The Response of Carbon Metabolism and Antioxidant Defenses of Alfalfa Nodules to Drought Stress and to the Subsequent Recovery of Plants[1,2][W][OA]

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Alfalfa (*Medicago sativa*) plants were exposed to drought to examine the involvement of carbon metabolism and oxidative stress in the decline of nitrogenase (N_2ase) activity. Exposure of plants to a moderate drought (leaf water potential of −1.3 MPa) had no effect on sucrose (Suc) synthase (SS) activity, but caused inhibition of N_2ase activity (−43%), accumulation of succinate (+36%) and Suc (+58%), and up-regulation of genes encoding cytosolic CuZn-superoxide dismutase (SOD), plastid FeSOD, cytosolic glutathione reductase, and bacterial MnSOD and catalases B and C. Intensification of stress (−2.1 MPa) decreased N_2ase (−82%) and SS (−30%) activities and increased malate (+40%), succinate (+68%), and Suc (+435%). There was also up-regulation (mRNA) of cytosolic ascorbate peroxidase and down-regulation (mRNA) of SS, homoglutathione synthetase, and bacterial catalase A. Drought stress did not affect *nifH* mRNA level or leghemoglobin expression, but decreased MoFe- and Fe-proteins. Rewatering of plants led to a partial recovery of the activity (75%) and proteins (>64%) of N_2ase, a complete recovery of Suc, and a decrease of malate (−48%) relative to control. The increase in O_2 diffusion resistance, the decrease in N_2ase-linked respiration and N_2ase proteins, the accumulation of respiratory substrates and oxidized lipids and proteins, and the up-regulation of antioxidant genes reveal that bacteroids have their respiratory activity impaired and that oxidative stress occurs in nodules under drought conditions prior to any detectable effect on SS or leghemoglobin. We conclude that a limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of N_2ase activity in alfalfa nodules.

Drought is a major factor limiting crop production and has a particularly negative impact on symbiotic N_2 fixation (Sprent, 1972; Zahran, 1999). However, the causes for the drought-induced inhibition of nitrogenase (N_2ase) activity are still uncertain. Studies on soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), and pea (*Pisum sativum*) have shown that the inhibitory effects may be mediated by a decrease in nodule O_2 permeability (Durand et al., 1987; Serraj and Sinclair, 1996; Ramos et al., 1999) and metabolic activity (Díaz del Castillo and Layzell, 1995). The finding that the activity of Suc synthase (SS), but not other carbon and nitrogen metabolism enzymes, rapidly declined in nodules upon imposition of stress provided strong evidence for a major role of SS in the inhibition of N_2 fixation (González et al., 1995; Gordon et al., 1997; Ramos et al., 1999). A detailed biochemical analysis of the *rug4* mutant of pea, which displays severely reduced SS activity, further demonstrated an essential role of SS in symbiosis (Gordon et al., 1999). These and subsequent results (Gálvez et al., 2005; Marino et al., 2006) led to the conclusion that SS is a critical regulatory enzyme in nodule carbon metabolism and in the early response of N_2 fixation to drought. The authors suggested that the inhibition of SS activity restricts the availability of malate and other dicarboxylic acids for bacteroid respiration (Fig. 1A), and that this is responsible for the inhibition of N_2ase activity (Gordon et al., 1997; Gálvez et al., 2005).

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. In plant

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[2] Dedicated to Dr. Frank R. Minchin on the occasion of his retirement.

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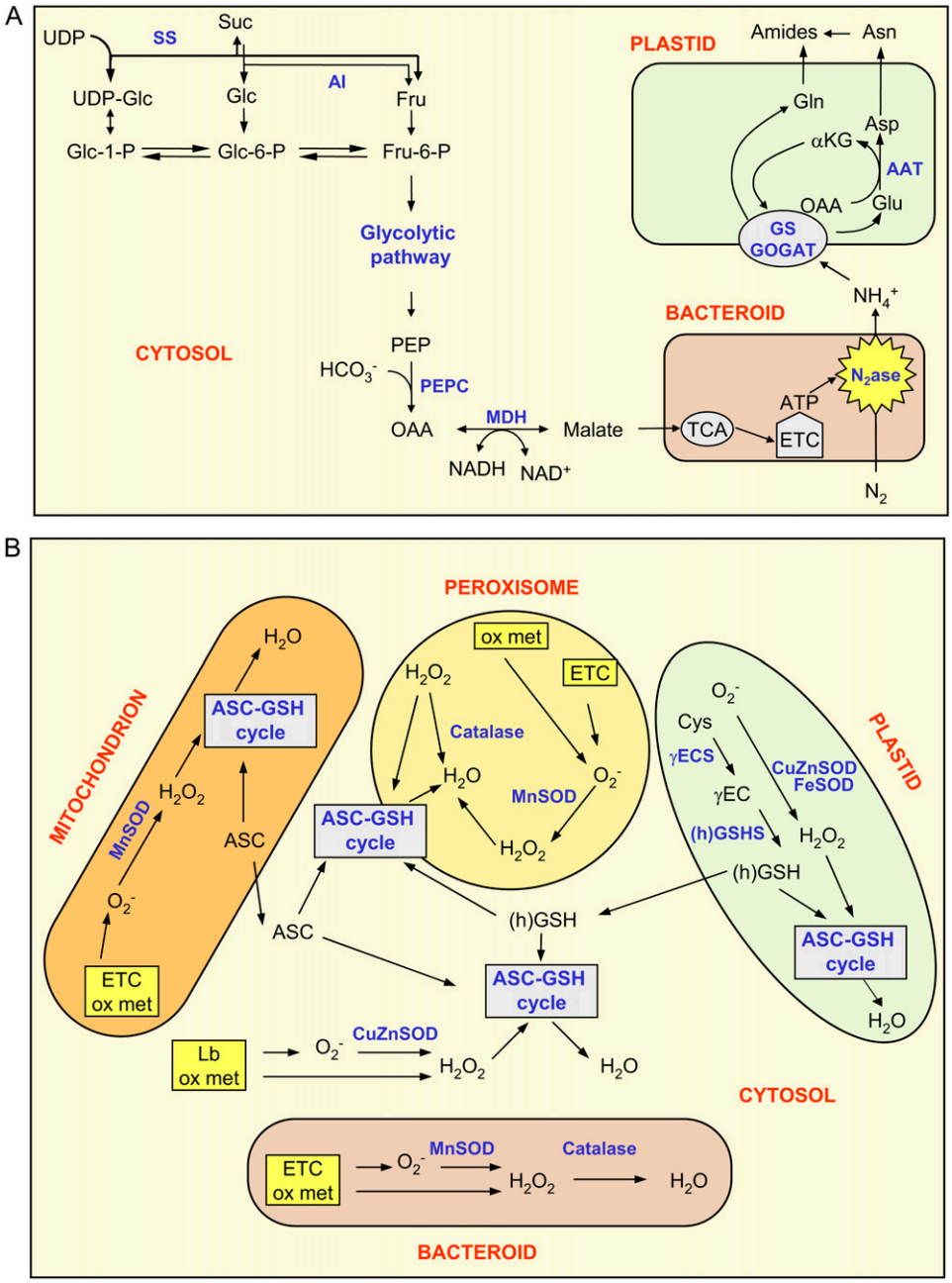

