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N_2 Fixation, Carbon Metabolism, and Oxidative Damage in Nodules of Dark-Stressed Common Bean Plants[1]

Yolanda Gogorcena, Anthony J. Gordon, Pedro R. Escuredo, Frank R. Minchin, John F. Witty, Jose F. Moran, and Manuel Becana*

Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Apdo 202, 50080 Zaragoza, Spain (Y.G., P.R.E., J.F.M., M.B.); and Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, United Kingdom (A.J.G., F.R.M., J.F.W.)

Common beans (*Phaseolus vulgaris* L.) were exposed to continuous darkness to induce nodule senescence, and several nodule parameters were investigated to identify factors that may be involved in the initial loss of N_2 fixation. After only 1 d of darkness, total root respiration decreased by 76% and in vivo nitrogenase (N_2ase) activity decreased by 95%. This decline coincided with the almost complete depletion (97%) of sucrose and fructose in nodules. At this stage, the O_2 concentration in the infected zone increased to 1%, which may be sufficient to inactivate N_2ase; however, key enzymes of carbon and nitrogen metabolism were still active. After 2 d of dark stress there was a significant decrease in the level of N_2ase proteins and in the activities of enzymes involved in carbon and nitrogen assimilation. However, the general collapse of nodule metabolism occurred only after 4 d of stress, with a large decline in leghemoglobin and antioxidants. At this final senescent stage, there was an accumulation of oxidatively modified proteins. This oxidative stress may have originated from the decrease in antioxidant defenses and from the Fe-catalyzed generation of activated oxygen due to the increased availability of catalytic Fe and O_2 in the infected region.

Experiments using dark-stressed plants have provided valuable information on the process of nodule senescence, which is commonly diagnosed by decreases in N_2 fixation, Lb, and total cytosolic protein (Sutton, 1983). Exposure of soybeans to prolonged darkness induces structural and catabolic changes in the nodules that mimic natural senescence, suggesting that the mechanisms underlying both processes are related (Pfeiffer et al., 1983; Cohen et al., 1986). The nodules of dark-stressed plants show decreased energy charge (Ching et al., 1975), increased proteolytic activity (Pfeiffer et al., 1983), and altered composition of the bacteroid population (Paau and Cowles, 1981). In addition, exposure of soybeans to short periods of darkness are sufficient to cause rapid declines in transcripts encoding key nodule proteins and in the activity of SS (Gordon et al., 1993). It is interesting that soybean bacteroids retain their viability during the dark-stress period (Cohen et al., 1986; Sarath et al.,1986).

Dark stress is also thought to disturb the O_2 relationships in nodules because N_2 fixation requires a fine adjustment of the O_2 flux to the bacteroids. This control is exerted at two levels: a variable O_2 diffusion barrier and Lb-facilitated diffusion within the infected cells (Hunt and Layzell, 1993). O_2 is consumed at high rates by mitochondria and bacteroids, and in both respiratory processes AOS, including the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are inevitably generated (Halliwell and Gutteridge, 1989). Also, the high concentration of Lb (1–3 mM) in infected cells and the tendency of its oxygenated form to autoxidize are conducive to the production of $O_2^{\cdot-}$ and H_2O_2 in the cytosol (Puppo et al., 1981). This may pose a serious threat to cellular activity because $O_2^{\cdot-}$ and H_2O_2, although only moderately reactive, can interact, in the presence of catalytic amounts of Fe, to form the hydroxyl radical (·OH) and the oxoferryl complex (Fe^{IV}=O). Both AOS are powerful oxidants (Halliwell and Gutteridge, 1989). To avoid oxidative damage, nodules contain an abundance of enzymes and low-molecular-weight compounds that scavenge or prevent the formation of AOS. These include the enzymes of the ASC-GSH pathway and their associated metabolites (Dalton et al., 1986).

Despite the criticality of O_2 for nodule activity and the usefulness of dark-stress treatments to study nodule senescence, very little is known about the effects of dark-stress-induced carbohydrate limitations on the mechanisms that prevent oxidative damage in nodules. Dark stress decreases respiration and N_2ase activity of soybean and lupine nodules (Layzell et al., 1990; Iannetta et al., 1993) and the activities of some antioxidant enzymes in cowpea and clusterbean nodules (Swaraj et al., 1988, 1994). Because it was found in a previous study (Becana and Klucas, 1992) that prolonged darkness promotes generation of toxic ·OH

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* Corresponding author; e-mail becana@eead.csic.es; fax 34–76–575620.

Abbreviations: AOS, activated oxygen species; ASC, ascorbate; GS, Gln synthetase; (h)GSH, (homo)glutathione in reduced form; (h)GSSG, (homo)glutathione in oxidized form; Lb, leghemoglobin; MDA, malondialdehyde; N_2ase, nitrogenase; SOD, superoxide dismutase; SS, Suc synthase; TBARS, 2-thiobarbituric acid-reactive substances.

radicals in bean nodules, which is a direct indication of oxidative stress, we have chosen this plant material to further investigate the role of oxidative damage in dark-induced nodule senescence, and to correlate senescence with changes in O_2 regulation and carbon metabolism.

MATERIALS AND METHODS

Chemicals and Sources of Antibodies

Organic solvents, inorganic acids, and salts for nutrient solutions were of analytical or HPLC grade from Panreac (Barcelona, Spain) or BDH-Merck (Lutterworth, UK). Metal-chelating resin (Chelex-100, Na^+ form) was obtained from Bio-Rad. All other chemicals were of analytical grade from Sigma or Aldrich. Deionized or single-distilled water was used for preparing nutrient solutions, and ultrapure water (Milli-Q system, Millipore) was used for all other purposes.

