the molecular and cellular levels, redox imbalances and oxidative damage are intimately linked to environmental constraints (Sassi *et al*. 2008; Zabalza *et al*. 2008), and also cause strong effects on nodule performance and BNF. For example, for alfalfa (*Medicago sativa* (L.)) nodules, Naya *et al*. (2007) showed that imbalanced redox and ROS states have major importance for maintenance of functionality. In other legumes, it still needs to be demonstrated whether oxidative stress is a primary response or whether it occurs later during the drought stress response, corresponding to a late, irreversible stage of nodule senescence (Arrese-Igor *et al*. 2011).

Peanut (*Arachis hypogaea* L.) is an economically important crop throughout the world. In Argentina, agricultural areas intermittently suffer from periods of water deficit almost every year (Collino *et al*. 2001) causing important yield losses. In the symbiotic association of peanut–*Bradyrhizobium* sp. SEMIA6144, nodules exposed to drought stress reach an osmotic potential value of –1.21 MPa, differing significantly from control and rehydrated ones (–0.87 and –0.74 MPa, respectively). Elsewhere, we have also demonstrated that nodule number, dry weight and nitrogen content per plant decreased significantly in stressed and rehydrated plants compared with controls. Furthermore, nodules turned into a state of oxidative stress, demonstrateds by H_2O_2 accumulation and lipid oxidation (Furlan *et al*. 2012). In the present work, BNF and the enzyme activity and gene expression patterns of the antioxidant system in response to drought stress and subsequent rehydration in the symbiotic association between peanut and *Bradyrhizobium* sp. were investigated in order to assess the capacity for recovery of the system. The rationale was that regulation of sensitive markers should reverse upon rehydration, and that reversibility and recovery mark important features determining productivity in a natural environment encountering episodes of 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 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 $\mu mol\ m^{-2}\ s^{-1}$, 16-h day/8-h night cycle, 28°C and a relative humidity of 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 colony forming units mL^{-1}. Plants were irrigated twice a week alternately with distilled water and Hoagland nutrient solution without nitrogen (Hoagland and Arnon 1950) in order to keep the field capacity (13%), which was determined through the pressure-plate method (Burk 1996). Thirty days after sowing, plants in the flowering phase (R1) (Boote 1982) were separated at random into three experimental groups: (a) control: plants were kept under water irrigation (osmotic potential (Ψo): –0.4 MPa); (b) drought stress: the irrigation was suspended for 14 days (Ψo: –0.6 MPa); (c) rehydration: plants subjected to 14 days of drought stress were re-irrigated for 3 days (Ψo: –0.4 MPa). Ψo was determined by measuring the freezing point of samples using an osmometer (Semi Micro K-700, Knauer, Berlin, Germany) as described by Furlan *et al*. (2012). Nodules were harvested into liquid nitrogen and stored at −80°C until use (Fig. 1).

Nitrogenase activity and Lb content

Nitrogenase activity was evaluated according to Hardy *et al*. (1973). Detached nodules were transferred to culture vessels capped with serum stoppers. A 10% acetylene atmosphere was injected to start the assay. After 1 h of incubation, the ethylene produced was measured by injecting 0.5 mL of the gas into a Konik gas chromatograph (KNK-3000 series; Konik, Sant Cugat del Vallès, Barcelona, Spain).

Lb content was determined by the pyridine–haemochrome method, measuring the absorbance at 556 nm and 539 nm from the reduced (+ dithionite) and the oxidised (+ ferricyanide) haemochromes, respectively. The concentration was calculated as the difference between absorbance at 556 nm and at 539 nm (extinction coefficient (ε): 23.4 $mM^{-1}\ cm^{-1}$) (Appleby and Bergersen 1980). Total protein content was assessed according to Bradford (1976) using BSA as the standard.

Antioxidant enzyme activities

For SOD (enzyme classification number (EC) 1.15.1.1) and CAT (EC 1.11.1.6) determination, nodule samples were 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 used to determine enzymatic activities.