Figure 1. Simplified representations of some metabolic routes examined in this work. A, Carbon and nitrogen metabolism in nodules of an amide-producing legume, such as alfalfa. Adapted from Arrese-Igor et al. (1999). B, Production of ROS and some major antioxidant systems in legume nodules. Adapted from Matamoros et al. (2003). AAT, Asp aminotransferase; ASC, ascorbate; ETC, electron transfer chain; GS, Gln synthetase; OAA, oxaloacetate; ox met, oxidative metabolism; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; MDH, malate dehydrogenase; TCA, tricarboxylic acid cycle.

cells, this occurs when the generation of reactive oxygen species (ROS) overwhelms the antioxidant defenses (Fig. 1B). Indeed, drought induces oxidative stress in pea nodules (Gogorcena et al., 1995), and other types of abiotic stress also lead to general decreases of antioxidant activities that are associated with nodule senescence (Swaraj et al., 1994; Gogorcena et al., 1997; Hernández-Jiménez et al., 2002; Porcel et al., 2003). In addition, the application of low concentrations of paraquat, a ROS generator, to the rooting medium of pea plants inhibits SS activity of nodules prior to N_2ase activity, suggesting that SS is an early target of oxidative stress in nodules (Marino et al., 2006). However, in all of these studies, the response of nodule antioxidants was not analyzed at the molecular level and in most of them N_2ase activity was not monitored, making it difficult to establish a relationship between the decrease of antioxidant protection and the loss of nodule function.

Alfalfa (*Medicago sativa*) is a perennial forage legume of great agronomical interest that produces indeterminate nodules upon infection of roots with *Sinorhizobium meliloti*. Previous agronomical, physiological, and biochemical studies strongly suggest that alfalfa is more drought tolerant than pea (Moran et al., 1994) and ureide-producing grain legumes (Sinclair and Serraj, 1995).

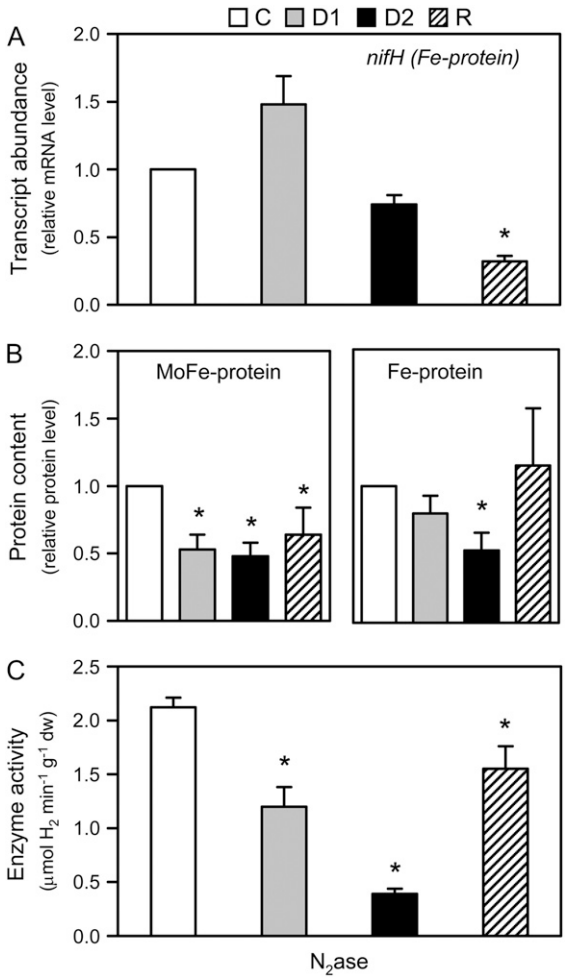

Figure 2. Expression of N_2ase in alfalfa plants exposed to drought stress and following recovery from drought. A, Steady-state mRNA levels of the *nifH* gene, encoding the Fe-protein (component 2), were quantified by qRT-PCR. Values are means ± SE of six biological replicates, each corresponding to RNA extracts from different plants. B, Protein levels of the MoFe-protein (component 1) and Fe-protein (component 2) of N_2ase. Values are means ± SE of four western blots that were analyzed densitometrically. C, Apparent N_2ase activity of intact plants measured as H_2 evolution with an open flow-through system. Values are means ± SE of five or six replicates. For all panels, treatments are designated as C (control), D1 (moderate drought stress), D2 (severe drought stress), and R (recovery). For A, means of D1, D2, and R are indicated with an asterisk when >2 (up-regulation) or <0.5 (down-regulation). For B and C, means of D1, D2, and R marked with an asterisk are significantly different from C, as determined by the Dunnett's *t* test ($P < 0.05$).

For example, photosynthesis was inhibited by 77% in pea leaves having a water potential (Ψ_w) of −1.3 MPa (Moran et al., 1994), but only decreased by 28% in alfalfa leaves at a Ψ_w of −1.8 MPa (Rubio et al., 2002). This inhibition was accompanied by consistent decreases in antioxidant activities and soluble protein in pea leaves, but by either minor or no changes in alfalfa leaves. On the basis of this relatively high drought tolerance, we have selected alfalfa as plant material to test the hypotheses that the SS activity and the antioxidant defenses of nodules are involved in the drought-induced inhibition of N_2ase activity.