Monoclonal antibodies to *Klebsiella pneumoniae* N_2ase component I (KpI) and polyclonal antibodies to component II (Kp2) were provided by Drs. M. Buck and S. Hill (IPSR, Sussex, UK). Polyclonal antibodies to GS and ASC peroxidase were gifts from Drs. J.V. Cullimore (Warwick, UK) and D.A. Dalton (Portland, OR), respectively. Antibodies to Lb and SS were raised as described by Gordon and Kessler (1990) and Gordon et al. (1992), respectively.

Plant Material and Dark Treatment

Nodulated beans (*Phaseolus vulgaris* L. cv Contender × *Rhizobium leguminosarum* biovar *phaseoli* strain 3622) were grown in controlled-environment chambers, with a day-length of 16 h, photon flux density of 350 to 400 μmol $m^{-2}s^{-1}$, and a day/night regime of 25/20°C and 70/85% RH. Plants were watered three times a week alternatively with distilled water and a nutrient solution composed of: (in g L^{-1}) $MgSO_4 \cdot 7H_2O$ (0.80), KH_2PO_4 (0.54), K_2HPO_4 (0.09), $CaSO_4 \cdot 2H_2O$ (0.50), NH_4NO_3 (0.04), and Sequestrene 330Fe (0.025; Ciba-Geigy, 6% Fe); and (in mg L^{-1}) $MnSO_4 \cdot H_2O$ (0.423), $CuSO_4 \cdot 5H_2O$ (0.063), $ZnSO_4 \cdot 7H_2O$ (0.073), H_3BO_3 (0.775), NaCl (1.475), $Na_2MoO_4 \cdot 2H_2O$ (0.030), and $CoCl_2 \cdot 6H_2O$ (0.013). The pH was adjusted to 6.5 with KOH.

Thirty days after sowing, plants were divided into four groups. Three of them were placed in continuous darkness for 1, 2, or 4 d, whereas the other group remained under a normal 16-h photoperiod. All other environmental conditions were identical for the four groups of plants. Control plants (0 d of darkness) were harvested in between those exposed to 2 and 4 d of darkness such that the maximum difference of plant age at harvest was 2 d. All plants were at the late vegetative growth stage when the nodules were harvested.

N_2 Fixation and Related Parameters

N_2ase activity and root respiration of intact plants was measured using a flow-through gas analysis system (Minchin et al., 1983) in which sealed roots were 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 containing 10% (v/v) C_2H_2 and 21% (v/v) O_2. Respiratory CO_2 production was measured using an IR gas analyzer, and N_2ase activity was measured as C_2H_4 production by flame ionization GC. Following exposure to C_2H_2, steady-state conditions were reached after 60 to 70 min, and the external O_2 concentration was then increased over the range of 21 to 60% (8.55–24.54 mmol O_2 L^{-1}) in steps of 5 or 10%. Each increase in O_2 took 5 to 6 min and was followed by a 20- to 25-min equilibration period.

O_2 Microelectrode Measurements

The O_2 concentration profiles of attached nodules were measured using O_2-sensitive microelectrodes, as described by Witty et al. (1987). As the electrode tip progressed into the nodule by step-wise (20 μm) insertions, the measured O_2 levels decreased until a steady-state value was achieved. This was taken as the O_2 concentration within the central infected zone.

Carbohydrates and Enzymes of Carbon and Nitrogen Metabolism

Nodules to be used for all biochemical determinations were harvested on ice, frozen in liquid N_2, and stored at −80°C for subsequent analysis. Carbohydrates were extracted from 0.2 to 0.4 g of nodules with 10 to 20 mL of boiling 80% ethanol. The extract was dried under vacuum at 40°C and the residue was re-dissolved in 4 mL of water. The contents of Glc, Fru, and Suc were determined spectrophotometrically at 340 nm in enzymic reactions coupled to the production of NADH, as described in Gonzalez et al. (1995). The starch content of the ethanol-insoluble residue was determined by measurement of Glc (as above), which was released following digestion with amyloglucosidase (MacRae, 1971).

Enzymes were extracted from approximately 0.2 g of nodules with a mortar and pestle in a buffer (5 mL g^{-1} fresh weight) composed of 50 mM Mops (pH 7.2), 5 mM $MgCl_2$, 20 mM KCl, 10 mM DTT, 1 mM EDTA, 200 mM sorbitol, and 1 mM PMSF (added just prior to extraction) at 2°C. After removing the host plant's soluble proteins by centrifugation at 20,000*g* for 30 min, the pellet containing the intact bacteroids was washed three times by resuspending it in the extraction buffer and centrifuged as above. The washed pellet was resuspended a fourth time in the extraction buffer lacking sorbitol and the bacteroids were broken by sonication (2 × 30-s pulses at 0°C) using an MSE soniprep 150 sonicator (Fisons, Loughborough, UK) at an amplitude of 10 μm. The bacteroid protein fraction (supernatant) obtained after centrifugation (as above) was prepared for PAGE and immunoblotting.

