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RESEARCH PAPER

Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.)

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Abstract

Despite its relevance, protein regulation, metabolic adjustment, and the physiological status of plants under drought is not well understood in relation to the role of nitrogen fixation in nodules. In this study, nodulated alfalfa plants were exposed to drought conditions. The study determined the physiological, metabolic, and proteomic processes involved in photosynthetic inhibition in relation to the decrease in nitrogenase (N_{ase}) activity. The deleterious effect of drought on alfalfa performance was targeted towards photosynthesis and N_{ase} activity. At the leaf level, photosynthetic inhibition was mainly caused by the inhibition of Rubisco. The proteomic profile and physiological measurements revealed that the reduced carboxylation capacity of droughted plants was related to limitations in Rubisco protein content, activation state, and RuBP regeneration. Drought also decreased amino acid content such as asparagine, and glutamic acid, and Rubisco protein content indicating that N availability limitations were caused by N_{ase} activity inhibition. In this context, drought induced the decrease in Rubisco binding protein content at the leaf level and proteases were up-regulated so as to degrade Rubisco protein. This degradation enabled the reallocation of the Rubisco-derived N to the synthesis of amino acids with osmoregulant capacity. Rubisco degradation under drought conditions was induced so as to remobilize Rubisco-derived N to compensate for the decrease in N associated with N_{ase} inhibition. Metabolic analyses showed that droughted plants increased amino acid (proline, a major compound involved in osmotic regulation) and soluble sugar (D-pinitol) levels to contribute towards the decrease in osmotic potential (Ψ_s). At the nodule level, drought had an inhibitory effect on N_{ase} activity. This decrease in N_{ase} activity was not induced by substrate shortage, as reflected by an increase in total soluble sugars (TSS) in the nodules. Proline accumulation in the nodule could also be associated with an osmoregulatory response to drought and might function as a protective agent against ROS. In droughted nodules, the decrease in N_2 fixation was caused by an increase in oxygen resistance that was induced in the nodule. This was a mechanism to avoid oxidative damage associated with reduced respiration activity and the consequent increase in oxygen content. This study highlighted that even though drought had a direct effect on leaves, the deleterious effects of drought on nodules also conditioned leaf responsiveness.

Key words: Drought, *Medicago sativa*, N_2 fixation, N remobilization, oxidative stress, photosynthesis, proteome, Rubisco.

Abbreviations: 2-CP, 2-Cys peroxiredoxin BAS1; 2-DE, two-dimensional electrophoresis; Ala, alanine; A_{sat}, light-saturated rate of CO_2 assimilation; Asn, asparagine; Asp, aspartic acid; CCI7, proteosome β1 subunit; C_i, intercellular CO_2 concentration; DH, dehydrogenase; DHAR, dehydroascorbate reductase; *DW*, dry weight; *E*, leaf transpiration; F_v/F_m, maximal photochemical efficiency; F_v'/F_m', efficiency of energy capture by open PSII reaction centres; *FW*, fresh weight; Gln, glutamine; G-3P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Glu, glutamic acid; cGAPDH, cytosolic glyceraldehyde 3-phosphate dehydrogenase; GC-MS, gas chromatography mass spectometer; g_s, leaf stomatal conductance; GS, glutamine synthetase; IPCC, Intergovernmental Panel on Climate Change; J_{max}, electron transport rate contributing to RuBP regeneration; *Je*(PSII), electron transport through photosystem II; *Je*(PCR), electron transport through photorespiratory carbon oxidation; LHCII, light-harvesting complex II; *LWC*, leaf water content; MDHAR, monodehydroascorbate reductase; N_{ase}, nitrogenase; *NPQ*, non-photochemical quenching; *PCR*, photosynthetic C reduction; *PPFD*, photosynthetic photon flux; Pro, proline; PSII, photosystem II; *qP*, photochemical quenching; QTL, quantitative trait locus; R_{leaf}, leaf respiration; R_{nodule}, nodule respiration; RuBP, ribulose *bis*phosphate; ROS, reactive oxygen species; Rubisco, ribulose 1,5 *bis*phosphate carboxylase oxygenase; Ser, serine; SOD, superoxide dismutase; TCA, tricarboxylic acid cycle; TFA, trifluoracetic acid; T_{leaf}, leaf temperature; TPI; triose phosphate isomerase; TSS, total soluble sugar; Vc_{max}, maximum photosynthetic rate; Φ_{PSII}, relative quantum efficiency of PSII photochemistry; Ψ_s, osmotic potential.

Introduction

The major environmental factor that constrains the productivity and stability of plants is water stress (Araus *et al.*, 2002). According to the different scenarios predicted by the Intergovernmental Panel on Climate Change (Alley *et al.*, 2007) it is expected that there will be a reduction in precipitation and rising evapotranspiration rates. The perceived need to gain further understanding of photosynthesis, so as to alleviate practical problems such as crop yield under drought conditions, has increased interest in 'water stress physiology' (Lawlor and Tezara, 2009).

Photosynthesis and cell growth are among the primary processes to be affected by drought (Chaves and Oliveira, 2004; Chaves *et al.*, 2009). The photosynthetic rates of plants exposed to drought decrease due to stomatal closure and non-stomatal processes (Lawlor and Cornic, 2002; Aranjuelo *et al.*, 2007; Lawlor and Tezara, 2009; Chaves *et al.*, 2009). Although it is generally accepted that stomatal closure is the main factor limiting photosynthetic activity under moderate water-limiting conditions (Chaves *et al.*, 2002, 2003), when water stress is more severe, metabolic impairment takes place (Medrano *et al.*, 2002). Deleterious effects of drought on photosynthesis will be mediated by the responsiveness of (i) the respiration system, electron transport, and ATP synthesis in the mitochondria (Atkin and Macherel, 2009), (ii) the accumulation of stress metabolites (Zhang *et al.*, 1999), and (iii) gene expression and protein synthesis (Lawlor and Tezara, 2009).

The balance between light capture and energy use are of great relevance to studies concerning the responsiveness of the photosynthetic apparatus under water-stress conditions (Sharp and Boyer, 1985, 1986; Cornic and Briantais, 1991; Chaves *et al.*, 2009; Lawlor and Tezara, 2009). When photosynthesis decreases and light excitation energy is in excess, photooxidative damage may occur. The excessive excitation energy in photosystem II (PSII) will lead to an impairment of photosynthetic function, progress to an accumulation of reactive oxygen species (ROS), and thereby result in oxidative stress. Plants have developed three main mechanisms to diminish photooxidative damage: (i) preventing the production of ROS by diminishing the electron transport chain (Lawlor and Tezara, 2009); (ii) scavenging ROS formed by an integrated system of enzymatic and non-enzymatic (ascorbate–glutathione cycle) antioxidants (Asada, 1999), and (iii) diminishing photooxidation through xanthophyll cycle-dependent thermal dissipation, which is an important photoprotective process in the light-harvesting antenna of PSII (Gilmore, 1997; Verhoeven *et al.*, 1999).