RESULTS

Effect of Drought on N_2ase and Nodule Respiration

The expression of N_2ase (mRNA, protein, and activity) was analyzed in nodulated alfalfa plants subjected to drought stress (Fig. 2). The mRNA level of the *nifH* gene (encoding the Fe-protein of N_2ase) of *S. meliloti* was determined by quantitative reverse transcription (qRT)-PCR, normalized with housekeeping genes, and expressed relative to values of control plants (Fig. 2A). Following the same criteria for significant gene up-regulation (ratio >2) or down-regulation (ratio <0.5) in the qRT-PCR analysis as those generally used in cDNA array studies (El Yahyaoui et al., 2004), our results show that the *nifH* mRNA level in alfalfa nodule bacteroids did not appreciably change under moderate or severe stress but decreased after the recovery period. Immunoblots revealed a significant decrease in the contents of the two proteins of N_2ase (MoFe-protein and Fe-protein), especially in nodules from severely stressed plants, and a partial (MoFe-protein) or complete (Fe-protein) recovery in nodules from reirrigated plants (Fig. 2B). The apparent N_2ase activity was measured as H_2 evolution in intact alfalfa plants 'Aragón' using an open flow-through system to minimize plant disturbance (Minchin et al., 1986). In alfalfa 'Aragón,' this activity consistently decreased upon application of moderate (−43%) and severe (−82%) drought stress, and recovered up to 75% of the control values after rewatering of plants (Fig. 2C).

An open flow-through system was also used to measure simultaneously H_2 and CO_2 evolution, and hence to determine N_2ase activity and some related parameters in control and drought-stressed plants of alfalfa 'N4' (Table I). A moderate drought stress induced a sharp decline (−81%) in total N_2ase activity, which was accompanied by less pronounced decreases in total root respiration (−54%) and in N_2ase-linked respiration (−66%). The discrepancy between the extent of inhibition of N_2ase activity and its associated respiration can be explained by major increases in the carbon cost of N_2ase (+126%) and in the O_2 diffusion resistance of nodules (+158%).

Effect of Drought on Organic Acids, Sugars, and Associated Enzymes

The effects of drought on the major dicarboxylic acids (succinate, α-ketoglutarate [αKG], and malate) and sugars (Suc) of nodules were examined (Fig. 3A), as they are used by the host cells and bacteroids for the production of the energy and reducing power required for N_2 fixation and other metabolic reactions (Temple et al., 1998). The application of a moderate drought caused an accumulation of Suc (+58%), no changes in malate, and a decrease (−23%) in αKG, relative to

Table I. *Effect of drought stress on parameters related to N_2ase activity, respiration, and O_2 diffusion resistance in alfalfa nodules*

Treatments are designated as C (control) and D1 (moderate drought stress). Values are means ± SE of six replicates, and those marked with an asterisk are significantly different based on the Dunnett's *t* test ($P < 0.05$). TRR, Total root respiration; GMR, nodulated root growth and maintenance respiration; NLR, N_2ase-linked respiration; *R* (−Ar), O_2 diffusion resistance in 79% N_2 + 21% O_2; *R* (+Ar), O_2 diffusion resistance in 79% Ar + 21% O_2.

Parameter	Units	C	D1	Δ
				%
N_2ase	μmol H_2 min^{-1} $plant^{-1}$	1.40	0.27*	−81
TRR	μmol CO_2 min^{-1} $plant^{-1}$	6.82	3.12*	−54
GMR	μmol CO_2 min^{-1} $plant^{-1}$	3.45	2.03*	−41
NLR	μmol CO_2 min^{-1} $plant^{-1}$	3.43	1.15*	−66
Carbon cost	mol CO_2 mol^{-1} H_2	1.96	4.42*	+126
R (−Ar)	s m^{-1}, × 10^{-6}	0.26	0.67*	+158
R (+Ar)	s m^{-1}, × 10^{-6}	0.56	1.04*	+86

values of control nodules. Intensification of stress had no further negative effect on the αKG content of nodules, but led to a consistent accumulation of succinate (+68%), malate (+42%), and Suc (+435%). Rewatering of plants caused the return of succinate and Suc to control values, whereas it sharply decreased (−48%) the nodule contents of αKG and malate.

Drought also affected the activities of some carbon and nitrogen metabolism enzymes in nodules (Fig. 3B), albeit the effects were less intense than those observed for carbon metabolites (Fig. 3A). In fact, the activities of SS, isocitrate dehydrogenase (ICDH), alkaline invertase (AI), and Glu synthase (GOGAT) in nodules of moderately stressed plants did not significantly differ from those of control plants. Application of severe drought stress only led to a minor decrease (−12%) in ICDH activity, but caused a significant decline (−30%) in SS, AI, and GOGAT activities. Upon rewatering of plants, SS activity returned to control values, ICDH and AI activities remained higher than the control (+20%), and GOGAT activity did not recover (Fig. 3B).

Effect of Drought on Antioxidant Enzymes of Nodule Host Cells

The high specificity and sensitivity afforded by qRT-PCR was central for the quantification of mRNAs encoding various isoforms of antioxidant enzymes, such as the superoxide dismutases (SODs), from the nodule host cells (Fig. 4A). Gene-specific primers (Supplemental Table S1) were designed for RT-PCR amplification of the cDNAs coding for CuZnSODc, CuZnSODp, plastidic FeSOD, and mitochondrial MnSOD. According to the criteria mentioned above for changes in gene expression to be considered significant, there was up-regulation of *CuZnSODc* and *FeSOD* under moderate drought and of *CuZnSODp* during recovery, but no changes in the expression of *MnSOD* for any of the treatments (Fig. 4A).

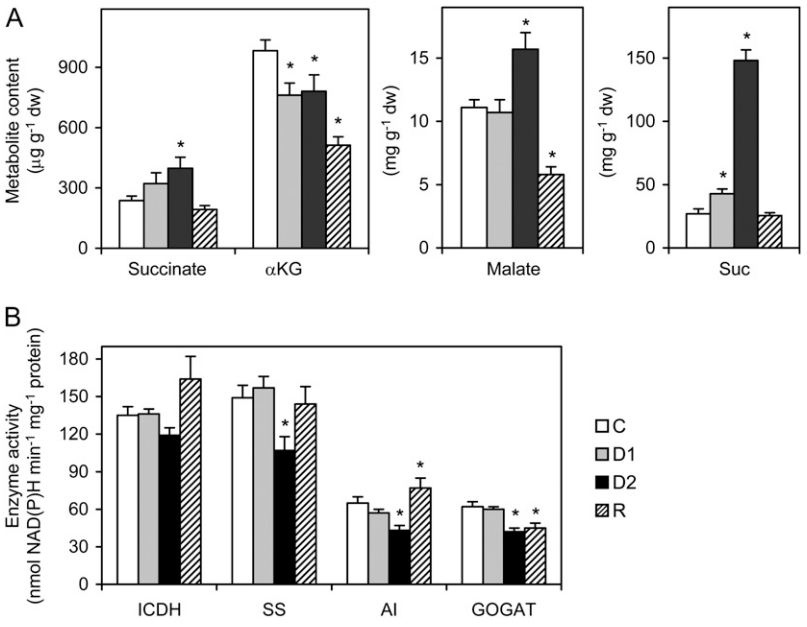

Figure 3. Contents of carbon substrates and associated enzyme activities in nodules of alfalfa plants exposed to drought stress and following recovery from drought. A, Contents of dicarboxylic acids and Suc in nodules. B, Specific activities of ICDH, SS, AI, and GOGAT in nodules. Plant treatments and statistical analysis are as described in Figure 2. Values are means ± SE of six to 10 replicates.