Samples (50 μL) of the crude, soluble host plant extracts were retained for the assay of PEP carboxylase (Gordon and Kessler, 1990), while 1 mL was desalted by centrifugation at 180*g* for 1 min through 5-mL columns of P6DG (Bio-Rad) equilibrated with the extraction buffer lacking sorbitol, PMSF, and EDTA, and with the concentration of DTT reduced to 2 mM. The desalted extract was used to

assay a range of enzymes. SS, GS, Asp aminotransferase, and alkaline invertase activities were determined as described in Gonzalez et al. (1995), whereas phosphofructokinase and PP_i:Fru-6-P phosphotransferase were assayed using the protocol of Gordon (1991). Glutamate synthase was assayed as in Groat and Vance (1981). Aldolase was assayed spectrophotometrically at 30°C by measuring the oxidation of NADH. The assay mixture contained, in a final volume of 1 mL, 50 mM each of Mops, Bicine, and Mes (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADH, 3 units mL^{-1} of glycerol-3-P dehydrogenase, 6 units mL^{-1} of triose phosphate isomerase, and 20 μL of the enzyme sample. The reaction was started by the addition to each cuvette of Fru-1,6-bisP to a final concentration of 1 mM.

Antioxidants, Pyridine Nucleotides, and Free Flavins

Antioxidant enzymes were extracted from 0.25 (catalase) or 0.5 g (other enzymes) of nodules with optimized media (Gogorcena et al., 1995). The homogenate was filtered through one layer of Miracloth (Calbiochem) and centrifuged at 15,000*g* for 20 min. All operations were performed at 0 to 4°C. Catalase activity was assayed by following the disappearance of H_2O_2 at 240 nm (Aebi, 1984). ASC peroxidase and dehydroascorbate reductase activities were determined following the oxidation of ASC at 290 nm (Asada, 1984) or the reduction of ASC at 265 nm (Nakano and Asada, 1981), respectively. Monodehydroascorbate reductase (Dalton et al., 1992) and (h)GSSG reductase (Dalton et al., 1986) activities were assayed by following the oxidation of NADH and NADPH at 340 nm, respectively. Where appropriate, controls were run for correcting nonenzymatic rates, and buffers and reagents were treated with Chelex resin to avoid contamination by trace amounts of transition metal ions.

For determination of SOD activity, 0.5 g of nodules were homogenized with 6 mL of 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA, 60 mg of soluble PVP, and 0.1% (v/v) Triton X-100. After centrifugation at 15,000*g* for 20 min, extracts were depleted of low-molecular-weight compounds by extensive dialysis against 5 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA. Total SOD activity was assayed by its ability to inhibit the reduction of ferric Cyt *c* by the O_2^- radical generated by a xanthine-xanthine oxidase system. The reaction mixture contained 10 μM potassium cyanide to inhibit Cyt *c* oxidase without affecting Cu plus Zn-SOD activity. One unit of activity was defined as the amount of enzyme required to inhibit ferric Cyt *c* reduction by 50% (McCord and Fridovich, 1969).

ASC was extracted from 0.25 g of nodules with 5 mL of 5% (w/v) metaphosphoric acid and quantified by formation of a dipyridyl-Fe^{2+} complex at low pH (Law et al., 1983). GSH and GSSG were extracted from 0.5 g of nodules with 5 mL of 5% (w/v) sulfosalicylic acid, and their concentrations were determined essentially by the method of Law et al. (1983). This is an enzymatic recycling protocol in which GSH is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH-dependent GSSG reductase; on the other hand, the assay is made specific for GSSG by masking the thiol group of GSH with 2-vinylpyridine (Griffith, 1980). This assay, however, cannot distinguish GSH (γGlu-Cys-Gly) from hGSH (γGlu-Cys-βAla). Because hGSH has been found in variable amounts in leaves, roots, and seeds of several legumes (Klapheck, 1988), and the same may occur in nodules, our measurements are likely to reflect the sum of both thiol tripeptides. Accordingly, the abbreviations (h)GSH and (h)GSSG are used in this work to denote the contents of GSH+hGSH and GSSG+hGSSG, respectively. For the same reason, the abbreviation (h)GSSG reductase has been used to refer to the nodule enzyme(s) catalyzing the reduction of (h)GSSG.

Pyridine nucleotides were extracted from 30 mg of nodules in alkaline (NADH and NADPH) or acidic (NAD^+ and $NADP^+$) medium (Gogorcena et al., 1995) and quantified by an enzymatic-cycling method (Matsumura and Miyachi, 1980). Free flavins were extracted from 0.3 g of nodules with 1.5 mL of 10% TCA at 0°C in the dark, as indicated by Cerletti and Giordano (1971) with some modifications. After centrifugation at 8,000*g* for 10 min, the pellet was re-extracted with 1 mL of 1% TCA and the suspension was centrifuged. The supernatants were pooled, the pH was adjusted to 6.1 with 2 M potassium phosphate (pH 7.0), and the volume was made up to 4 mL with distilled water. Free flavins in the samples were separated and quantitated by HPLC as described by Light et al. (1980) using an analytical μBondapak-C_{18} column (Waters) and fluorescence detection (excitation at 445 nm; emission at 520 nm).