SOD isozymes were individualised and identified on 11% polyacrylamide native gels by incubation with specific inhibitors (5 mM 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 were assigned to Mn-SOD isozymes. SOD activity was determined according to Beauchamp and Fridovich (1973) using nitrotetrazolium blue chloride (NBT; Sigma-Aldrich) in

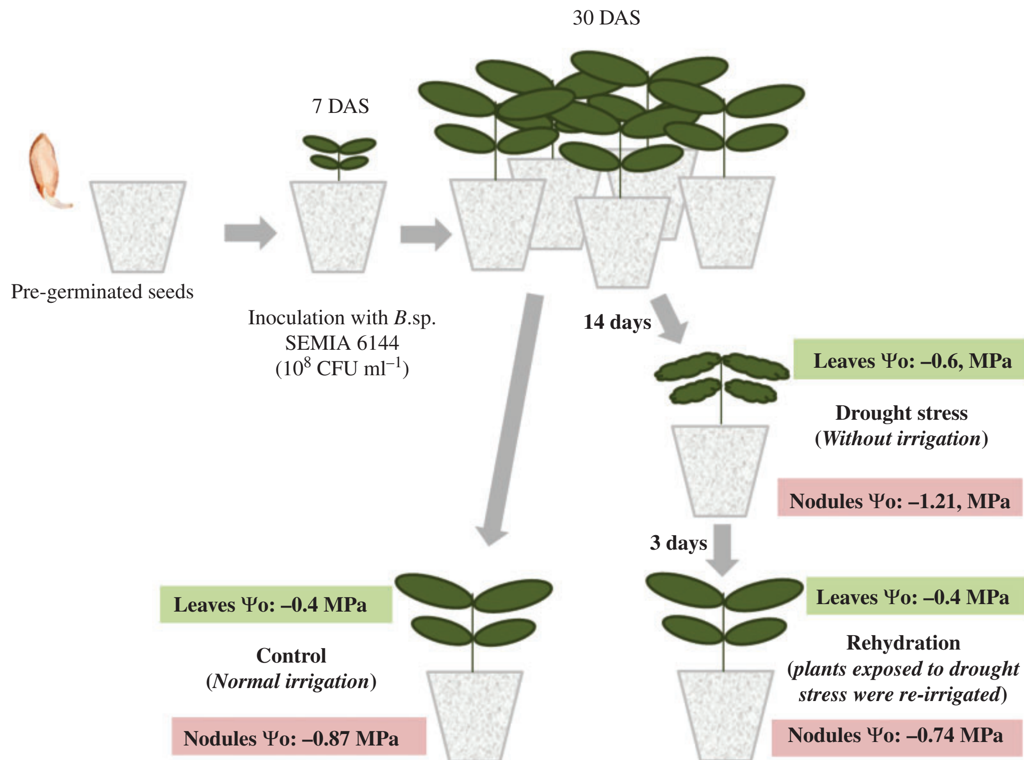

Fig. 1. Experimental set-up. Pregerminated peanut seeds were transferred to pots filled with sterile volcanic sand. Seven days after sowing (DAS), plants were inoculated with *Bradyrhizobium* sp. SEMIA6144. Plants were irrigated twice a week alternately with distilled water and Hoagland nutrient solution without nitrogen in order to keep the field capacity (13%). Thirty DAS plants in flowering phase (R1) were separated at random into three experimental groups: control: plants were kept under water irrigation (osmotic potential (Ψo): –0.4 MPa); drought stress: irrigation was suspended for 14 days (Ψo: –0.6 MPa); rehydration: plants subjected to 14 days of drought stress were reirrigated for 3 days (Ψo: –0.4 MPa). Nodules were harvested into liquid nitrogen and stored at −80°C until use. CFU, colony forming units.

the presence of riboflavin. One mL of 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 were placed under fluorescent light for 15 min. SOD-specific activity was determined spectrophotometrically at 560 nm and expressed as units mg^{-1} protein. One unit of SOD activity is defined as the amount of enzyme required to inhibit the reduction of NBT by 50%.

CAT activity was measured using the method described by Aebi (1984). One mL of the assay mixture contained 25 µg protein extract, 50 mM phosphate buffer at pH 7.5 and 5 mM H_2O_2. The reaction was measured using 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 ascorbate, which allows us to measure the total activity since APXs from organelles are inactive in extraction media without ascorbate (Dalton *et al*. 1986; Amako *et al*. 1994).

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 ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2 in a total volume of 1 mL. The oxidation of ASC was followed at 290 nm. To calculate APX-specific activity the extinction coefficient of ASC was used (2.8 mM^{-1} cm^{-1}). One unit of APX produces 1 mmol oxidised ASC min^{-1}.

GR activity was determined as described by Shaedle and Bassham (1977). One mL of the assay mixture contained 50 µL protein extract, 0.5 mM GSSG and a 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 the NADPH oxidation at 340 nm. One unit of GR is defined as the quantity of enzyme needed to produce 1 µmol $NADP^+$ min^{-1}.

GPX-like activity was determined as described by Flohé and Günzler (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. Then 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 is defined as the quantity of enzyme needed to produce 1 µmol $NADP^+$ min^{-1}. This assay likely describes glutathione- or glutaredoxin-dependent thiol

peroxidase activity since plants do not contain Se-dependent GPX. Plant GPX appears to function as a TRX-linked thiol peroxidase (Navrot *et al*. 2006).