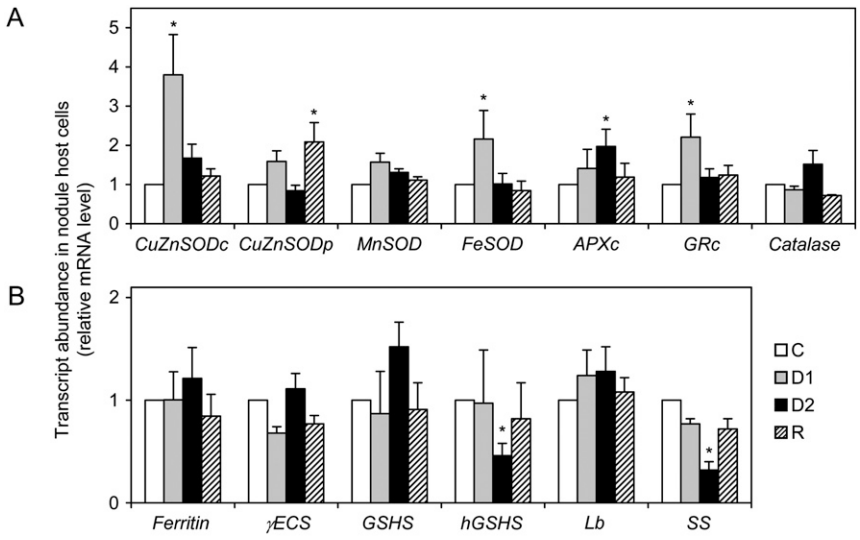

Figure 4. Steady-state levels of mRNAs encoding antioxidant enzymes (A) and other important proteins (B) in the nodule host cells of alfalfa plants exposed to drought stress and following recovery from drought. Plant treatments are designated as in Figure 2. Values are means ± SE of four to six biological replicates, each corresponding to RNA extracts from different plants. Means of D1, D2, and R are indicated with an asterisk when >2 (up-regulation) or <0.5 (down-regulation).

The expression of other antioxidant enzymes (Fig. 4A), as well as of other important proteins (Fig. 4B), was investigated in nodules. Cytosolic ascorbate peroxidase (APXc) and catalase are involved in the direct scavenging of H_2O_2; cytosolic glutathione reductase (GRc) in the detoxification of H_2O_2 and maintenance of glutathione in the reduced form; ferritin in the binding of free iron in a nontoxic form; and γ-glutamylcysteine synthetase, glutathione synthetase, and homoglutathione synthetase (hGSHS) in the synthesis of thiol compounds (Matamoros et al., 2003; Puppo et al., 2005). The only relevant findings were the up-regulation of *GRc* under moderate drought and of *APXc* under severe drought (Fig. 4A), and the down-regulation of *hGSHS* and *SS* under severe drought (Fig. 4B). Also, the imposition of stress or the subsequent recovery of plants from drought had no significant effect on the content of leghemoglobin (Lb), which was approximately 0.12 mg mg^{-1} protein for all treatments, and on the activities of CuZnSODc, CuZnSODp, MnSOD, FeSOD, APXc, GR, and catalase (data not shown). However, the protein level of the CuZnSODc isoform increased slightly in nodules of plants exposed to a moderate or severe (+25%) drought stress and more markedly in nodules of plants recovered from drought (+148%).

Effect of Drought on Antioxidant Enzymes of Bacteroids

Free-living and symbiotic *S. meliloti* contain two SOD and three catalase isoforms. The MnSOD (*sodA*) is located in the cytosol (Santos et al., 1999), whereas the CuZnSOD (*sodC*) is located in the periplasm (Ampe et al., 2003). Catalase A (*katA*) is mainly expressed in the bacteroids, catalase C (*katC*) in the bacteria within the infection threads, and catalase B (*katB*) in both the bacteria and bacteroids (Jamet et al., 2003). Using specific primers (Supplemental Table S1), we examined the effect of drought stress on the expression of the five genes in the bacteroids (Fig. 5). Application of a moderate water deficit to plants led to a modest, yet significant, increase in the *sodA* mRNA level, which returned to control values under severe stress and consistently decreased in the recovery treatment. In contrast, no changes were observed in *sodC* expression for any treatment. Interestingly, the *kat* genes were differentially expressed with drought stress, although the changes were modest in many cases (Fig. 5). Whereas *katB* and *katC* were up-regulated in nodules of moderately stressed plants, the mRNA level of *katA* markedly decreased during drought stress and remained low upon rewatering of plants. Also, in nodules of severely stressed plants, the *katB* gene remained up-regulated, but the mRNA level of *katC* did not differ significantly from the control.

Effect of Drought on Antioxidant Metabolites

The major redox metabolites of nodules were quantified using enzymatic methods and high-performance capillary electrophoresis (CE). This enabled us to determine not only the total amounts of ascorbate and thiol tripeptides, but also the proportions of their oxidized forms (Fig. 6). The content of total ascorbate (ascorbate + dehydroascorbate) in control alfalfa nodules was 5.9 μmol g^{-1} dry weight (0.9 μmol g^{-1} fresh weight), which is in the range reported for other legume nodules (Dalton et al., 1986; Gogorcena et al., 1995, 1997). Ascorbate decreased with moderate (−30%) and severe (−58%) drought stress and partially recovered (79% of control) upon rewatering of plants, whereas dehydroascorbate did not significantly vary with any of the treatments (Fig. 6). Dehydroascorbate accounted for approximately 19% of total ascorbate in nodules of control and moderately stressed plants and increased to 33% during severe stress and recovery, proportions below the range of 40% to 80% observed by Groten et al. (2006) and Marino et al. (2007) in pea

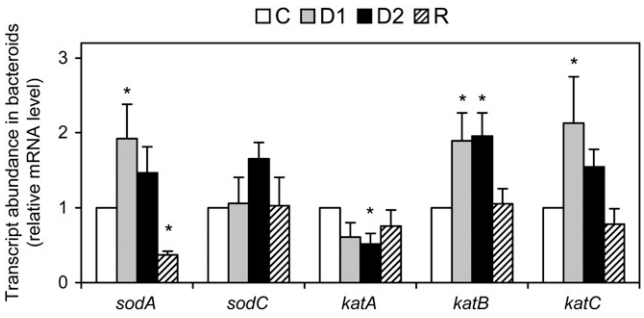

Figure 5. Steady-state levels of mRNAs encoding antioxidant enzymes in the bacteroids of alfalfa plants exposed to drought stress and following recovery from drought. Plant treatments are designated as in Figure 2 and statistical analysis is as described in Figure 4. Values are means ± SE of four to six biological replicates, each corresponding to RNA extracts from different plants.

nodules of a comparable developmental stage. In this respect, we should note that, in our hands, the proportion of dehydroascorbate increased up to 50% to 60% if alfalfa or common bean nodules were not harvested directly into liquid nitrogen but left instead to stand on ice for less than 1 h (J. Loscos, M.A. Matamoros, and M. Becana, unpublished data).