The effect of water limitation on proteins involved in C metabolism in legumes is not well understood. Analysis on this topic has been limited, and mainly focused on the characterization of the quantity and activity of Rubisco (Parry *et al.*, 2003; Aranjuelo *et al.*, 2007). Proteomics addresses analytical questions about the abundance and distribution of proteins in organisms, the expression profiles of different tissues and the identification and localization of individual proteins of interest (Kersten *et al.*, 2002). This method has developed as an important approach in evaluating plant responsiveness under limited growth conditions (Desclos *et al.*, 2008, 2009).

Although the influence of water availability on plant growth and photosynthetic activity has been studied extensively (Bushby, 1982; Fellows *et al.*, 1987; Irigoyen *et al.*, 1992), less attention has been given to the role of nodule activity in plant performance under drought conditions. Nodule activity depends on photosynthates supplied by the plant, which are used by the nitrogenase enzyme as a source of energy and reducing power to fix N_2 (Gálvez *et al.*, 2005; Larrainzar *et al.*, 2009). Similarly, the products of N_2 fixation, which are either amides or ureides, are exported throughout the plant via the xylem to other organs where N is required, for example, protein synthesis (Ladrera *et al.*, 2007). This coupling results in the regulation of nitrogenase activity in plants by photosynthesis (carbon supply), nitrogen availability (N source strength), and N demand (N sink strength).

The main objective of this study was to characterize the responsiveness of the photosynthetic apparatus of nodulated alfalfa (*Medicago sativa* L.) plants during exposure to drought conditions. The relationship between plant and nodule metabolism was also studied to determine its possible implication in alfalfa responsiveness to drought. The combination of physiological and proteomic analyses may constitute an original contribution to understanding the drought effect on photosynthetic activity.

Materials and methods

Experimental design

Seeds of alfalfa (*Medicago sativa* L, Magali variety) were germinated on plates. After 3–4 d, seedlings were transplanted into 7.0 l white plastic pots filled with sand and grown in a greenhouse at 25/15 °C (day/night) with a photoperiod of 14 h under natural daylight. During the first month, plants were inoculated three times with *Sinorhizobium meliloti* strain 102F78. Plants were watered twice a week with Hoagland N-free full nutrient solution and once a week with deionized water to avoid salt accumulation in pots. When the plants were 91-d-old, half of the plants (randomly selected) were exposed to drought conditions (with water withholding) whereas the others were maintained in optimal water availability conditions. Over 7 d, drought plants were grown without any watering, whereas control plants were watered until pot capacity. Plant water status was evaluated by measuring the leaf water content (*LWC*) and osmotic potential (Ψ_s). Leaf water content was calculated as $LWC=(FW–DW)/FW$, where *FW* refers to fresh weight and *DW* refers to dry weight. Osmotic potential was determined using a Wescor 5500 osmometer (Wescor, Logan, Utah, USA) as described by Ball and Oosterhuis (2005).

Gas exchange and chlorophyll fluorescence determinations

Fully expanded apical leaves were enclosed in a Li-Cor 6400 gas exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA). The gas exchange response to CO_2 was measured from 0 to 1000 μmol mol^{-1} CO_2. The light-saturated rate of CO_2 assimilation (A_{sat}) was estimated at a photosynthetic photon flux density (*PPFD*) of 1200 μmol m^{-2} s^{-1} using equations developed

by von Caemmerer and Farquhar (1981). Stomatal conductance (g_s) was determined as described by Harley *et al.* (1992). Estimations of the maximum carboxylation velocity of Rubisco (Vc_{max}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were made using the method of Ethier and Livingston (2004). Plants were dark adapted for 30 min before dark respiration (R) measurements. Nodule respiration was studied by placing them in a respiration chamber ($20\times12\times6\times10^{-6}$ m^3) connected in parallel to the sample air hose of a LI-COR-6400 according to Aranjuelo *et al.* (2009).

The maximal quantum efficiency of PSII (F_v/F_m) and the relative quantum efficiency of PSII photochemistry (Φ_{PSII}) were simultaneously measured with a fluorescence chamber (LFC 6400-40; Li-COR) coupled to the Li-Cor 6400 portable photosynthesis system. For F_v/F_m determinations, leaves were dark-adapted for 30 min. Non-photochemical quenching (*NPQ*) was calculated as $\left(\frac{F_m}{F'_m}\right)$–1 as described by Bilger and Björkman (1990) Photochemical quenching (*qP*) was calculated according to Andrews *et al.* (1993). The rate of electron transport through PSII [*Je*(PSII)] was measured as described by Harley *et al.* (1992). The rate of oxygenation by Rubisco (V_o) was estimated as described by von Caemmerer and Farquhar (1981) as $V_o=(V_c\times pO_2)/(S_r\times C_i)$ where V_c refers to the rate of carboxylation of RuBP, pO_2 refers to the ambient partial pressure of O_2, S_r refers to the relative specificity of Rubisco, and C_i refers to the intercellular CO_2 concentration. The rate of carboxylation by Rubisco (V_c) was estimated as $V_c=(A+R_d)/[1-pO_2/(2\times S_r\times C_i)]$, where R_d refers to the rate of day respiration (Miyake and Yokota, 2000). The electron fluxes in the two cycles, expressed as $Je(PCR)=4\times V_c$ and $Je(PCO)=4\times V_o$, respectively (Krall and Edwards, 1992), were conducted at growth conditions corresponding to each treatment.

Nitrogenase activity

Alfalfa nodule activity was estimated by the C_2H_2 reduction technique (Hardy *et al.*, 1973). Intact nodulated roots were enclosed in a 1.0 l glass flask and 100 ml of C_2H_2 was added. The flask was incubated at room temperature for 10 min. Afterwards, eight samples of 5 ml were withdrawn from the flask and the ethylene content in the samples was quantified using a Fractovap 4200 (Carbo Erba Strumentazione, Milan, Italy) gas chromatograph equipped with a hydrogen flame ionization detector and a column of Poropak R30/100 (2 m×1/8). Determinations were conducted at 90 °C (45 °C detector and injector) with a carrier gas flow rate of 25 ml min^{-1}. Acetylene reduction protocol allows the meaningful analyses of growth condition effects in nitrogenase (N_{ase}) activity (Streeter *et al.*, 2003; King and Purcell, 2005).