GST activity was determined by measuring the conjugation of glutathione 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 protein extract and a phosphate buffer (0.1 M, pH 7). The reaction was measured following the absorbance at 340 nm for 5 min. To calculate GST-specific activity, the extinction coefficient of CDNB was used (9.6 mM cm^{-1}). One unit of GST produces 1 µmol CDNB–GSH conjugate min^{-1}.

Total and reduced ascorbate contents

Total ascorbate [reduced ascorbate (ASC) + dehydroascorbate (DHA)] and ASC content were measured based on the method of Law *et al*. (1983) with modifications. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ASC in an acidic solution. The Fe^{2+} then forms complexes with bipyridyl, giving a pink colour that is absorbed at 525 nm. Total ascorbate was determined through a reduction of DHA to ASC by dithiothreitol. Nodules (0.1 g) were homogenised in liquid nitrogen with 10% (p/v) trichloroacetic acid (1 : 10) and centrifuged at 10 000*g* for 20 min. Half of the supernatant of nodule extract was assayed for total ascorbate content and the other half was assayed for ASC only. DHA concentration was then deduced from the difference. A sample (100 µL) of the supernatant was added to an equal volume of 150 mM NaH_2PO_4 buffer at pH 7.4 and 2 mM DTT. After vortex-mixing, it 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) orthophosphoric acid, 2% (w/v) trichloroacetic acid, 0.8% (w/v) 2–2'-bypiridil in 70% (v/v) ethanol and 0.3% (w/v) $FeCl_3$. *N*-ethylmaleimide was omitted for ASC determination and this volume added to the volume of DTT was replaced with distilled water. 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 0–50 nmol of ASC was used for calibration.

Total and oxidised glutathione contents

Nodules (0.1 g) were pulverised in liquid nitrogen, suspended in 5% (w/v) 5-sulfosalicylic acid (1 : 10) and centrifuged at 10 000*g* for 10 min at 4°C to remove cell debris. The supernatants were used for measuring the GSH content by the method of Anderson (1985) using glutathione Reductase from baker's yeast (*Saccharomyces cerevisiae*; Sigma-Aldrich). 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 a final volume of 1 mL. The mixture was incubated at 30°C for 15 min and then 5 µL GR (266 units mL^{-1}) were added. The reaction was followed at 412 nm and the total glutathione content comprises the reduced form (GSH) and the oxidized form (GSSG) and 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, the latter being placed on the side of the tube above the level of the liquid. 2-Vinylpyridine and triethanolamine were added to ensure that GSSG was the only form of total glutathione 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 g^{-1} dry weight (DW).

RNA isolation, cDNA synthesis and transcript profiling

RNA isolation and the subsequent cDNA synthesis were performed according to Wormuth *et al*. (2006). Semiquantitative reverse transcription–PCR analysis was carried out as previously described (Finkemeier *et al*. 2005). The used primer combinations are listed in Table 1. PCR amplification was performed by 1 cycle at 94°C for 3 min, and for the optimised number of cycles for each gene product for 15 s at 94°C, 30 s at 57°C or 58°C, and 45 s at 72°C. Equal loading of each amplified cDNA was determined using the control actin PCR product. Following separation of the PCR products on ethidium bromide-stained agarose gels, the transcript levels were estimated from densitometric readings of three independent experiments and expressed as relative expression ratios. The identity of all PCR products was confirmed by sequence analysis at the Center of Biotechnology of Bielefeld University, Bielefeld, Germany. Quantitative real-time PCR (q-PCR) analysis was carried out on the iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 20 µL according to the manufacturer's instructions. The iCycler was programmed to 94°C for 3 min, 45× (94°C for 15 s, 57°C or 58°C for 30 s, 72°C for 45 s) and 72°C for 10 min followed by a melting curve program (55−95°C in increasing steps of 0.5°C). The efficiency of each reaction was calculated using LinRegPCR software (Ruijter *et al*. 2009). Signal values were subsequently derived from the threshold cycles (the average background was subtracted) using the equation of Pfaffl (2001).

Statistical analysis

Experiments were conducted in a completely randomised design and 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 homogeneity of variance was not given, data were transformed using an appropriate function.