Mature alfalfa nodules have been reported to contain approximately two-thirds of GSH (γGlu-Cys-Gly) and one-third of hGSH (γGlu-Cys-βAla), a structurally related homolog that is present exclusively in some legume species and tissues (Matamoros et al., 2003). This was confirmed by our determinations in control nodules of 4 μmol g^{-1} dry weight (0.7 μmol g^{-1} fresh weight) of GSH and 2.1 μmol g^{-1} dry weight (0.3 μmol g^{-1} fresh weight) of hGSH (Fig. 6). Moderate or severe drought stress had no effect on the contents of GSH or hGSH, whereas, during the recovery of plants, GSH significantly decreased (−30%) but hGSH remained constant. As a result, the proportion of GSH declined from 67% in control plants to 52% in drought-recovered plants, and the proportion of hGSH increased concomitantly from 33% to 48% (Fig. 6). The oxidized forms of the two thiol tripeptides remained fairly constant and at a low level (<4%) during drought and subsequent recovery.

Effect of Drought on Lipid Peroxidation and Protein Oxidation

The accumulation of oxidatively damaged lipids and proteins is a marker of oxidative stress in plant and animal tissues. These products arise, among other mechanisms, by the oxidative attack of lipids and proteins by ROS (Stadtman, 1992; Halliwell and Gutteridge, 1999) and can be conveniently detected and quantified by HPLC and immunological methods. To determine if alfalfa nodules were experiencing oxidative stress under drought conditions, malondialdehyde (MDA), a product of membrane lipid peroxidation, was quantified by reaction with thiobarbituric acid (TBA) and subsequent HPLC analysis of the corresponding colored adduct (Fig. 7A). Moderate and severe drought stress caused similar consistent increases (60%–73%) in the nodule content of MDA, which remained at that high level upon rewatering of plants. The pattern of oxidized proteins in nodules was also examined in gels upon derivatization of the protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNP) using a commercial antibody raised against the hydrazone derivatives (Fig. 7B). Such immunoblots revealed that, relative to controls, the levels of oxidatively modified proteins were higher in nodule extracts from drought-stressed plants and similar in nodule extracts from plants recovered from drought. Taken together, these results provide conclusive evidence that drought induces oxidative stress in alfalfa nodules, a situation previously reported for pea nodules (Moran et al., 1994) and leaves (Iturbe-Ormaetxe et al., 1998).

DISCUSSION

The results of this study with alfalfa reveal that the decline of N_2ase activity with drought does not involve an inhibition of SS activity but a metabolic limitation of bacteroids. This may be caused by alterations in the O_2 availability and respiratory capacity of bacteroids and by the oxidative damage of nodule cell components. Two major differences in the response of

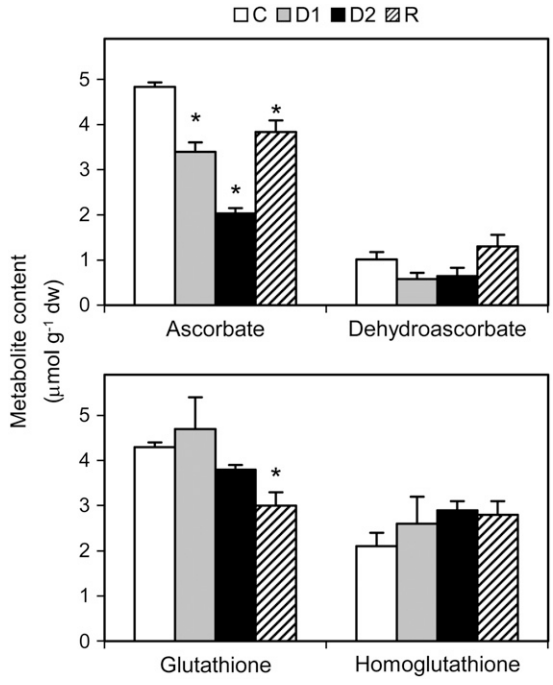

Figure 6. Contents of antioxidant metabolites in nodules of alfalfa plants exposed to drought stress and following recovery from drought. Plant treatments and statistical analysis are as described in Figure 2. Values are means ± SE of five to seven replicates.

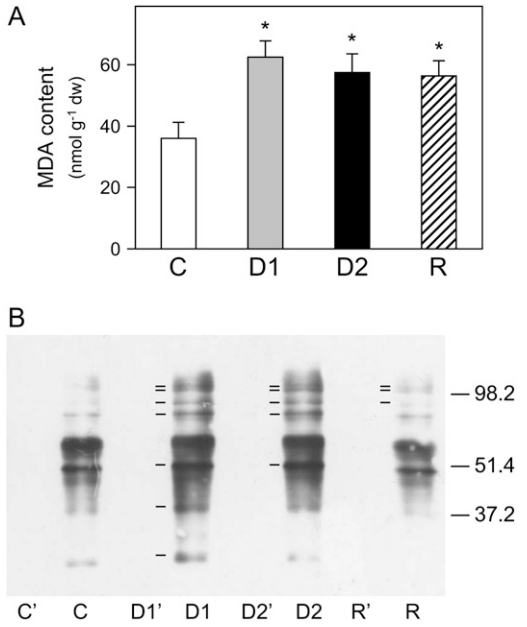

Figure 7. Oxidative damage of lipids and proteins in nodules of alfalfa plants exposed to drought stress and following recovery from drought. For both sections, treatments are designated as C (control), D1 (moderate drought stress), D2 (severe drought stress), and R (recovery). A, Content of MDA in nodules. Values are means ± SE of four replicates and are statistically compared as described in Figure 2. B, Immunoblot analysis of carbonyl derivatives of proteins from nodules. Lanes labeled C′, D1′, D2′, and R′ were loaded with aliquots of the corresponding C, D1, D2, and R extracts, in which the derivatization step was omitted (negative controls). All lanes of the 10% SDS gel contain 15 μg of nodule protein. Approximate molecular masses in kilodaltons are given on the right and bars mark immunoreactive proteins with an enhanced signal in D1, D2, or R as compared to C. The immunoblot shown is representative of four of them, each loaded with nodule extracts from plants grown in different series.