Oxidative Damage to Lipids and Proteins

Lipid peroxides were extracted from 0.5 g of nodules using an ice-cold mortar and pestle with 5 mL of 5% (w/v) metaphosphoric acid and 100 μL of 2% (w/v) butyl hydroxytoluene. Homogenates were filtered through one layer of Miracloth and centrifuged at 12,000*g* for 20 min. The chromogen was formed by mixing 0.5 mL of supernatant, 50 μL of 2% butyl hydroxytoluene (in ethanol), 0.25 mL of 1% (w/v) thiobarbituric acid (in 50 mM NaOH), and 0.25 mL of 25% (v/v) HCl, and by incubating the reaction mixtures at 95°C for 30 min. A blank was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing thiobarbituric acid with 50 mM NaOH. The reaction was stopped in an ice-bath and the chromogen was extracted with 1-butanol. Lipid peroxides in the butanol phase were quantified as the concentration of TBARS (Minotti and Aust, 1987) or MDA after separation by HPLC (Draper et al., 1993).

Oxidatively modified proteins were extracted from 0.5 g of nodules, as described elsewhere (Levine et al., 1990; Gogorcena et al., 1995). Protein oxidation was measured as the total content of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine after the removal of possible contaminating nucleic acids with 1% (w/v) streptomycin sulfate (Levine et al., 1990). Volumes of samples were adjusted so that the amount of protein assayed for carbonyl content was 0.5 mg for all of the samples.

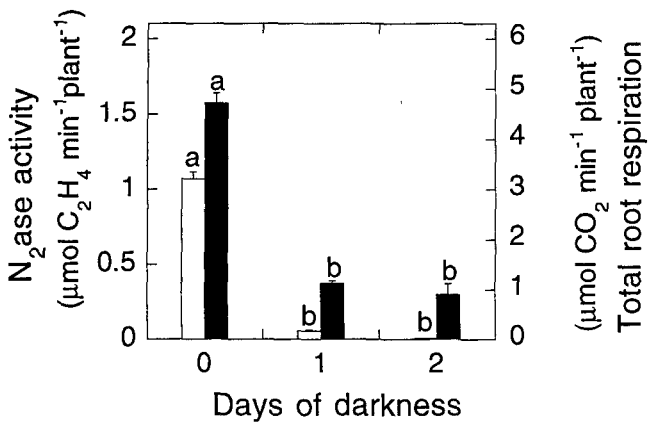

Figure 1. N_2ase activity (□) and total root respiration (■) of beans exposed to continuous darkness for 0 to 2 d. Values are means ± SE (n = 3). For each parameter, statistical analysis was performed as for Table I.

Immunoblot Analyses

Samples of the bacteroid protein fraction (100 μL) were treated with 25 μL of 250 mM Tris-HCl (pH 6.5) containing 8% SDS, 20% glycerol, and 20% 2-mercaptoethanol. Samples were then boiled for 2 min, and aliquots containing equal amounts of protein were loaded into sample wells of 0.75-mm thick, 12.5% polyacrylamide gels (Laemmli, 1970) and separated by SDS-PAGE using a mini-gel system (Bio-Rad). Electrophoretic transfer of proteins to nitrocellulose membranes was achieved using a mini-transblot system (Bio-Rad). Blots were then probed for N_2ase components 1 and 2 (Gordon and Kessler, 1990).

Soluble host plant proteins, denatured and blotted as in Cresswell et al. (1992), were identified with antibodies to SS (Gordon et al., 1992), GS (Gordon and Kessler, 1990), Lb (Gordon and Kessler, 1990), and ASC peroxidase (Dalton et al., 1993).

Other Biochemical Analyses

Nodules to be used for the quantitation of protein-bound Fe, catalytic Fe, free heme, and Lb were extracted and fractionated with the precautions described earlier (Escuredo et al., 1996). The concentration of protein-bound Fe in the >3-kD fraction was determined by atomic absorption spectrophotometry using a graphite furnace atomizer (AA-670G and GFA-4A, Shimadzu, Kyoto, Japan), whereas that of catalytic Fe in the <3-kD fraction was estimated by the bleomycin assay (Evans and Halliwell, 1994). Free heme in the <3-kD fraction and Lb in the >3-kD fraction were quantified by a fluorometric method (LaRue and Child, 1979), as described previously (Escuredo et al., 1996). Protein concentrations of the nodule cytosol and bacteroids were determined with a commercial dye (Bio-Rad) using BSA as the standard.

Total lipids were extracted from 0.25 g of nodules at room temperature, as indicated by Folch et al. (1957) or by Bligh and Dyer (1959) with minor modifications. In the former case, nodules were homogenized with 5 mL of chloroform:methanol (2:1, v/v), and the organic phase was washed three times with 1 mL of 0.9% (w/v) NaCl. In the latter case, nodules were homogenized with 2 mL of chloroform:methanol:water (1:2:0.8, v/v/v) and the nodule residue was re-extracted with 2 mL chloroform. In both cases, the solvent of the organic phase was evaporated with N_2 and subsequently in vacuo (Savant Instruments, Farmingdale, NY), and the lipids were quantified gravimetrically.

RESULTS

N_2 Fixation and Related Parameters

Exposure of nodulated beans to prolonged darkness caused a decrease of total root respiration by 76% and a decrease of N_2ase activity by 95% after 1 d, and the complete cessation of N_2 fixation after 2 d (Fig. 1). There were also marked differences in the relationship between N_2ase activity and external O_2 concentration in control and 1-d dark-stressed plants (Fig. 2). For control plants, raising external O_2 up to 40% increased C_2H_2 reduction activity, followed by decreases at 50 and 60%, whereas for 1-d dark-stressed plants, each increase in external O_2 caused a decrease in N_2ase activity such that it reached zero at 50% O_2. N_2ase-linked respiration and O_2 diffusion resistances could not be accurately estimated after 1 or 2 d of darkness because of the very low or nonexistent N_2ase activity.