Pigments and soluble sugar determinations

Extracts for pigment analysis were prepared by grinding 100 mg fresh weight in a cold mortar with 10 ml of ethanol (95%, v/v). The homogenate was centrifuged at 3165 *g* for 10 min at 4 °C. An aliquot of 1 ml from the supernatant was taken, 4 ml of 95% ethanol were added, and the absorbance measured at 750, 665, 649, and 470 nm. Absorbance determinations were carried out with a Spectronic 2000 (Bausch and Lomb, Rochester, USA) spectrophotometer. Extinction coefficients and equations used to calculate pigment contents were those described by Liechenthaler (1987). For sugar extraction about 50 mg of lyophilized and ground leaves were suspended with 1 ml of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). The solution was mixed and centrifuged at 12 000 *g* for 5 min at 5 °C. The supernatant was heated at 100 °C for 3 min and afterwards centrifuged at 12 000 *g* for 5 min at 5 °C. After centrifugation, the supernatant containing the soluble fraction was purified with a solid phase extraction column (Oasis MCX 3cc, Waters) to separate sugars from the other soluble compounds. Total soluble sugars were determined with the Spectronic 2000 spectrophotometer according to the method proposed by Yemm and Willis (1954). Sucrose, glucose, and fructose contents were analysed using a Waters 600 high performance liquid chromatograph (Waters Millipore Corp., Milford, Massachusetts, USA). The HPLC refractive index detector (Waters 2414) was set at 37 °C. Samples were eluted from the columns at 85 °C (connected in series Aminex HPX-87P and Aminex HPX-87C, 300 mm×7.8 mm, Bio-Rad) with water at a flow rate of 0.6 ml min^{-1} and retention time run up to 45 min.

Quantification of D-pinitol was conducted by liquid chromatography and mass spectrometry. LC–MS detection was achieved using an Applied Biosystems/PE SCIEX API 150 EX single quadrupole mass spectrometer equipped with a turbo ionspray source (PerkinElmer Series 200 Pump). Two columns, an Aminex HPX-87P and Aminex HPX-87C, were serial connected and eluted at 0.6 ml min^{-1} with water as the mobile phase. Column temperatures were maintained at 85 °C. A post-column addition of 18 μM of sodium acetate in acetonitrile at a flow rate of 0.6 ml min^{-1} was undertaken with the isocratic pump to obtain adducts of sodium. A 1:3 split was done before placing in the mass spectrometer. Typically, 50 μl of standards and samples diluted in water were injected to columns.

Free amino acid determinations by GC-MS

Frozen leaf and nodule samples were ground to a fine powder in liquid N and a sub-sample was lyophilized. Trifluoracetic Acid (TFA) 10% (v/v) was added to the sample to avoid enzymatic activity and to extract the soluble fraction. The homogenate was centrifuged at 6000 *g* for 15 min at 4 °C. The supernatant was purified with Ultrafree-MC 10000 NMWL (Millipore, Billerica, Massachusetts, USA) in an Eppendorf tube and centrifuged (13 000 *g* for 45 min at 4 °C). L-norleucine (Sigma-Aldrich, St Louis, Missouri, USA) was added as internal standard to the filtered samples and the mixture was dried with a Speed Vac desiccator overnight. Then the samples were re-suspended in 1 ml of HCl 0.1 N (v/v) and passed through a chromatographic column (Dowex 50W X8 H^+, 16–40 mesh size, Sigma®) as previously described by Owen *et al.* (1999. The mixture of amino acids eluted from the column was completely evaporated and derivatized with N-methyl-N-(*tert*-butyildimethylsilyl)-trifluoroacetamide (MTBSTFA, Aldrich®) as outlined by Woo and Chang (1993) and Woo and Lee (1995). The amino acid derivatives were then injected directly into a gas chromatography-mass spectrometer (GC-MS). The concentrations of amino acids in the samples were calculated using external calibration curves for each amino acid and values were recalculated against the internal standard (L-norleucine).

Proteomic characterization

Frozen and ground leaf samples (200 mg fresh weight) were resuspended in 2 ml of cold acetone containing 10% TCA (v/v). After centrifugation at 16 000 *g* for 3 min at 4 °C, the supernatant was discarded and the pellet was rinsed with methanol, acetone, and phenol solutions as previously described by Wang *et al.* (2003). The pellet was stored at –20 °C or immediately resuspended in 200 μl of R2D2 rehydratation buffer [5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate, 2% N-decyl-N,N-dimethyl-3-ammonio-1-propanesulphonate, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine, 0.5% IPG buffer (GE Healthcare, Saclay, France), pH 4 to 7 (Mechin *et al.*, 2003)]. The total soluble protein (*TSP*) concentration was determined by the method of Bradford (1976) using BSA as standard. The two-dimension electrophoresis was conducted according to what described by Desclos *et al.* (2008).

Image analysis of 2-DE gels

Images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System and analysed using

Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Gels from four independent biological replicates were used. An average gel, representative of each group, was automatically selected by the software with a parameter for spots to be present on more than two-thirds of the gels. The software automatically selected the average gel with the most spots as the image for the reference gel, and unmatched spots from the remaining average gel were added to the reference gel which was subsequently used for spot matching to average gels. Warping and matching were automatically performed and only adjusted on those gels where darker images led to both incorrect warping and matching. M_r and pI were calculated using Samespots software calibrated with commercial molecular mass standards (precision protein standards prestained Bio-Rad) run in a separate marker lane on the 2-DE gel. ANOVA (P <0.05) was performed using MiniTAB to compare the relative abundance of the total volume of all detected spots for each gel.

Protein identification by ESI-LC MS/MS

Excised spots were washed several times with water and dried for a few minutes. Peptide extracts were then dried and dissolved in starting buffer for chromatographic elution, which consisted of 3% CH_3CN and 0.1% HCOOH in water. Peptides were enriched and separated using lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine. Tandem mass spectrometry spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 for MS/MS fragments.

The LC MS/MS data were converted into DTA-format files which were further searched for proteins with MASCOT Daemon. Only peptides matching an individual ion score >51 were considered. Proteins with two or more unique peptides matching the protein sequence were automatically considered as a positive identification. Among the positive matches based on one unique peptide, the fragmentation spectrum from each peptide was manually interpreted using the conventional fragmentation rules. In particular, we looked for a succession of at least five *y*- and/or *b*-ions, specific immonium ions, specific fragment ions (proline and glycine), and signatures of any modifications carried by the peptides. For protein identification, two strategies were used to mine the maximum information. Measured peptides were searched in the NCBInr-protein sequence database viridiplantae (green plants). Once the proteins were identified, we proceeded to their presumed biological function according to Bevan *et al.* (1998). This classification showed that up-regulated proteins belonged to the energy and protein destination and storage functional groups, whereas the down-regulated proteins belonged to metabolism, energy, protein destination and storage, transport, cell structure and disease/defence groups.

Statistical analyses

Data was processed by two-factor analysis of variance (ANOVA). Means ±standard errors (SE) were calculated, and when the F-ratio was significant, least significant differences were evaluated by Tukey's test using the statistical software package SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The results were accepted as significant at P <0.05. All values shown in the figures and tables are means ±SE.