Results

Effect of drought stress on BNF and Lb content

Table 2 shows that nitrogenase activity was inhibited by 90% in stressed plants, whereas rehydrated ones recovered 30% of this activity in 3 days. The Lb content was decreased by over 40% in drought-stressed nodules compared with control and rehydrated ones; the protein content remained unchanged in response to different treatments. Lb content represented 37% of the soluble protein content in control conditions. As water stress was

Table 1. Primers used in PCR reactions

Name	Sequence	Product size (bp)	Annealing temperature (°C)	Cycle number	Reference
Actin	5′-GAGCTGAAAGATTCCGATGC-3′5′-GCAATGCCTGGGAACATAGT-3′	178	57	30	Morgante *et al.* (2011)
Ubiquitin	5′-AAGCCGAAGAAGATCAAGCAC-3′5′-GGTTAGCCATGAAGGTTCCA-3′	145	57	30	Morgante *et al.* (2011)
RPR-10	5′-GCCCTGGAACTGTCAAGAAG-3′5′-CTCTTGGAACCCTGTTCCTC-3′	134	58	30	Luo *et al.* (2005)
Fer1	5′-TTCGTCGATCAGTGTGAAGC-3′5′-CTCCCTTTCCTCTGCACTTG-3′	159	57	30	This work
AREB1	5′-TCGCAAGCAGGCCTATACTT-3′5′-TGGACCTGTAACCGTCCTTC-3′	181	58	40	This work
CuZn-SOD	5′-AACTGGACCGCATTTCAATC-3′5′-ACAACAACAGCCCTTCCAAC-3′	180	57	30	This work
GST	5′-CTTGGGCCAAAAGGTGTATG-3′5′-TTTCCATCACCGGAAAACAC-3′	130	58	30	Luo *et al.* (2005)
GR	5′-TCAGGCCACTAAAGGCTACG-3′5′-TGGGTGAATCCCCACAGTAG-3′	197	57	40	This work
cAPX	5′-TGGTCACACTCTTGGAGCTG-3′5′-TCAACAAGAGGGCGGAATAC-3′	183	57	30	This work

Table 2. Effect of drought stress and rehydration on nitrogenase activity, leghaemoglobin (Lb) and soluble protein contents in peanut nodules

Values are means ± s.e. ($n = 6$). Different letters in each row indicate significant differences at $P < 0.05$ according to Duncan's test

	Control	Drought stress	Rehydration
Nitrogenase activity (µmol N_2 reduced h^{-1} $plant^{-1}$)	1.97 ± 0.30c	0.25 ± 0.09a	0.54 ± 0.01b
Lb content (mg g^{-1} DW)	48.46 ± 2.78b	29.39 ± 1.61a	51.51 ± 4.95b
Soluble protein content (mg g^{-1} DW)	130.96 ± 6.62a	149.79 ± 4.92a	137.96 ± 2.74a
Lb per soluble protein × 100	36.98 ± 0.46b	19.72 ± 1.62a	37.25 ± 3.08b

imposed, this value declined to 20% and recovered to control levels upon rehydration (Table 2).

Nodule antioxidant enzyme activities in response to drought stress

Three SOD isozymes were detected according to their mobility and differential inhibition to H_2O_2 or KCN (data not shown). They were classified as Mn-SOD I, Mn-SOD II and CuZn-SOD (Fig. 2). The total SOD activity of nodules remained unchanged during drought stress. However, drought stress had a distinct effect on the activities of several enzymes involved in the H_2O_2 removal in nodules, namely CAT, APX and GPX, which increased. CAT-specific activity increased by ~85% of the control at severe stress and decreased at rewatering. APX and GPX activities increased by ~40% of the control and later decreased, reaching control values upon rehydration. GR activity increased by doubling its activity under drought stress conditions compared with control and rehydrated conditions. GST activity did not show changes in response to drought stress and rehydration (Fig. 3).

Nodule antioxidant metabolite contents in response to drought stress

Total ascorbate (ASC and DHA) content in peanut nodules remained unchanged in response to different treatments (Table 3). However, drought-stressed and rehydrated nodules revealed an increase in ASC of 35% and a consequent decrease in DHA values. Thus, DHA content corresponded to ~25% of the total ascorbate in drought-stressed and rehydrated plants, whereas it was close to 45% in control conditions. Severe