The concentration of O_2 within the infected zone of the control nodules was below the level of detection (0.0025%) for the microelectrode (Table I). After 1 or 2 d of prolonged darkness the internal concentrations rose to 0.9 to 1.5% and showed a further increase to 4% after 4 d of darkness. The response of other parameters of nodule functioning to dark stress is shown in Table I. Thus, total cytosol protein decreased by 13 and 54% after 2 and 4 d of darkness, respectively, and the corresponding decreases in Lb content were 18 and 75%, indicating that Lb is a cytosolic protein particularly sensitive to dark stress. The decline in Lb was not reflected by an increase in free heme, which remained

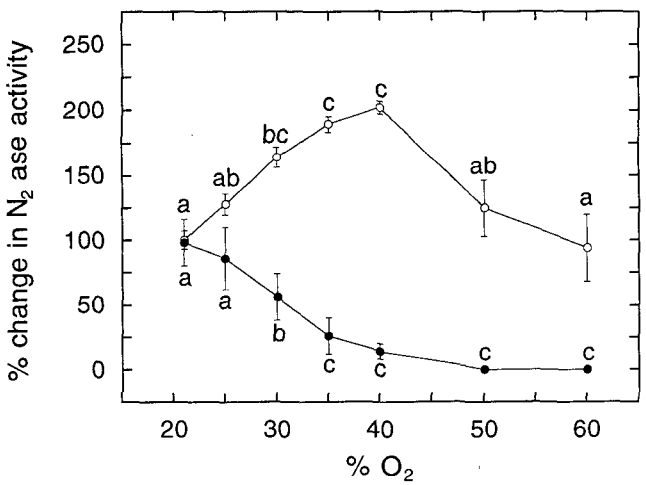

Figure 2. Effect of external O_2 concentration on N_2ase activity of beans exposed to continuous darkness for 0 d (○) or 1 d (●). Values are means ± SE (n = 3). For each parameter, statistical analysis was performed as for Table I.

Table I. *Parameters of nodule functioning in bean plants exposed to continuous darkness for 0 to 4 d*

Parameter[a]	Days of Darkness			
	0	1	2	4
O_2 in infected zone (%)	ND[b]	0.9 a	1.5 a	3.9 b
Free heme (nmol)	2.6 a	2.0 a	2.4 a	2.0 a
Lb (nmol)	227 a	234 a	186 b	57 c
Cytosol protein (mg)	13.4 a	12.3 a	11.1 b	6.1 c
Bacteroid protein (mg)	8.0 a	7.6 ab	7.6 ab	6.2 b
Protein-bound Fe (μg)	19.5 a	19.3 a	19.1 a	12.4 b
Lipids (mg)	20.0 a	20.4 a	24.7 b	24.4 b

[a] Except for O_2 concentrations, values are given in units g^{-1} fresh weight. Means (n = 8–12 for O_2 concentrations; n = 3–8 for other parameters) were compared by one-way analysis of variance and Duncan's multiple range test. Those denoted by the same letter do not differ significantly at $P < 0.05$. [b] ND, Not detectable (<0.0025%).

constant over the time course. In contrast, bacteroid protein was relatively insensitive to dark stress, with a decrease of only 23% after 4 d. The concentration of Fe in the high-molecular-mass (>3-kD) fraction of nodules, which represents mostly Fe bound to proteins, was not affected after 2 d of darkness and only declined by 36% after 4 d. However, unlike the negative effect of dark stress on nodule proteins, the total lipid content of nodules increased by 22% (Table I).

Antioxidants, Pyridine Nucleotides, and Free Flavins

Exposure of plants to continuous darkness decreased the antioxidant defenses and the pyridine nucleotide content of nodules (Tables II and III). In general, the declines were in the range of 10 to 20%, 20 to 40%, and 50 to 70% after 1, 2, and 4 d of darkness, respectively. For antioxidants, the overall decreases after 4 d ranged from 39% for ASC to 65 to 70% for catalase and (h)GSH, and for pyridine nucleotides from 30% for NADPH to 68% for NAD^+ (Tables II and III). However, the decreases were not progressive for parameters such as dehydroascorbate reductase activity, which declined by 20 and 63% after 2 and 4 d, and for (h)GSSG reductase and catalase activities, which remained

Table II. *Antioxidant enzymes in nodules from bean plants exposed to continuous darkness for 0 to 4 d*

Enzyme[a]	Days of Darkness			
	0	1	2	4
	μmol min^{-1} g^{-1} fresh wt			
ASC peroxidase	11.7 a	10.5 b	9.9 b	6.3 c
DHA reductase[b]	0.74 a	0.69 ab	0.59 b	0.27 c
(h)GSSG reductase	0.47 a	0.50 a	0.46 a	0.24 b
MDHA reductase[c]	3.33 a	2.46 b	2.19 b	1.23 c
Catalase	1660 a	1900 a	1590 a	500 b
SOD[d]	384 a	382 a	332 b	263 c

[a] Statistical analysis of means (n = 7–12) was performed as for Table I. [b] DHA, dehydroascorbate. [c] MDHA, monodehydroascorbate. [d] SOD activity is expressed as units g^{-1} fresh weight (×1000), as defined in "Materials and Methods."