Results

Suppression of irrigation over 7 d reduced leaf water content by 33% (*LWC*; Table 1) and increased osmotic potential of leaves in alfalfa plants subjected to drought (Table 1). Photosynthesis (A) also decreased dramatically (58%; Table 1) in droughted plants as a consequence of stomatal closure (as reflected by stomatal conductance and transpiration decrease), a reduction in carboxylation capacity of Rubisco (Vc_{max}), and a reduction in the potential rate of electron transport contributing to RuBP regeneration (J_{max}). Although no significant differences were observed in leaf dark respiration (R_{leaf}), drought decreased nodule respiration (R_{nodule}) as shown in Table 1. Intercellular CO_2 concentration (C_i) data confirmed that drought-treated plants had lower intercellular CO_2 available (Table 1).

Table 1. The water availability effect (control versus drought) in terms of leaf water content (*LWC*), leaf osmotic potential (Ψ_s), stomatal conductance (g_s), transpiration (E), leaf temperature (T_{leaf}), leaf respiration (R_{leaf}), saturating maximum photosynthetic rate (A_{sat}), maximum carboxylation velocity of Rubisco (Vc_{max}), and the maximum electron transport rate contributing to RuBP regeneration (J_{max}), intercellular CO_2 concentration (C_i), the maximal photochemical efficiency (F_v/F_m), relative quantum efficiency of PSII photochemistry (Φ_{PSII}), efficiency of energy capture by open PSII reaction centres (F'_v/F'_m), photochemical quenching (*qP*), non-photochemical quenching (*NPQ*), total chlorophyll (Chl *a*+*b*), electron transport through Photosystem II [*Je*(PSII)], electron transport through photosynthetic carbon reduction [*Je*(PCR)], electron transport through photorespiratory carbon oxidation [*Je*(PCO)], nodule respiration (R_{nodule}), and acetylene reduction assay (ARA) of *Medicago sativa* plants
Measurements were conducted at the end of the experiment, when plants were 3 months old. Each value represents the mean ±SE (n=8). The different letters indicate significant differences (P <0.05).

Parameter	Control	Drought
Leaf		
LWC	76.31±0.97 a	51.68±8.31 b
Ψs_{leaf} (MPa)	–1.45±0.05 a	–3.85±0.61 b
g_s (mmol H_2O m^{-2} s^{-1})	527.14±13.83 a	98.14±15.81 b
E (mmol H_2O m^{-2} s^{-1})	0.65±0.12 a	0.33±0.05 b
T_{leaf} (°C)	24.04±0.31 b	26.78±0.6 a
R_{leaf} (μmol m^{-2} s^{-1})	–2.30±0.29 a	–1.6±0.38 a
A_{sat} (μmol m^{-2} s^{-1})	25.83±3.03 a	10.83±2.77 b
Vc_{max}(μmol m^{-2} s^{-1})	139.15±11.7 a	106.11±9.00 b
J_{max} (μmol m^{-2} s^{-1})	150.30±7.48 a	126.84±5.12 b
C_i (μmol mol^{-1})	297.83±27.47 a	204.25±16.21 b
F_v/F_m	0.76±0.01 a	0.78±0.02 a
Φ_{PSII}	0.29±0.03 a	0.20±0.02 b
F'_v/F'_m	0.50±0.03 a	0.41±0.03 b
qP	0.61±0.03 a	0.44± 0.06 b
NPQ	1.22±0.1 b	2.03± 0.08 a
Chl *a*+*b* (mg g^{-1} DM)	8.89±0.8 a	9.87±90.05 a
Je(PSII) (μmol e^{-2} s^{-1})	149.3±2.81 a	117.45±4.54 b
Je(PCR) (μmol e^{-2} s^{-1})	115.48±2.8 a	74.00±3.31 b
Je(PCO) (μmol e^{-2} s^{-1})	33.82±1.48 b	43.44±1.67 a
Nodule		
Ψs_{nodule} (MPa)	–0.88±0.005 a	–1.20±0.01 b
R_{nodule} (μmol m^{-2} s^{-1})	–5.41±1.49 a	–2.11±0.62 b
Nase (μmol C_2H_2 g^{-1} DW h^{-1})	41.80±7.84 a	10.05±1.23 b