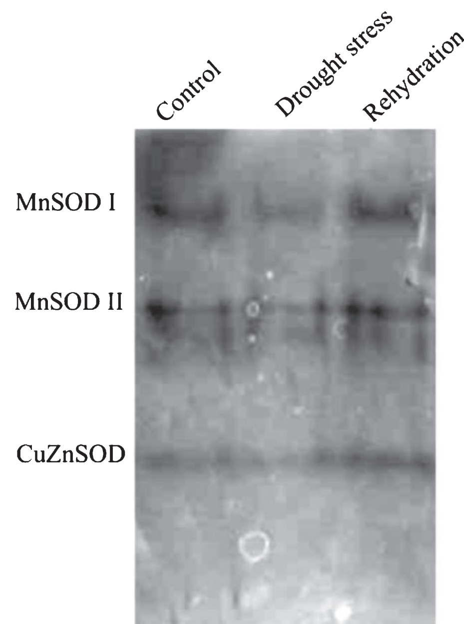

Fig. 2. Superoxide dismutase (SOD) isozyme activity in peanut following drought stress and rehydration in nondenaturing polyacrylamide gels. All lanes were loaded with 25 µg protein.

stress and rehydration had no effect on total glutathione (GSH + GSSG) content in peanut nodules compared with control condition, whereas the GSSG content increased (38%) in response to drought stress and reversed upon rehydration (Table 3).

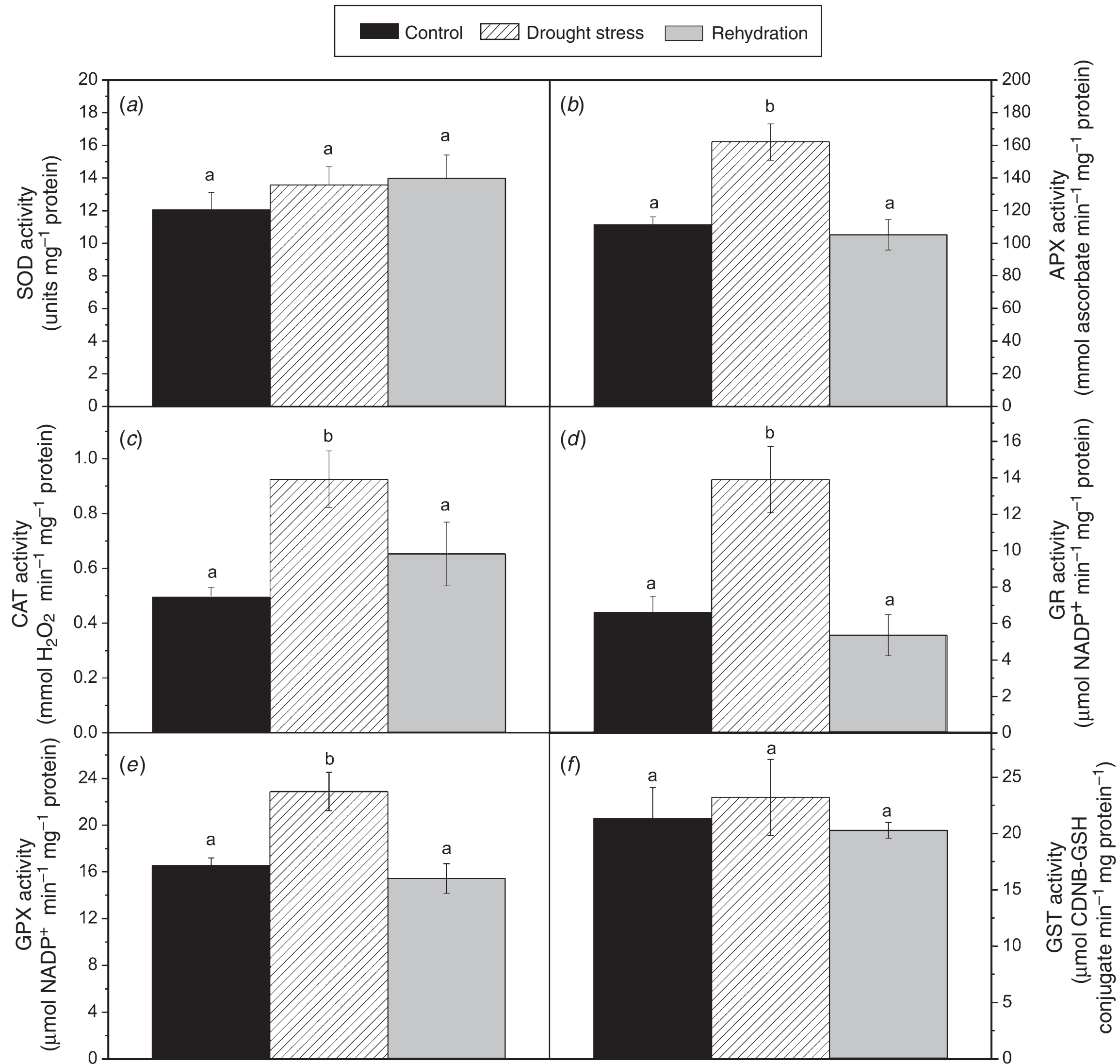

Fig. 3. Effect of drought stress and rehydration on nodule antioxidant enzyme activities. Values are means ± s.e. ($n = 5$). Different letters in each column indicate significant differences at $P < 0.05$ according to Duncan's test. (*a*) Superoxide dismutase (SOD); (*b*) ascorbate perioxidase (APX) (*c*) catalase (CAT); (*d*) glutathione reductase (GR); (*e*) glutathione perioxidase (GPX); (*f*) glutathione S-transferase (GST).