Table III. *Low-molecular-weight antioxidants, pyridine nucleotides, and free flavins in nodules from bean plants exposed to continuous darkness for 0 to 4 d*

Metabolite[a]	Days of Darkness			
	0	1	2	4
	nmol g^{-1} fresh wt			
ASC	940 a	770 b	690 c	570 d
(h)GSH	690 a	540 b	440 b	240 c
(h)GSSG	30 a	30 a	60 b	10 c
NAD^+	35.2 a	27.1 b	18.8 c	11.4 d
NADH	6.6 a	5.6 b	4.5 c	3.8 c
$NADP^+$	10.3 a	9.0 b	7.2 c	5.9 d
NADPH	6.3 a	5.3 b	5.7 ab	4.4 c
Riboflavin	40.0 a	ND[b]	38.9 a	54.2 b
FMN	3.3 a	ND	2.7 b	3.0 b
FAD	6.8 a	ND	5.1 b	3.4 c

[a] Statistical analysis of means (n = 5–8) was performed as for Table I. [b] ND, Not determined.

unaffected for up to 2 d of darkness but were reduced by 49 and 70%, respectively, after 4 d (Table II). On the other hand, dark stress for up to 2 d had only a minor, if any, effect on the nodule content of free flavins, but 4 d of prolonged darkness caused a 50% decrease in FAD and a 36% increase in riboflavin (Table III).

Catalytic Fe and Oxidative Damage

Catalytic Fe represents the fraction of Fe in a tissue that is active in the generation of free radicals and possibly other AOS through Fenton chemistry, and its concentration can be assessed by its ability to promote DNA degradation. Catalytic Fe was not detectable in the nodule cytosol (<3-kD fraction) of control plants, but it was clearly present after 1 d and further increased after 2 or 4 d of darkness (Fig. 3A).

The nodule content of lipid peroxides, estimated as TBARS by the conventional thiobarbituric acid test, declined by 18% after 1 d of darkness and by 25% after 4 d (Fig. 3B; □). When lipid peroxides were measured by HPLC as content of MDA, a main toxic product of peroxidation, the corresponding decreases after 1 d and 4 d were 39 and 61% (Fig. 3B; ■). In contrast, the content of oxidized proteins in the nodules did not vary for the first 2 d of darkness but was almost doubled after 4 d (Fig. 3C).

Carbon and Nitrogen Metabolism

Placing plants in complete darkness for up to 4 d reduced the nodule content of soluble sugars and starch (Table IV). The most abundant carbohydrate was Suc, which declined by 97% within 1 d; Fru, which was present in low concentrations, declined to a similar extent. Starch declined by 50% by 1 d, but then remained constant over the time course. A similar pattern was also shown by Glc, which did not vary following an initial decline of 64% within 1 d (Table IV).

Many enzyme activities decreased to a much greater extent than cytosolic protein (Tables I and V). Thus, PEP carboxylase, GS, and glutamate synthase declined signifi-

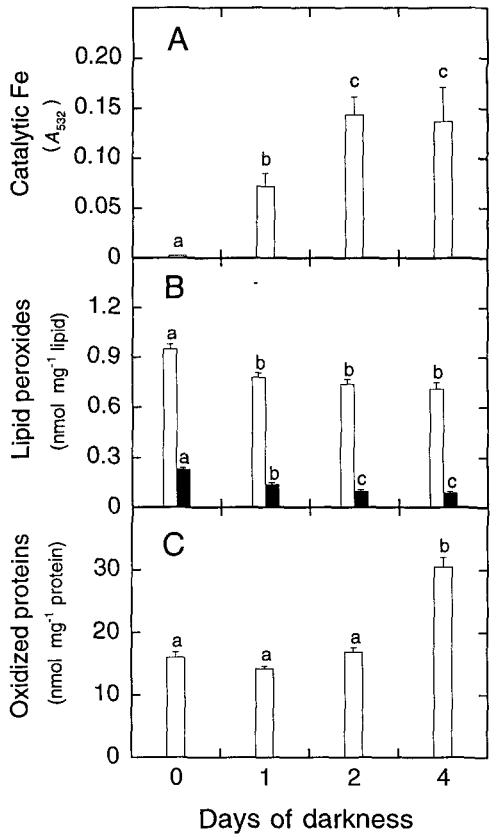

Figure 3. Contents of catalytic Fe (A), lipid peroxides (B), and oxidized proteins (C) in nodules from bean plants exposed to continuous darkness for 0 to 4 d. Catalytic Fe was expressed as A_{532}; in our experimental conditions (25-μL sample; 120-min incubation at 37°C), 0.20 absorbance units correspond approximately to 1 μM of catalytic Fe. Lipid peroxides were expressed as nmol TBARS mg^{-1} lipid (□) and nmol MDA mg^{-1} lipid (■), and oxidized proteins as nmol carbonyl groups mg^{-1} protein. Values are means ± SE (n = 4–10). For each parameter, statistical analysis was performed as for Table I.

cantly after 1 d of darkness, and SS, alkaline invertase, and phosphofructokinase after 2 d. Asp aminotransferase, PP_i:Fru-6-P phosphotransferase, and aldolase decreased in parallel with the general decline in protein content. After 4 d many of these key enzymes had activities of less than 10% of the control values (Table V).