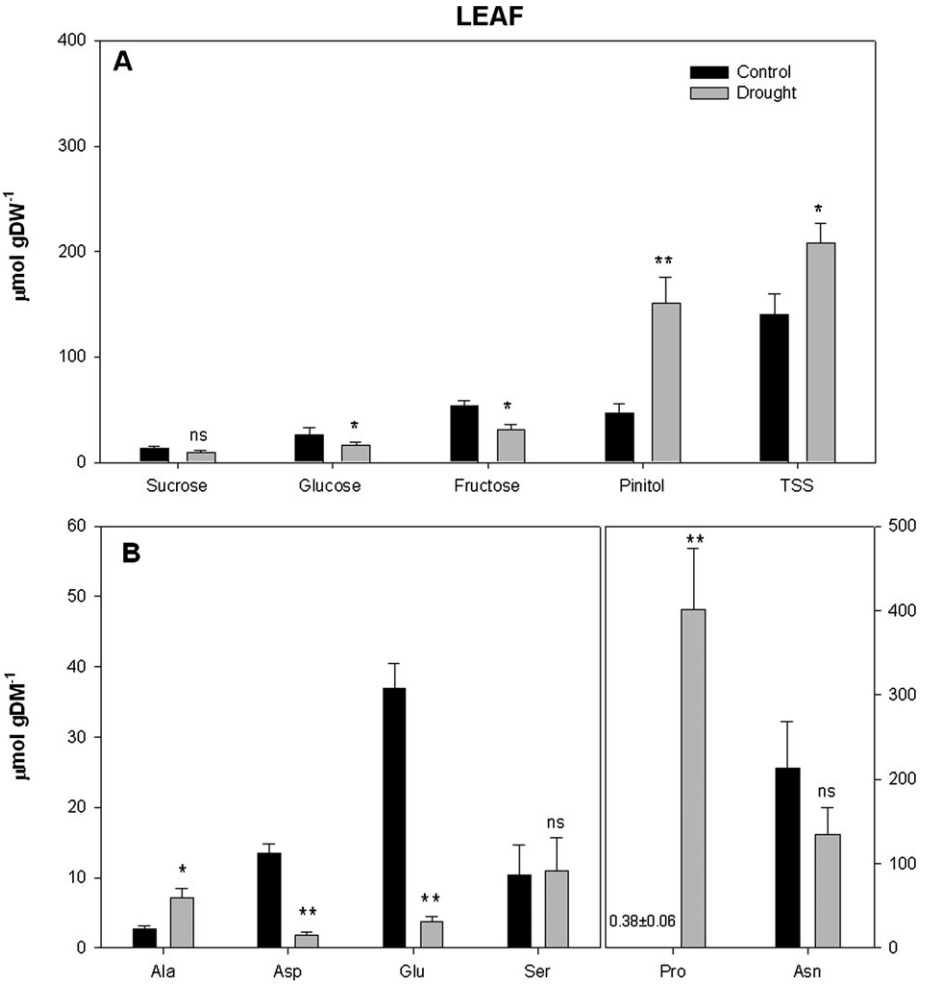

Fig. 1. Water availability effect on (A) leaf soluble sugar (sucrose, glucose, fructose, pinitol, and total soluble sugar, TSS) and (B) free amino acid (Ala, alanine; Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Asn, asparagine, and Pro, proline) content in *Medicago sativa* plants. Measurements were conducted at the end of the experiment. The *y*-axis scale for asparagine and proline was modified to make the text more understandable. In addition, since the proline values detected in control plants were low, the average value ±SE was added to the figure. Each value represents the mean ±SE (n= 4). Different letters indicate significant differences (P <0.05) between treatments.

Nitrogenase (N_{ase}) activity was markedly decreased under drought conditions (Table 1).

Under drought conditions Φ_{PSII} was negatively affected (Table 1). Furthermore, the lower rate of electron transport through PSII ([*Je*(PSII)]) detected in droughted plants could have contributed to a reduction in the photosynthetic capacity of these plants. Electron transport through photosynthetic carbon reduction, *Je*(PCR), confirmed that fewer electrons were delivered to photosynthetic carboxylation processes in droughted plants. The electron flux for photorespiratory carbon oxidation, *Je*(PCO), was increased under drought conditions (Table 1). Photochemical quenching (*qP*) was lower in droughted plants, whereas non-photochemical quenching (*NPQ*) was stimulated by 27% in low water availability treatments (Table 1). No statistical differences were observed for total chlorophyll content associated with water availability (Table 1). Interestingly, leaf soluble sugar determinations (Fig. 1A) highlighted that glucose and fructose concentrations were diminished under low water availability conditions, even though total soluble sugar (TSS) and D-pinitol content were stimulated. No statistical differences were observed for sucrose content. The analyses of free amino acid content in leaves showed that although drought increased alanine and proline content, glutamic and aspartic acid content decreased in droughted plants (Fig. 1B). On the other hand, although no statistical differences were observed for sucrose, glucose and D-pinitol, in droughted nodules fructose and TSS increased (Fig. 2A). Concerning the nodule free amino acid content, drought increased proline content, whereas glutamic acid and asparagine (marginally significant; P=0.063) decreased. No significant differences at P <0.05 were detected in alanine, aspartic acid, and serine levels (Fig. 2B).

The drought effect on the leaf protein pattern in alfalfa plants was studied using 2-DE (Fig. 3). Our protocol enabled the identification of 26 proteins that differed in their expression under control and drought conditions. Twelve of those proteins were down-regulated by drought,

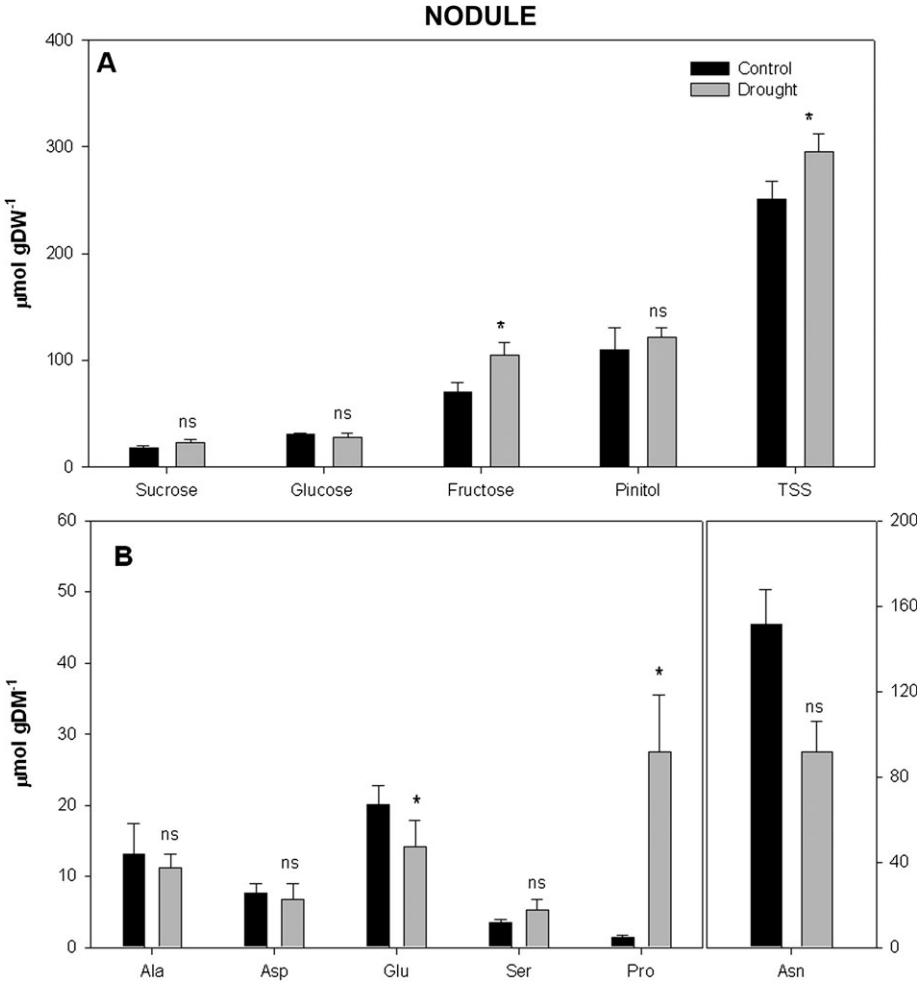

Fig. 2. Water availability effect on (A) nodule soluble sugar (sucrose, glucose, fructose, pinitol, and total soluble sugar, TSS) and (B) free amino acid (Ala, alanine; Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Asn, asparagine, and Pro, proline) content in *Medicago sativa* plants. Measurements were conducted at the end of the experiment. Each value represents the mean ±SE (n=4). The different letters indicate significant differences (P <0.05) between treatments.

with the remaining 16 being up-regulated (Table 2). These proteins were classified into different groups according to their presumed biological function. The down-regulated proteins were classified into two groups: energy processes (11 proteins identified) and protein destination and storage processes (1 protein identified). Among the up-regulated proteins, six groups were detected: metabolism (2 proteins identified), energy (4 proteins identified), protein destination and storage (3 proteins identified), transport (1 protein identified), cell structure (1 protein identified) and the proteins involved in disease/defence processes (5 proteins identified). The roles of these proteins are discussed in the following section with regard to changes in physiological traits in response to drought.