carbon metabolism to drought were found with respect to previous results with other legumes. First, in alfalfa nodules, a moderate drought stress inhibited N_2ase activity by 43% (Fig. 2) but had no effect on SS mRNA (Fig. 4B) and activity (Fig. 3), whereas concomitant decreases in SS and N_2ase activities were observed in common bean (Ramos et al., 1999), pea (Gálvez et al., 2005), and soybean (González et al., 1995). Second, in alfalfa nodules subjected to severe drought stress, when N_2ase was inhibited by 82%, the concentrations of malate and succinate increased by 40% and 68%, respectively (Fig. 3), whereas they declined progressively with drought in pea nodules (Gálvez et al., 2005). Consequently, in alfalfa nodules under stress conditions, SS activity was sufficient to sustain the production of both organic acids from Suc. The accumulation of Suc under drought stress was also reported for other legume nodules and was mainly attributed to the inhibition of SS activity (González et al., 1995; Ramos et al., 1999; Gálvez et al., 2005). Our results, especially with moderately stressed nodules, are in sharp contrast with this explanation and suggest that the accumulation of Suc in alfalfa nodules is caused by a still active import of Suc from the shoot, together with a limitation of Suc consumption in the nodules due to impairment of respiratory activity. Furthermore, the accumulation of dicarboxylic acids and Suc with drought stress (Fig. 3) indicates that nodule metabolism and, in particular, N_2ase activity and respiration are not limited by the provision of reduced carbon.

The existence of metabolic limitations underpinning the drought-induced decline of N_2ase activity was initially proposed on the basis that it was only partially restored by raising external O_2 concentration (Díaz del Castillo and Layzell, 1995; Serraj and Sinclair, 1996). A potential limiting factor is Lb degradation (Guerin et al., 1990; Irigoyen et al., 1992), but there was no effect of drought on the Lb content of alfalfa nodules, in agreement with earlier studies on pea (González et al., 1998) and soybean (Gordon et al., 1999). Another metabolic constraint for nodule activity under drought stress conditions could be a decrease in the respiratory capacity of the bacteroids (Díaz del Castillo and Layzell, 1995). This conclusion is supported by the accumulation of malate and succinate (Fig. 3), the main respiratory substrates of bacteroids (Lodwig and Poole, 2003), and by the reduction of N_2ase-linked respiration (Table I). In accordance with this hypothesis, the increase in resistance to O_2 diffusion would be in response to an increase in O_2 concentration in the infected zone, due to reduced bacteroid respiration. The decline of organic acids in nodules of drought-recovered plants would then be explained by a reactivation of both N_2ase activity and respiration in the bacteroids. Although our results cannot discern whether an alteration of bacteroid respiration is a cause or an effect of the inhibition of N_2ase activity, the molecular analysis of N_2ase expression provides some insight (Fig. 2). Moderate and severe drought stress did not significantly affect the mRNA levels of *nifH*, but did decrease, to different extents, the contents of MoFe-protein and Fe-protein in bacteroids. These observations suggest that the loss of N_2ase activity is, at least in part, caused by protein degradation, in addition to other probable factors such as a decrease in ATP and reducing power. Interestingly, rewatering of plants decreased the *nifH* mRNA level but allowed the partial (MoFe-protein) or total (Fe-protein) recovery of the N_2ase proteins, which may be indicative of an increased translation of the *nifH* transcript or of a higher stability of the Fe-protein in the drought-recovered plants. In any case, N_2ase activity was not completely restored, which suggests that nodule metabolism remains affected even after 2 d of plant rewatering. This metabolic limitation is evident by the significantly lower content of MoFe-protein in bacteroids and by the declines in malate content and GOGAT activity in nodules of drought-recovered plants (Fig. 3). In fact, GOGAT, which is a primary enzyme for nitrogen assimilation in alfalfa nodules (Temple et al., 1998),

may be limited by a lower availability of αKG. This enzyme activity is associated with nodule development and N_2 fixation (Groat and Vance, 1981) and appears to be particularly sensitive to drought stress (Ramos et al., 1999).

Another potential, and frequently overlooked, metabolic constraint of drought-stressed nodules is the oxidative damage of nodule components. Our results reveal that this occurs in alfalfa nodules. The upregulation during drought of a number of genes involved in antioxidant protection, namely, *CuZnSODc*, *FeSOD*, *APXc*, *GRc*, *sodA*, *katB*, and *katC* (Figs. 4A and 5), indicates that both the host cells and bacteroids are probably experiencing oxidative stress. This was confirmed by the accumulation of peroxidized lipids (estimated as MDA) and oxidatively modified proteins (estimated as carbonyl groups) in nodules during drought stress and subsequent recovery (Fig. 7). At least two factors may contribute to ensuing oxidative stress in alfalfa nodules. First, although the activities of APX and associated enzymes, as assayed in vitro, remained fairly constant with drought, a large decrease in the concentrations of ascorbate (Fig. 6) and probably of NAD(P)H (Gogorcena et al., 1995) may compromise H_2O_2 detoxification through the ascorbate-GSH cycle in vivo. Second, nonenzymatic formation of cytotoxic aldehydes and protein carbonyl derivatives (Fig. 7) requires the generation of highly oxidizing ROS, such as the hydroxyl radical, which in turn depend on trace amounts of metal ions (Stadtman, 1992; Halliwell and Gutteridge, 1999). Indeed, catalytic iron (Gogorcena et al., 1995; Evans et al., 1999) and hydroxyl radical production (Becana and Klucas, 1992) have been found to increase in senescing nodules.

In summary, we conclude that a decrease in SS expression (mRNA and activity) is not the cause of the drought-induced loss of N_2ase activity in alfalfa. Interestingly, a similar response of nodule metabolism to drought was found in *Medicago truncatula* (R. Ladrera, E.M. González, and C. Arrese-Igor, unpublished data). This raises the possibility that the decrease in SS activity observed in other legumes is not directly responsible for the inhibition of N_2ase, but rather that the negative effects of drought on both activities are concomitant and mediated through another factor, possibly oxidative damage. Alternatively, it is possible that, depending on legume species, two models exist concerning the role of carbon metabolism in the inhibition of N_2ase activity by drought. In soybean, pea, and common bean, the inhibition would be mediated by SS, leading to a decrease in the organic acid levels of nodules. In alfalfa and *M. truncatula*, the inhibition would not involve SS activity and organic acids would accumulate in nodules. In any case, the accumulation of respiratory substrates, lipid peroxides, and oxidized proteins and the decrease of N_2ase proteins and N_2ase-linked respiration reveal that both impairment of bacteroid function and oxidative stress take place in alfalfa nodules before any detectable effect on SS expression. Further studies are required to determine if oxidative stress and effects on N_2ase protein content occur prior to, and therefore may be responsible for, the observed reduction in bacteroid respiration.