Transcript levels of marker genes and antioxidant enzymes

A literature search resulted in the identification of transcripts that have been used or could be indicative of the involvement of specific signalling pathways in the drought stress response. Sets of marker transcripts were selected to address signalling-related effectors, namely to drought (RPR-10) (Luo *et al*. 2005), ABA (AREB1) (Hong *et al*. 2013) and H_2O_2 (Fer1) (op den Camp *et al*. 2003). All marker genes showed the expected upregulation with significant $\log_2$ fold change >0.5 relative to nontreated plants (Fig. 4). Transcript levels of four selected antioxidant enzymes were quantified by quantitative real-time PCR for their response to drought stress and rehydration. The transcript level of CuZn-SOD, GR and GST was upregulated by drought stress, whereas cAPX remained unaltered. In response to rehydration, all increases were reversed (Fig. 5).

Discussion

Legume root nodules are at high risk of redox imbalance and oxidative damage due to their intense aerobic metabolism, which results in ROS generation. It may be assumed that redox and ROS imbalances are exacerbated under drought stress conditions, causing an oxidative stress (Becana *et al*. 2010). Imaging of ROS ($O_2^{\cdot-}$ and H_2O_2) accumulation as a consequence of drought stress indicates that nodules turned into a state of increasing oxidative stress (Fig. S1, available as Supplementary Material to this paper), which is in line with the results from quantitative biochemical determination of accumulating H_2O_2 and lipid peroxides (estimated as malondialdehyde) under drought stress (Furlan *et al*. 2012). Currently, oxidative damage is considered to be a potent factor that negatively influences BNF (Naya *et al*. 2007). The responses of nodule functioning to most

Table 3. Contents of low molecular mass antioxidants in peanut nodules 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, oxidized glutathione

Antioxidant metabolite	Control	Drought stress (μmol g^{-1} DW)	Rehydration
Total ASC	10.40 ± 0.61a	10.56 ± 0.88a	10.85 ± 0.39a
ASC	5.94 ± 0.46a	8.17 ± 0.69b	7.88 ± 0.34b
DHA	4.79 ± 0.62b	2.49 ± 0.52a	3.33 ± 0.36ab
ASC $(ASC + DHA)^{-1}$	0.54 ± 0.05a	0.77 ± 0.06b	0.71 ± 0.03b
Total GSH	17.17 ± 3.32a	13.31 ± 1.40a	18.93 ± 2.98a
GSH	16.78 ± 3.33a	12.77 ± 1.36a	18.53 ± 2.95a
GSSG	0.38 ± 0.02a	0.54 ± 0.04b	0.40 ± 0.04a
GSH $(GSH + GSSG)^{-1}$	0.97 ± 0.01b	0.96 ± 0.00a	0.98 ± 0.00b

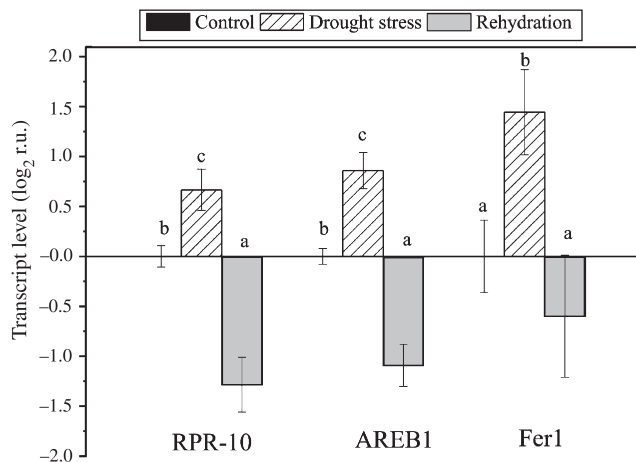

Fig. 4. Transcript levels of marker genes in peanut in response to drought stress and rehydration. Marker transcripts responsive to drought (RPR-10), ABA (AREB1) or ROS (H_2O_2) (Fer1), were quantified and expressed as a $\log_2$ fold change relative to the control (well irrigated plants). Data are means ± s.e. of three biological experiments with replicate each (quantitative real-time PCR); P-value <0.05, Duncan's test, normalised to the control with actin and ubiquitin.