Discussion

The inhibitory effect of drought on photosynthetic activity has been widely described and is mainly associated with stomatal and metabolic limitations (Chaves *et al.*, 2009; Lawlor and Tezara, 2009). The decrease in leaf water content (from 76.32% to 51.68%) and the increase in leaf osmotic potential (Ψ_s) confirmed the deterioration of leaf water status in droughted plants.

Physiological characterization parameters revealed that diminishment of the Rubisco carboxylation (Vc_{max}) and the RuBP regeneration activities (J_{max}), together with stomatal closure (Table 1) explained the photosynthetic decrease in droughted plants (Allen *et al.*, 1997; Nogués and Baker, 2000; Nunes *et al.*, 2008). Although the drought effect on Rubisco activity is a long known phenomenon, the analysis of its regulation is complex (Parry *et al.*, 2002; Chaves *et al.*, 2009; Lawlor and Tezara, 2009), especially in N_2-fixing plants.

At the leaf level, the negative effects of drought on the large subunits of the four different Rubisco spots (Table 2) revealed that diminishment of Rubisco activity can be explained, in part, by lower Rubisco availability (Table 2). The down-regulation observed for proteins involved in Rubisco assembly (putative Rubisco, and the subunit binding-protein) confirmed this point. The reduction in N_{ase} activity observed in droughted plants (Table 1), together with the general depletion of the main leaf amino acid

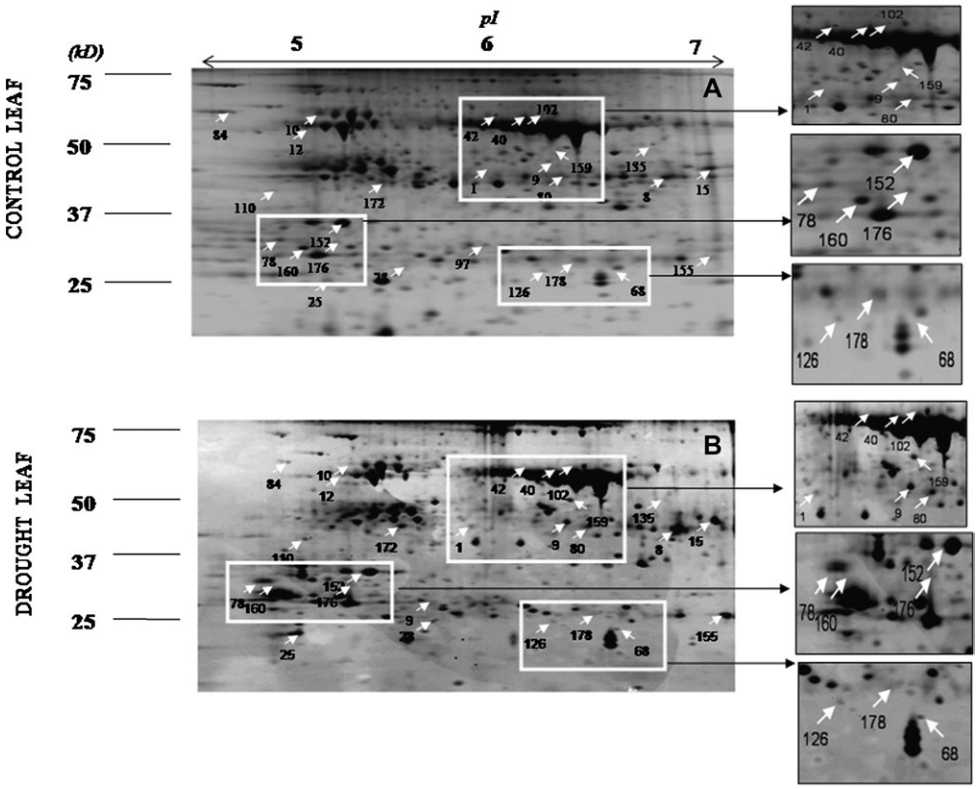

Fig. 3. Silver-stained two-dimensional gel of proteins extracted from *Medicago sativa* leaves grown under control (A) and drought (B) conditions. In the first dimension, 125 mg of total protein was loaded on a 18 cm IEF strip with a linear gradient of pH 4–7. The second dimension was conducted in 12% polyacrylamide (w/v) gels (20×20 cm) (for details, see Materials and methods). The gel image analyses conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 26 proteins (marked by arrows) that, statistically, were involved in the plant response to drought.

content (with the exception of proline and alanine; Fig. 1B) suggests that drought negatively affected plant N availability (Gordon *et al.*, 1997; Ramos *et al.*, 1999; Ladrera *et al.*, 2007; Larrainzar *et al.*, 2009). Under these unfavourable conditions, the mobilization of N from the main leaf N reservoir (i.e. Rubisco) would contribute towards alleviating the N deficiency (Feller *et al.*, 2008). This idea was reinforced by the up-regulation under drought conditions of enzymes with proteolytic activity, such as the proteosome β1 subunit (CCI7) (Table 2), which could have contributed to the degradation of Rubisco (Desclos *et al.*, 2009). In accordance with this point, the up-regulation of glutamine synthetase (the enzyme involved in the GS-GOGAT cycle where assimilated NH_3 is converted to glutamic acid, Glu, and glutamine, Gln) under drought conditions suggests that there was reallocation of N derived from Rubisco to other processes and organs (Ochs *et al.*, 1999; Diaz *et al.*, 2008; Desclos *et al.*, 2009). The fact that, in droughted leaves, Glu levels decreased suggests that this amino acid was rapidly redirected to proline synthesis (Delauney and Verma, 1993; Fougère *et al.*, 1991; Hare *et al.*, 1999). The increase in free proline under water stress (Figs 1B, 2B) is associated with its role as an osmoregulant to prevent dehydration and maintaining negative water potential to avoid water loss (Irigoyen *et al.*, 1992; Hare *et al.*, 1998). The fact that Rubisco activase content (Table 2) diminished under droughted conditions revealed that the lower activation state could also have negatively affected the above-mentioned Rubisco activity (Chaves *et al.*, 2002; Reddy *et al.*, 2004; Parry *et al.*, 2008).