MATERIALS AND METHODS

Biological Material and Plant Treatments

Nodulated plants of alfalfa (*Medicago sativa* 'Aragón' or 'N4' × *Sinorhizobium meliloti* 102F78) were grown in 4-L pots (six to eight plants per pot), containing 2:1 (v/v) perlite:vermiculite, under controlled-environment conditions (16-h photoperiod, 350 μmol m^{-2} s^{-1}, 25°C/18°C day/night regime, 70% relative humidity). Plants were watered three times a week, alternatively with distilled water and with a mineral nutrient solution containing 0.5 mM NH_4NO_3 (Gogorcena et al., 1997). Plants of alfalfa 'Aragón' were grown for 50 to 55 d and were then separated at random into four groups. One of them (control) was kept under optimal water conditions and two other groups were subjected to drought stress by withholding irrigation until the plants reached a leaf Ψ_w (mean ± SE) of −1.3 ± 0.1 MPa (moderate drought; 5–7 d) and −2.1 ± 0.2 MPa (severe drought; 8–10 d). The fourth group of plants was also subjected to severe drought and then allowed to recover by reirrigation for 2 d (recovery). Control and recovery plants had a similar leaf Ψ_w of −0.6 ± 0.2 MPa. Leaf Ψ_w was measured 1 h after the beginning of the photoperiod in representative leaves, situated in the upper third of the shoot, with a pressure bomb (Soil Moisture Equipment). Nodule Ψ_w was measured in the same plants as leaf Ψ_w using C52 sample chambers coupled to a HR-33T microvoltmeter (Wescor). Values of nodule Ψ_w (mean ± SE) were −0.8 ± 0.1, −1.5 ± 0.1, −2.5 ± 0.1, and −0.7 ± 0.1 MPa for the control, moderate stress, severe stress, and recovery treatments, respectively. Plants of alfalfa 'N4' were subjected to only a moderate drought stress (Ψ_w of −1.5 ± 0.1 MPa) and used to assess the respiration and carbon cost associated with N_2ase activity. Nodules were harvested into liquid nitrogen and stored at −80°C, except for samples to be used for dry weight determination, which were dried for 48 h at 80°C.

N_2ase Activity and Root Respiration

Total N_2ase activity and nodulated root respiration were measured on intact plants using a flow-through gas system that incorporated H_2 detectors (Witty and Minchin, 1998). Root systems of alfalfa 'N4' were sealed in their growth pots, allowed to stabilize for 18 h in a stream of air enriched with 500 μL CO_2 L^{-1}, and then exposed to a gas stream of 79% (v/v) argon (Ar) and 21% (v/v) O_2. Respiratory CO_2 production was measured using an IR gas analyzer, and N_2ase activity was measured as H_2 production using an electrochemical hydrogen sensor (City Technology Ltd.). After steady-state conditions had been reached following exposure to Ar/O_2 (within 65 min), the O_2 concentration in the gas stream was increased over the range 21% to 50% (8.55–20.45 mmol O_2 L^{-1}). N_2ase-linked respiration was calculated from the linear relationship between changes in total root respiration and H_2 production during the O_2 stepping period (Witty et al., 1983).

For alfalfa 'Aragón,' the apparent N_2ase activity was measured in intact plants, as indicated above, by measuring H_2 evolution with an electrochemical sensor (Qubit Systems) in an open flow-through gas system under a stream of 79% (v/v) N_2 and 21% (v/v) O_2 (Witty and Minchin, 1998). The H_2 sensor was calibrated with high-purity gases flowing at the same rate as the sampling system (500 mL min^{-1}).

Enzyme Activities, Soluble Protein, and Lb

All enzymes were extracted from nodules at 0°C to 4°C, and activities were measured at 30°C within the linear range. The enzymes involved in carbon and nitrogen metabolism were extracted in an optimized medium (Marino et al., 2006). Desalted extracts were used to assay SS (EC 2.4.1.13), AI (EC 3.2.1.26), NADP-dependent ICDH (EC 1.1.1.42), and NADH-dependent GOGAT (EC 1.4.1.14) by following the reduction of NAD^+ (SS, AI), the reduction of $NADP^+$ (ICDH), or the oxidation of NADH (GOGAT) at 340 nm. The protocols used for determination of SS and AI (González et al., 1995), ICDH (Gálvez et al., 2005), and GOGAT (Groat and Vance, 1981) activities have been described in detail in the corresponding references.

The SODs (EC 1.15.1.1) were extracted in a medium consisting of 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 1% (w/v) soluble polyvinylpyrrolidone, and 0.1% (v/v) Triton X-100. Total SOD activity was determined by the ferric cytochrome *c* method with modifications (Rubio et al., 2002). The CuZnSODc, CuZnSODp, plastidic FeSOD, and mitochondrial MnSOD isoforms were individualized in native gels and identified by differential inhibition with KCN or H_2O_2 (Rubio et al., 2002). APX (EC 1.11.1.11) and catalase (EC 1.11.1.6) were extracted in a medium containing 50 mM potassium phosphate, pH 7.0, and 0.5% soluble polyvinylpyrrolidone, and the activities were assayed, respectively, following the disappearance of ascorbate at 290 nm (Asada, 1984) and the decomposition of H_2O_2 at 240 nm (Aebi, 1984). GR (EC 1.6.4.2) was extracted in a medium consisting of 50 mM Tricine, pH 7.8, 0.2 mM EDTA, 10 mM β-mercaptoethanol, and 1% soluble polyvinylpyrrolidone, and was assayed by following the oxidation of NADPH at 340 nm (Dalton et al., 1986). Because organelle APXs are inactivated in extraction media lacking ascorbate, whereas the APXc is stable (Dalton et al., 1986; Amako et al., 1994), the activity assayed in nodule extracts was only due to APXc. However, the GR activity assayed corresponded to the sum of the cytosolic and plastidic isoforms.