Immunoblot Analyses

Antibodies to the two N_2ase components were used to determine whether dark stress had effects on the amount of the enzyme protein. Both components were found to decline significantly over the 4 d of continuous darkness (Fig. 4). Little change had occurred after 1 d. However, after 2 d both components were greatly reduced and after 4 d neither component was detectable (Fig. 4).

Immunoblot analyses were also used to quantify the levels of key cytosolic proteins (Lb) and enzymes of the ASC-GSH cycle (ASC peroxidase), carbon metabolism (SS), and nitrogen assimilation (GS). The levels of these proteins (Fig. 4) corresponded to the amount (Lb) or activities determined by other means (Tables I, II, and V). Only the apparently unchanged amount of the GS protein after 2 d of dark treatment conflicted with the measured reduction in GS activity (Table V).

Table IV. *Carbohydrates in nodules from bean plants exposed to continuous darkness for 0 to 4 d*

Metabolite[a]	Days of Darkness			
	0	1	2	4
	μg g^{-1} fresh wt			
Glc	157 a	60 b	51 b	60 b
Fru	42.2 a	1.0 b	4.5 b	0.1 b
Suc	4690 a	126 b	68 b	49 b
Starch	994 a	529 b	476 b	464 b

[a]Statistical analysis of means (n = 3) was performed as for Table I.

DISCUSSION

Results presented in this work reveal that the decline in N_2ase activity during the first 2 d of continuous darkness was not related to a failure of the antioxidant protection of nodules. The activities of ASC peroxidase and monodehydroascorbate reductase, as well as the contents of ASC, (h)GSH, and pyridine nucleotides, declined significantly after 1 d, but the decreases were in the range of 13 to 26%, which are probably physiologically irrelevant. Swaraj et al. (1994) found that dark treatment of clusterbean plants for 18 h resulted in a 55% loss in apparent N_2ase activity but only in a 22 to 30% decrease in ASC and (h)GSH content and in the ASC peroxidase activity of nodules. Although these results were interpreted as indicative of the close association between N_2 fixation and antioxidants, it is clear from the data shown in this work that this is not the case. Rather, the impairment of the ASC-GSH cycle and the ensuing oxidative stress in the nodules lag well behind the fall in N_2ase activity and may be a common feature of senescence (Escuredo et al., 1996).

The initial rapid decline in N_2 fixation and root respiration within 1 d of darkness appears to be related to the 98% depletion of Suc in the nodules, which in turn is likely to

Table V. *Enzymes of some key enzymes involved in carbon and nitrogen metabolism in nodules from bean plants exposed to continuous darkness for 0 to 4 d*

Enzyme[a]	Days of Darkness			
	0	1	2	4
	μmol min^{-1} g^{-1} fresh wt			
PEP carboxylase	10.03 a	7.62 b	1.36 c	0.33 c
Phosphofructokinase	2.61 a	2.30 a	0.58 b	0.26 b
PP_i:Fru-6-P phosphotransferase	1.07 a	0.86 ab	0.64 bc	0.36 c
Aldolase	0.19 a	0.17 a	0.15 a	0.07 b
Invertase	2.96 a	2.74 a	1.02 b	0.31 c
SS	7.63 a	6.97 a	2.42 b	0.64 c
GS	2.62 a	2.22 b	1.42 c	0.22 d
Glutamate synthase	0.36 a	0.31 b	0.08 c	0.02 d
Asp aminotransferase	7.00 a	6.83 a	6.16 a	2.90 b

[a] Statistical analysis of means (n = 3) was performed as for Table I.

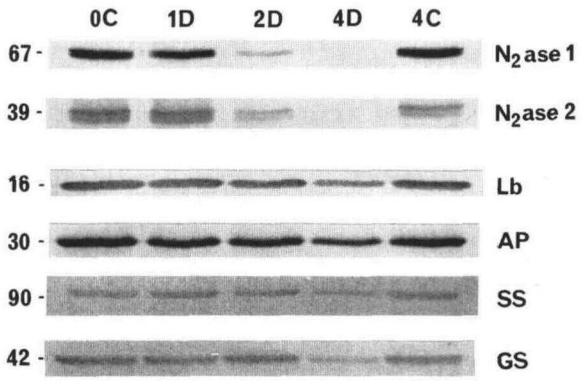

Figure 4. Immunodetection of bacteroid proteins (N_2ase 1 and N_2ase 2) and host plant proteins (Lb, AP [ASC peroxidase], SS, and GS) from nodules of beans exposed to continuous darkness for 1, 2, and 4 d (1D, 2D, and 4D). Control samples taken at 0 d (0C) and 4 d (4C), corresponding to plants grown under normal photoperiod conditions, are included. Shown on the left of the blots are molecular masses in kilodaltons estimated from the relative migration of prestained markers.

limit the formation of ATP (Ching et al., 1975) and the supply of respiratory substrates to bacteroids. As shown for other legumes, a consequence of carbohydrate depletion is a marked increase in the O_2 diffusion resistance of the nodules (Layzell et al., 1990; Iannetta et al., 1993). However, this mechanism to protect N_2ase can only function effectively if there is sufficient carbohydrate to fuel the infected cell respiration that is required to maintain low-internal O_2 concentrations.