The decrease in J_{max} under drought conditions suggests that the diminishment of Rubisco activity and photosynthetic activity could also be associated with limitations of RuBP regeneration. The down-regulation of enzymes involved in the C regeneration processes (sedoheptulose-1,7-*bis*phosphatase, ribulose-phosphate 3-epimerase and phosphoribulokinase) in drought conditions (Table 2) may have caused the depletion in RuBP regeneration. The repression of genes involved in the synthesis of Calvin Cycle enzymes under drought conditions has been associated with an increase in sugar concentration in the leaf (Krapp *et al.*, 1991; Koch, 1996; Stitt *et al.*, 2007) suggesting that sugars may act as signalling molecules (Chaves *et al.*, 2009). The larger leaf total soluble sugar (TSS, Fig. 1A) levels that were detected in drought conditions could have been involved in the inhibition of Calvin Cycle enzymes. The enhancement in soluble sugar content was related to an increase in pinitol content (Fig. 1A). Alongside proline (Fig. 1B), pinitol was central in maintaining water balance in alfalfa plants. As recently described by Obendorf *et al.* (2008), glucose is one of the precursors of pinitol synthesis and therefore the decrease in glucose content in droughted leaves could have

Table 2. Annotation of up/down-regulated identified spots following drought in silver stained two-dimensional electrophoresis gels of leaves

Spot no. represents the number of proteins assigned. Spot volume (%) is an estimation of relative protein abundance. The pI and molecular mass (M_r) values shown are the theoretical and experimental values. SC represents the protein sequence coverage (%) score, which is the Mascot score of the in-solution digestion protocol. Function, the predicted protein function is assigned according to the NCBInr-protein sequence database.

Spot no.	Spot % volume variations	Experimental pI/M_r	Theoretical pI/M_r	PM	SC (%)	Score (P <0.05 corresponding to score >51)	Protein name/organism/NCBI accession no.	Regulation
01. Metabolism								
159	641.02	6.31/51.0	6.06/49.71	5	17	198	Dihydrolipoamide dehydrogenase/*Pisum sativum*/gil9955321	**Up-regulated**
172	720.36	5.35/40.7	6.29/47.08	14	19	466	Glutamine synthetase/*Medicago sativa* /gil17367236	**Up-regulated**
02. Energy								
8	170.34	6.85/47.8	9.75/49.63	2	28	62	Glyceraldehyde 3-phosphate dehydrogenase/*Ficus wassa*/gil87300266	**Up-regulated**
10	16.92	4.85/56.5	4.99/61.78	2	4	153	β subunit of mitochondrial ATP synthase/*Chlamydomonas reinhardtii* /gil159466892	**Down-regulated**
12	16.84	4.77/52.8	5.25/52.68	2	5	60	β subunit of ATP synthase/*Eurya* sp./gil20269410	**Down-regulated**
15	167.44	7.19/46.0	8.93/43.411	5	20	218	(NADP-dependent glyceraldehydephosphate) Glyceraldehyde-3-phosphate dehydrogenase A/*Pisum sativum*/gil120658	**Up-regulated**
40	16.33	6.34/63.0	6.09/52.11	3	8	138	Rubisco large subunit/*Pieris phillyreifolia* /gil1352804	**Down-regulated**
42	16.29	6.30/62.1	6.0/51.00	4	10	159	Rubisco large subunit/*Heisteria cauliflora*/gil112408850	**Down-regulated**
80	155.37	6.37/41.5	5.92/35.47	5	21	191	Cytosolic malate dehydrogenase/*Cicer arietinum*/gil10334493	**Up-regulated**
97	48.84	5.99/35.7	5.83/38.63	5	18	258	Fructose-*bis*phosphate aldolase 1/*Pisum sativum*/gil399024	**Down-regulated**
102	15.01	6.30/62.1	6.22/51.85	6	14	233	Rubisco large subunit/*Akania bidwillii*/gil2500654	**Down-regulated**
135	57.1	6.70/51.0	6.73/29.96	2	10	60	Rubisco large subunit/*Echeandia* sp./gil1865802	**Down-regulated**
152	14.01	5.04/35.2	5.63/29.99	37	65	1057	Rubisco activase/*Medicago sativa*/gil23320705	**Down-regulated**
155	1381.95	7.02/34.0	8.80/43.31	14	24	607	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor/*Pisum sativum*/gil120658	**Up-regulated**
160	13.37	4.20/30.5	5.83/42.21	10	19	306	Sedoheptulose-1,7-*bis*phosphatase precursor/*Oryza sativa* (indica cultivar-group)/gil27804768	**Down-regulated**
176	24.45	5.05/30.7	5.41/39.00	3	11	73	Phosphoribulokinase/*Pisum sativum*/gil1885326	**Down-regulated**
178	76.01	6.17/27.2	8.23/30.34	3	9	107	Ribulose-phosphate 3-epimerase/*Spinacia oleracea*/gil2833386	**Down-regulated**
06. Protein destination and storage								
78	155.68	4.86/33.9	8.80/43.31	11	35	514	14-3-3-like protein/*Cicer arietinum*/gil148612111	**Up-regulated**
84	15.47	4.85/62.5	5.20/61.18	21	35	985	Putative rubisco subunit binding-protein alpha subunit/*Trifolium pratense*/gil84468288	**Down-regulated**
110	148.88	4.61/40.0	4.60/38.37	5	11	178	Plastoglobulin-1/*Pisum sativum*/gil62900628	**Up-regulated**
126	145.18	6.31/27.5	6.30/24.60	1	8	69	Proteasome subunit beta type-1 (20S proteasome alpha subunit F) (20S proteasome subunit beta-6)/*Petunia×hybrida*/gil17380185	**Up-regulated**
07. Transport								
9	169.45	6.33/46.5	6.04/35.10	2	9	62	Putative chloroplast inner envelope protein/*Oryza sativa*/gil15341602	**Up-regulated**
09. Cell structure								
1	178.63	5.95/40.2	6.01/40.82	3	11	149	Reversibly glycosylated polypeptide/*Gossypium hirsutum*/gil18077708	**Up-regulated**

Table 2. *Continued*

Spot no.	Spot % volume variations	Experimental pI//M_r	Theoretical pI//M_r	PM	SC (%)	Score (P <0.05 corresponding to score >51)	Protein name/organism/NCBI accession no.	Regulation
11. Disease/defence								
25	161.22	4.96/24.9	4.93/21.84	4	24	211	2-cys peroxiredoxin-like protein/*Hyacinthus orientalis*/gil47027073	**Up-regulated**
28	165.27	5.49/25.0	5.47/24.04	2	8	111	Dehydroascorbate reductase/*Zinnia elegans*/gil50058092	**Up-regulated**
68	158.47	6.45/25.6	7.16/26.62	1	6	67	Superoxide dismutase/*Medicago sativa*/gil23534609	**Up-regulated**
78	155.68	4.86/33.9	8.80/43.31	11	35	514	14-3-3-like protein/*Cicer arietinum*/gil148612111	**Up-regulated**
155	1381.95	7.02/34.0	8.80/43.31	14	24	607	Glyceraldehyde-3-phosphate dehydrogenase A, subunit/*Pisum sativum*/gil120658	**Up-regulated**
159	641.02	6.31/51.0	6.06/49.71	5	17	198	Dihydrolipoamide dehydrogenase/*Pisum sativum*/gil9955321	**Up-regulated**

been a consequence of glucose being directed towards pinitol synthesis. This is confirmed by the up-regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which implies that more 3-phosphoglycerate was formed. 3-phosphoglycerate is a glucose (Buchanan *et al.*, 2000) and pinitol precursor. Pinitol has been previously described in legumes as a major carbohydrate (up to the 50–60% of soluble sugars), especially under water stress conditions (Ford, 1984; McManus *et al.*, 2000; Streeter *et al.*, 2001) and it may act as an osmolyte (Reddy *et al.*, 2004).