environmental constraints, including drought, were related to the operation of the oxygen diffusion barrier. Oxygen diffusion barrier closure lowers oxygen exposure for bacteroids and therefore causes a lack of energy to support the highly energy-dependent BNF. On the other hand, it was demonstrated that BNF can be directly disrupted by a drought-induced accumulation of N compounds, which was provoked by an inhibition of nodular export (Arrese-Igor *et al*. 2011). A strong correlation was established between drought, nodule sucrose synthase activity and BNF for several grain legumes (Arrese-Igor *et al*. 1999). Here, it is shown that peanut nodules exposed to drought had impaired BNF (estimated as nitrogenase activity). The subsequent rehydration partially restored the ability to fix nitrogen. Under stress conditions, peanut experienced an oxidative damage that was reversed upon rehydration, tentatively supporting the view that the decline in nitrogenase activity was related to oxidative stress. Further studies are

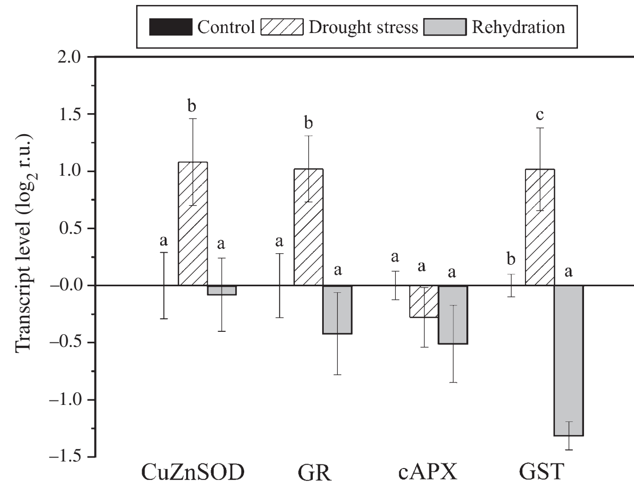

Fig. 5. Transcript levels of selected antioxidant enzymes in peanut in response to drought stress and rehydration. CuZn superoxide dismutase (CuZn-SOD), glutathione reductase (GR), cytosolic ascorbate peroxidase (cAPX) and glutathione S-transferase (GST) transcripts were quantified and expressed as a $\log_2$ fold change relative to the control (well irrigated plants). Data are means ± s.e. of three biological experiments with replicate each (quantitative real-time PCR); P-value <0.05, Duncan's test, normalised to the control with actin and ubiquitin.

required to elucidate if alterations in nitrogen or carbon metabolism (or both) contribute to the low BNF.

Lb represents the main reservoir of O_2 in the nodule cytosol and its autoxidation generates $O_2^{\cdot -}$ (Puppo *et al*. 1981). Moreover, Lb has been shown to be extremely sensitive to redox changes *in vitro* and *in vivo* (Marino *et al*. 2009), and the degradation of Lb is a useful oxidative stress marker in nodules (Dalton 1995; Marino *et al*. 2006). Thus Asensio *et al*. (2011) showed that the amount of Lb was more affected after treatment with the oxidative stress inductor methyl viologen than the total soluble proteins in pea (*Pisum sativum* L.) nodules. Likewise in alfalfa nodules, severe water stress reduced total soluble protein, but Lb was the primary protein that was affected at moderate water deficits (Irigoyen *et al*. 1992). In this study, peanut nodules revealed no differences in total soluble protein content but a marked decrease in Lb content that could be associated with the onset of oxidative stress under drought conditions. A remarkable dynamics was seen upon rehydration, with full restoration of Lb levels after 72 h. It will be interesting to study the precise kinetics of the recovery at the molecular levels of gene expression, transcript accumulation and protein synthesis.

Structural or regulatory genes that are expressed in a stimulus-specific manner can be used as marker transcripts to assess the possible involvement of signalling pathways. The protein RPR-10, which is homologous to RPR-10 from *Retama raetam* (Forssk.) Webb & Berthel, is strongly expressed in peanut plants under drought stress (Pnueli *et al*. 2002; Luo *et al*. 2005). It has been suggested that RPR-10 has a ribonucleolytic function, but this may not be exclusive, since other examples of pathogenesis-related (PR) proteins indicate their involvement in multiple cellular functions. The possibility that RPR-10 acts as a dehydrin or chaperone similar to small heat-shock proteins gains support from the high number of polar residues per total