Our data indicate that the lack of carbohydrate in bean nodules resulted in the cessation of effective respiration, leading to an increase in mean internal O_2 concentration from undetectable to 0.9% after only 1 d of darkness. Such levels may be high enough to inactivate N_2ase because this activity was reduced to 5% of control values, and this low activity could not be increased, and was in fact decreased, by raising external O_2. The almost complete depletion of Suc in bean nodules after 1 d of darkness indicates that any supply from host reserves must have been significantly below the respiratory requirements of nodules. Nevertheless, the nodulated root system must still contain some respiratory substrates after 1 and 2 d of darkness, as evidenced by the measurable rate of CO_2 production. A low level of nodule respiration during the first 2 d of dark stress is also evidenced by the fact that mean internal O_2 concentrations were held at 0.9 to 1.5%.

These observations differ substantially from those reported for soybean. After 4 d of darkness, soybeans had only 8% of total N_2ase activity but isolated bacteroids still retained 50% of the activity (Sarath et al., 1986). Furthermore, bacteroids were found to fully recover their N_2ase activity upon adding succinate to the assay medium, indicating that the loss of whole-plant N_2ase activity was due to a deficiency in carbohydrates rather than to a decrease in N_2ase protein (Schuller, 1986). In soybeans exposed to darkness for 2 d, N_2ase activity could still be increased by raising external O_2 (Schuller, 1986), whereas in beans N_2ase protein levels and activity were greatly reduced. This is consistent with the fact that soybean nodules showed a reduced internal O_2 concentration after 3 d of darkness (Layzell et al., 1990), whereas it was markedly increased in bean nodules. These differences indicate that bean nodules are more sensitive to dark stress than soybean nodules, and could reflect a lower availability of reserve carbohydrates in bean plants. Also, bean nodules seem to be more sensitive to low light than those of soybean. Antoniw and Sprent (1978) reported that transfer from high- to low-light intensity caused nodule senescence and reduced N_2ase activity within 24 h.

Nodules from beans deprived of light for 2 d showed a rapid decline in many biochemical parameters, but some others that are related to nodule activity, such as total bacteroid and cytosol protein, Lb, and flavins, and antioxidant enzyme activities, remained constant or declined only slightly. This suggests that protection against oxidative damage was still operative after 2 d of darkness. There was also a sharp increase in catalytic Fe, which is consistent with previous data showing a greater capacity of the nodule cytosol of dark-stressed beans, relative to that of control beans, to catalyze deoxyribose and linolenic acid degradation in vitro (Becana and Klucas, 1992). In the presence of H_2O_2, catalytic Fe can potentially give rise to ·OH radicals and other highly reactive species (Halliwell and Gutteridge, 1989), damaging important molecules within the nodules. However, after 2 d of darkness there were no signs of oxidative damage in vivo using lipid or protein oxidation as markers. A logical explanation for this finding is that mean internal O_2 concentrations of 0.9 to 1.5% are sufficiently high to cause loss of N_2ase protein, but are below a threshold level that induces oxidative stress due to uncontrolled formation of AOS. Also, the presence of substantial amounts of antioxidant defenses (70–80% of controls) could buffer against the appearance of oxidative damage at this stage. The fact that the internal O_2 levels were still substantially below atmospheric levels indicates the combination of an operational O_2 diffusion barrier and a residual respiratory activity within the infected zone after 2 d of stress.

After 4 d of dark stress the amount of oxidatively modified proteins was doubled, indicating a shift from a reductive to an oxidative state in nodules. This shift is supported by an increase (from 0.9–1.5% to 4%) in internal O_2 concentration and by a decrease (30–65%) in the contents of some reductants and redox cofactors (ASC, [h]GSH, NAD[P]H, and FAD). This oxidative stress may have originated from the rise in internal O_2 concentration, coupled to the high levels of catalytic Fe and the lowering in antioxidant defenses. However, the nodule contents of TBARS, and especially of MDA, which are commonly used as markers of oxidative stress, decreased significantly. We do not have any explanation for this yet, but lipid peroxidation was found to be enhanced only in nodules exposed to water stress (Gogorcena et al., 1995) and not in those induced to senesce by treatment with nitrate (Escuredo et al., 1996) or dark stress (this work).

CONCLUSIONS

Time-course studies of the dark-stress-induced decline in bean root nodule metabolism allow us to discern fac-

tors that may be causative from those that accompany or are a consequence of the cessation of N_2 fixation. After 1 d of darkness, in vivo N_2ase activity was almost zero and carbon substrates were almost completely exhausted. The resulting decline in respiratory activity may have induced an increase in O_2 diffusion resistance, but this was insufficient to prevent an increase in central zone O_2 to about 1%. This concentration may have caused the inactivation of N_2ase; however, the specific activities of key enzymes of carbon and nitrogen metabolism remained high. After 2 d of dark stress, there were substantial decreases in the levels of N_2ase proteins, and activities of enzymes involved in carbon and nitrogen metabolism were also reduced, but the O_2 diffusion barrier appeared to be still partially functional. It was not until after 4 d that there was a general collapse of nodule metabolism, with a subsequent decline in Lb content and antioxidant protection. This final senescent stage involved oxidative stress, as evidenced by the accumulation of damaged proteins. This oxidative damage correlated with a marked decline in antioxidant protection and probably resulted from the interaction between catalytic Fe and the AOS originating from the increased access of O_2 to the infected region. In this scenario an increase in internal O_2 concentration from 0.9–1.5% to 4% would be a critical factor. This increase was almost certainly a consequence of a continued reduction in residual respiratory activity, possibly combined with a reduced efficiency of the O_2 diffusion barrier.

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