The reported inhibition of Rubisco and other enzymes involved in the Calvin cycle under drought (see above) implies a reduction in the demand for ATP and NADPH. Such a decrease was also reflected in the reduction in electron transport (Lawlor and Tezara, 2009) through photosystem II [*Je*(PSII); Table 1]. Drought increased the electron flux destined for photorespiratory carbon oxidation [*Je*(PCO); Table 1]. This enhancement was confirmed by the up-regulation of two protein isoforms of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the up-regulation of dihydrolipoamide dehydrogenase (Table 2), both involved in photorespiration (Bourguignon *et al.*, 1996; Wingler *et al.*, 1999). The excess of electrons was transferred to oxygen at PSI or via the Mehler reaction, with the consequent generation of reactive oxygen species (ROS; Mehta *et al.*, 1992; Ort *et al.*, 2002; Chaves and Oliveira, 2004; Bogeat-Triboulot *et al.*, 2007; Moreno *et al.*, 2008; Parry *et al.*, 2008). In order to protect PSII against increased production of ROS, drought-stressed plants improved the mechanism of excess electron removal by dissipating part of this energy through heat emission (Ort and Baker, 2002) as observed by the enhancement of NPQ (Table 1). Moreover, the proteomic profile revealed that drought induced the up-regulation (Table 2) of well-known proteins involved in detoxification of ROS such as superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), 2-cys peroxiredoxin-like protein (BAS1) (Baier *et al.*, 2000; Dietz *et al.*, 2002; Desclos *et al.*, 2009) and GAPDH, which is also involved in ROS scavenging (Hancock *et al.*, 2005).

Deleterious effects of drought on N_{ase} activity (Table 1) have confirmed that the legume–*Sinorhizobium* symbiosis is very sensitive to water stress (Antolín and Sánchez-Díaz, 1992; Irigoyen *et al.*, 1992; Streeter, 2003; Aranjuelo *et al.*, 2007; Ladrera *et al.*, 2007; Larrainzar *et al.*, 2009). Under drought conditions, symbiotic nitrogen fixation (SNF) was more affected by drought than CO_2-photosynthetic assimilation rates (Durand *et al.*, 1987; Serraj *et al.*, 1999). The deleterious effect of drought on N_{ase} activity decreased asparagine (Asn) content in the nodules (Fig. 2B), which is the major N-transporting amino acid. Furthermore, since ammonia (another major form of N transport) content is also dependent on N_2 fixation, drought might have negatively affected its availability and limited its partitioning to leaves with a consequent limitation to plant N availability. The enhancement of nodule TSS levels under drought conditions (Fig. 2A) means that sugar availability limitations were discarded as an explanation of decreased N_2 fixation (Ramos *et al.*, 1999; Gálvez *et al.*, 2005; Naya *et al.*, 2007). However, reduced nodule respiration rates (Table 1), in the form of lower N_{ase}-linked respiration, reflects the possible impairment of C metabolism that could have contributed to the depletion of N_{ase} activity (Gordon *et al.*, 1997; Ramos *et al.*, 1999). The lower respiration rates observed in nodules (Table 1) suggest that the TSS increase under drought conditions was associated with the decrease in carbohydrate requirements (Fellows *et al.*, 1987; Aguirreolea and Sánchez-Díaz, 1989; Irigoyen *et al.*, 1992). According to Gálvez *et al.* (2005), the down-regulation of the glycolytic pathway might provoke a shortage of substrates for bacteroid respiration, and, as a consequence, a transient accumulation of oxygen in the affected region would occur leading to an increase in the resistance of the oxygen diffusion barrier in order to avoid N_{ase} damage.

Other studies (Hartwig, 1998; Serraj *et al.*, 1998, 1999; King and Purcell, 2005) have suggested that N_2 fixation is regulated by the N feedback mechanism. However, proline was the only amino acid that increased in droughted

nodules (Fig. 2B) and its increase is associated with osmoregulatory mechanisms that maintain nodule turgor (Fougère *et al.*, 1991; Irigoyen *et al.*, 1992; Hare *et al.*, 1999). Our data suggest that, as a consequence of N_{ase} inhibition, droughted nodules invested large resources in the synthesis of proline osmoregulant. Another mechanism that could play a role in the drought-induced inhibition of N_2 fixation, but has received much less attention, is oxidative stress (Marino *et al.*, 2006; Naya *et al.*, 2007). High proline levels in the nodules have been observed to have a protective role against ROS (Koca *et al.*, 2007; Türkan and Demiral, 2009). The increase observed in proline levels of droughted nodules suggests that these nodules could have been subjected to oxidative stress and that proline increase played an important role in averting oxidative damage in nodules under water stress.

Conclusions

In summary, the analysis of the leaf proteome and nodule metabolism that was conducted in this work has provided new insights into the drought impairment of photosynthetic activity as revealed by physiological studies. This study showed that droughted plants invested a large quantity of C and N resources into the synthesis of osmoregulants (i.e. pinitol and proline) in order to maintain osmotic turgor in droughted leaves and nodules. Although stomatal closure initially limited the photosynthetic activity of drought-stressed alfalfa plants, proteomic analyses revealed important metabolic constraints. The deleterious effect of drought on photosynthetic activity was targeted to Rubisco and N_{ase} activities. Drought negatively affected the availability of Rubisco binding protein content (involved in the assembly of Rubisco large and small subunits) and this could be related to the lower Rubisco availability. The lower activation state (as reflected by the lower Rubisco activase content) of this protein may have also contributed to the photosynthetic decrease. Furthermore, the proteomic characterization also revealed that the down-regulation of three Calvin cycle proteins involved in the C regeneration contributed to limiting RuBP regeneration and, consequently, Rubisco activity. The deleterious effect of drought on N_{ase} activity was involved in the down-regulation of Rubisco protein content. The up-regulation of proteases and glutamine synthetase, together with the depletion of aspartic and glutamic acid under drought conditions, highlighted the fact that Rubisco-derived N was targeted to the synthesis of osmoregulant compounds (i.e. proline) and may be transported to the taproot. The increase in nodule TSS and the reduced respiration observed in droughted nodules suggests that N_{ase} activity decreased due to enhancement of the oxygen diffusion barrier resistance that prevents oxidative damage to N_{ase}. Although more research is required in this area, the increase in proline content reveals that the potential for oxidative stress damage in droughted nodules should also be considered.

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