number of side chains found in PR-10 (~40%) compared with dehydrin (~50%). Therefore PR-10 may function as a late embryogenesis abundant (LEA)-like protein to protect other proteins and cellular structures during stress due to its high number of polar residues (Pnueli *et al*. 2002). Closely related to ABA signalling, acclimation to drought exploits the ABA-dependent activation of target genes via the ABA response element (Seki *et al*. 2007). ABA response element binding (AREB) protein and the ABA response element binding transcription factor are plant-specific, and mediate plant responses to hormones and stresses (Bensmihen *et al*. 2002; Finkelstein *et al*. 2002). The present study demonstrated the accumulation of RPR-10 and AREB1 transcripts during the stress period, which indirectly indicates ABA accumulation in peanut plants (Furlan *et al*. 2012). Increased transcript levels of AREB1 in stressed peanut were also described by Hong *et al*. (2013). Following rehydration, AREB1 transcript levels were lower than in control conditions. As mentioned above, AREB1 might be downregulated as consequence of declining ABA accumulation.

In peanut, drought stress initiated ABA accumulation, which may trigger H_2O_2 production (Fig. S1; Furlan *et al*. 2013). Upregulation of ferritin, a protein involved in Fe sequestration, may be instrumental to prevent the formation of hydroxyl radicals in the presence of H_2O_2 under this stress condition (op den Camp *et al*. 2003). Thus the marker transcript Fer1 is activated in response to excess light and is associated with oxidative stress (Oelze *et al*. 2012). In peanut nodules, increased expression of Fer1 was correlated with H_2O_2 accumulation, suggesting, for the time being, that Fer1 is a suitable marker of oxidative stress in our plant system as well. Accepting RPR-10, AREB1 and Fer1 as powerful stress markers allows us to describe efficient recovery after relief of the water deficit: accumulation of each transcript was reversed after rehydration.

Induction of the components of the antioxidant system is a well-known mechanism in plants that is associated with ROS accumulation under abiotic stress. However, different species subjected to drought frequently show contrasting responses. Pea nodules exposed to drought stress revealed a general decrease in the activity of antioxidant enzymes such as APX, GR, SOD and CAT (Gogorcena *et al*. 1995). Soybean (*Glycine max* (L.) Merr.) nodules showed a decrease in APX and GR activities but an increase in SOD and CAT activities (Porcel *et al*. 2003). However, Naya *et al*. (2007) found an increase in CuZn-SOD (cytosolic), Fe-SOD, APX and GR transcripts in alfalfa. These authors also studied the rehydration process and reported that increased transcripts were reversed after rehydration for 48 h, whereas a new CuZn-SOD isoform (plastid) was induced. In agreement with our results, in legume nodules, several isoforms of CuZn-SOD as well as mitochondrial Mn-SOD are highly expressed, whereas cytosolic Fe-SOD expression is considerably lower (Becana *et al*. 2010). The variability of responses underlines the need to study the transcript and activity regulation in more detail, especially in different species. Here, similar results were found in peanut nodules, where drought stress and rehydration affected antioxidant enzyme gene transcription. Thus, CuZn-SOD, GR and GST gene expression were increased during drought and reversed upon rehydration. For GR, these changes were associated with increased enzyme activity, whereas SOD and GST activities did not change. In contrast, increased APX activity was not correlated with cAPX transcript levels. GST transcript levels in rehydrated plants were lower than in control plants. It is proposed that the increased antioxidant activity linked to ABA accumulation may be responsible for GST downregulation. To fully recover plant homeostasis, an overshooting response may transiently cause a situation where enzyme transcripts are lower than those in the control plants. In peanut, increased antioxidant enzyme activity may be linked to ABA accumulation, since the antioxidant system was induced by external application of ABA (Zhou *et al*. 2005; Bright *et al*. 2006; Zhang *et al*. 2007; Lu *et al*. 2009).

In conclusion, this study establishes a framework with which peanuts respond to drought cycling. Drought stress drastically reduced BNF and Lb content. Simultaneously, ROS accumulated and selected components of the antioxidant system were induced in peanut nodules. It appears reasonable to assume that the increase of antioxidant enzyme activities during drought is crucial for the restoration of a normal redox metabolism within the short period of 3 days after the onset of rehydration. The strong upregulation of ABA- and H_2O_2-dependent markers in drought-stressed nodules tentatively indicates that both metabolites are involved in triggering the expression of antioxidant enzyme genes. The restoration of normal Lb amounts also appears of prime importance for returning nitrogen metabolism to normal. The established framework opens the path for future work, namely to dissect the time-resolved kinetics in detail, to compare genotypes with different drought tolerance levels and progression to an omics-driven systematic approach.

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