Soluble protein was quantified by the dye-binding microassay (Bio-Rad) using bovine serum albumin as the standard. Lb concentration was determined by the pyridine-hemochrome method, using an extinction coefficient (556 nm minus 539 nm) of 23.4 mM^{-1} cm^{-1} for the difference spectrum between the reduced (+dithionite) and the oxidized (+ferricyanide) hemochromes (Appleby and Bergersen, 1980).

Carbon and Antioxidant Metabolites

Organic acids and sugars were extracted from nodules in 5% (w/v) trichloroacetic acid, and samples were processed as described by Wilson and Harris (1966) with minor modifications (Gálvez et al., 2005). Succinate, malate, and αKG were quantified by ion chromatography in a DX-500 system (Dionex) by gradient separation with an IonPac AS11 column (Dionex) according to the method recommended by the supplier. Suc was analyzed by CE in a Coulter PACE system 5500 (Beckman) coupled to a diode array detector (Marino et al., 2006).

Ascorbate and dehydroascorbate were measured as described by Bartoli et al. (2000) with some modifications (Matamoros et al., 2006) using nodules harvested directly into liquid nitrogen to avoid artifactual oxidation of ascorbate. Extracts were made with 1 M $HClO_4$, cleared by centrifugation, neutralized with 1 M K_2CO_3 to pH 5.6, and centrifuged again. Two aliquots were made and one of them was treated with 0.4 mM dithioerythritol for 15 min at room temperature. The aliquots were incubated with 0.05 units of ascorbate oxidase (Sigma), and the decrease in A_{265} was monitored until stable and used to calculate ascorbate concentration based on an extinction coefficient of 14.3 mM^{-1} cm^{-1}. Ascorbate was quantified by direct analysis of the aliquots not treated with dithioerythritol, and dehydroascorbate as the difference in ascorbate concentration between the treated and untreated aliquots.

Thiol compounds were extracted from nodules with 2% (w/v) metaphosphoric acid and 1 mM EDTA. The extracts were cleared by centrifugation and treated with 65 mM dithiothreitol for 15 min at room temperature. The concentrations of the thiol tripeptides (reduced plus oxidized forms) were measured by CE (Marino et al., 2006) using GSH (Sigma) and hGSH (Bachem) for calibration. The proportion of oxidized thiols was determined in extracts prepared in the same way, prior to dithiothreitol treatment, by an enzymatic recycling procedure using yeast GR to reduce the disulfide forms and vinylpyridine as thiol blocking agent (Law et al., 1983).

Oxidative Damage of Lipids and Proteins

The nodule content of MDA, a major product formed from decomposition of lipid peroxides, was quantified by HPLC essentially as described elsewhere (Iturbe-Ormaetxe et al., 1998). The $(TBA)_2$-MDA adduct was resolved on a C_{18} column (4.6 × 250 mm, 5 μm; Baker), eluted at 1 mL min^{-1} with 5 mM potassium phosphate, pH 7.0, containing 15% acetonitrile and 0.6% tetrahydrofurane, and was detected at 532 nm. Calibration curves were made with 1,1,3,3-tetraethoxypropane (Sigma) as the standard, which is stoichiometrically converted into MDA during the acid-heating step of the TBA reaction. The purity of the peak corresponding to the $(TBA)_2$-MDA adduct was routinely monitored by scanning between 400 and 600 nm with a photodiode-array detector. The peaks in the samples and standard showed identical spectra, with a maximum of A_{532} and a shoulder at 495 nm (Iturbe-Ormaetxe et al., 1998).

The oxidative damage of proteins was measured by derivatization of carbonyl groups with DNP and subsequent separation of proteins on SDS gels, using the OxyBlot protein oxidation kit (Chemicon) according to the supplier's recommendations. The DNP-hydrazone derivatives of proteins were detected on membranes using rabbit specific anti-DNP as the primary antibody (1:150 dilution), a peroxidase conjugate of goat anti-rabbit IgG as the secondary antibody (1:3,000 dilution), and the SuperSignal West Pico chemiluminescence kit (Pierce).

Immunoblot Analysis

Total proteins of nodules were denatured and prepared for electrophoresis as described by Gordon et al. (1999). Proteins were resolved in 10% (N_2ase) or 12.5% (CuZnSODc) SDS gels, and transferred to polyvinylidene difluoride membranes (Bio Trace PVDF; PALL Life Sciences) using a Mini Trans-Blot transfer cell (Bio-Rad) following standard protocols. The primary antibodies against the *Klebsiella pneumoniae* Kp1 and Kp2 N_2ase proteins (1:5,000) and spinach (*Spinacia oleracea*) CuZnSODc (1:3,000) were generously provided by P.W. Ludden (Madison, WI) and S. Kanematsu (Miyazaki, Japan), respectively. The secondary antibody (1:10,000 for Kp1 and Kp2; 1:20,000 for CuZnSODc) was goat anti-rabbit IgG peroxidase conjugate (Sigma). Proteins were detected on blots with the SuperSignal West Pico chemiluminescent substrate (Pierce). Densitometric semiquantitative analysis was performed with the Quantity One software (Bio-Rad).

Transcript Levels

Total RNA was isolated from nodules using the RNAqueous kit (Ambion), treated with DNaseI (Roche) at 37°C for 30 min, and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). qRT-PCR analysis was carried out with the iCycler iQ system (Bio-Rad) using iQ SYBR-Green Supermix reagents (Bio-Rad) and specific primers for genes expressed in the nodule host cells and bacteroids (Supplemental Table S1). The PCR program consisted of an initial denaturation and *Taq* activation step of 5 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. A melting curve analysis was performed after every PCR reaction to confirm the accuracy of each amplified product. All reactions were set up in duplicate. The mRNA levels of alfalfa genes were normalized against *EF1-α* (El Yahyaoui et al., 2004) and those of *S. meliloti* genes against *smc00324* and *smc02641* (Becker et al., 2004). Values of treated plants were expressed relative to those of control plants using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The absence of contamination with genomic DNA was tested by qRT-PCR in all RNA samples, after the DNase treatment but prior to reverse transcription, using the primers of the housekeeping genes.

Statistical Analysis

Each measurement of N_2ase activity and associated parameters was made with a pool of three to five intact plants. Each biochemical assay was made with nodules harvested from plants growing in different pots. Data were obtained from two (N_2ase activity, carbon metabolism, and antioxidant enzymes and metabolites) or four to six (mRNAs of antioxidant enzymes) series of plants that were grown independently. The total numbers of replicates are stated in each figure. For each treatment, the measurements from the different series of plants were pooled for statistical analysis. For each parameter, the mean values of the drought and recovery treatments were compared with the controls by ANOVA and Dunnett's *t* test.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table S1. Primers used for qRT-PCR.

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