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Involvement of Activated Oxygen in Nitrate-Induced Senescence of Pea Root Nodules[1]

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The effect of short-term nitrate application (10 mM, 0–4 d) on nitrogenase (N_2ase) activity, antioxidant defenses, and related parameters was investigated in pea (*Pisum sativum* L. cv Frilene) nodules. The response of nodules to nitrate comprised two stages. In the first stage (0–2 d), there were major decreases in N_2ase activity and N_2ase-linked respiration and concomitant increases in carbon cost of N_2ase and oxygen diffusion resistance of nodules. There was no apparent oxidative damage, and the decline in N_2ase activity was, to a certain extent, reversible. The second stage (>2 d) was typical of a senescent, essentially irreversible process. It was characterized by moderate increases in oxidized proteins and catalytic Fe and by major decreases in antioxidant enzymes and metabolites. The restriction in oxygen supply to bacteroids may explain the initial decline in N_2ase activity. The decrease in antioxidant protection is not involved in this process and is not specifically caused by nitrate, since it also occurs with drought stress. However, comparison of nitrate- and drought-induced senescence shows an important difference: there is no lipid degradation or lipid peroxide accumulation with nitrate, indicating that lipid peroxidation is not necessarily involved in nodule senescence.

Nitrate at concentrations >2 to 3 mM has a detrimental effect on the symbiosis between *(Brady)rhizobium* and crop legumes, because it inhibits several steps of the infection process, nodule growth, and nitrogen fixation (Streeter, 1988). A number of hypotheses have been proposed to account for the inhibition of N_2ase activity by nitrate. These include an increase in the resistance of an O_2 diffusion barrier (Minchin et al., 1986, 1989) and the formation of a nitrosyl complex between Lb and NO generated during nitrate metabolism (Kanayama et al., 1990). The precise mechanism is not known at present, but according to both hypotheses the nitrate-induced loss of N_2ase activity would result from impairment in O_2 supply to the bacteroids. Indeed, the control of O_2 concentration within the nodules is critical for optimal N_2ase activity (Hunt and Layzell, 1993). Therefore, it is not surprising that the effects of nitrate on the O_2 relationships of nodules have been examined mainly from a quantitative perspective, whereas other aspects of O_2, such as its metabolism and toxicity, have often been overlooked.

Most of the O_2 consumed during respiration of bacteroids and nodule mitochondria is reduced to water, but activated O_2 species, including the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), are always formed as toxic by-products. Other reactions generating activated O_2 are also expected to occur in the nodules. The autoxidation of oxygenated Lb to ferric Lb yields O_2^-, and this reaction is favored by the pH of nodules (approximately 6.4), which becomes more acidic (approximately 5.5) during senescence (Pladys et al., 1988). The O_2^- radical can also be formed by oxidation of key proteins of bacteroids, such as Fd, hydrogenase, and N_2ase (Dalton, 1995). This radical can dismutate to H_2O_2, which in turn may attack Lb, releasing free Fe and producing the highly devastating hydroxyl radical ($^{\cdot}OH$) through Fenton chemistry (Puppo and Halliwell, 1988). However, nodules possess an array of enzymes and small molecules ("antioxidants") to dispose of O_2^- and H_2O_2. Because $^{\cdot}OH$ is extremely short-lived (1 ns) in cells, there are no specific mechanisms to scavenge this radical, and its formation has to be prevented by minimizing the concentrations of O_2^-, H_2O_2, and catalytic Fe, which appear to be required to generate $^{\cdot}OH$ in biological systems (Halliwell and Gutteridge, 1989).

A commonly used protocol to study the negative effects of nitrate on nodule metabolism is to supply legumes with a relatively high but physiological nitrate concentration (10 mM) for a short period of time (≤4 d) and to follow the changes in nodule activity along with those produced in the parameters of interest. Using this short-term approach,

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Abbreviations: ASC, ascorbate; DHA, dehydroascorbate; Lb, leghemoglobin; MDA, malondialdehyde; MDHA, monodehydroascorbate (ascorbate free radical); N_2ase, nitrogenase; SOD, superoxide dismutase; TBARS, 2-thiobarbituric acid-reactive substances.

we have investigated the effect of nitrate on antioxidant defenses and markers of oxidative stress in pea (*Pisum sativum*) nodules.

MATERIALS AND METHODS

Chemicals

Organic solvents, inorganic acids, and salts used for preparing nutrient solutions were analytical or HPLC grade from Panreac (Barcelona, Spain). All other chemical and biochemicals were of the highest quality available from Sigma, Aldrich, or Calbiochem. Chelex-100 (200–400 mesh, Na^+ form) resin was obtained from Bio-Rad. Deionized or single-distilled water was used for preparing the nutrient solutions, and ultrapure water, obtained through a Milli-Q system (Millipore), was used for all other purposes.

Plant Culture and Nitrate Treatments

Pea (*Pisum sativum* L. cv Frilene) plants, inoculated with *Rhizobium leguminosarum* biovar *viceae* strain NLV8, were grown in pots containing perlite and vermiculite in controlled-environment chambers under conditions previously described (Moran et al., 1994). After approximately 36 d of growth on a nutrient solution (Becana et al., 1991) supplemented with 0.5 mM NH_4NO_3, plants were separated at random into four groups. Two groups were treated with 10 mM KNO_3 for 2 and 4 d; the two other groups served as controls (untreated). Nodules of one control group were harvested immediately and those of the other control group were harvested 4 d later; the latter control plants received 10 mM KCl. All plants were at the late-vegetative-growth stage when nodules were harvested.

Initial and final controls were included to take account of the minor growth effects during the 4-d study period. For all parameters where no differences occurred between the two sets of controls, all percent comparisons were made against the initial controls; where differences occurred between the control sets, comparisons after 2 d of nitrate were made against the initial controls, and comparisons after 4 d of nitrate were made against the final controls.

Nitrogen Fixation and Related Parameters

N_2ase activity and nodulated root respiration were measured on intact plants using a flow-through gas system (Minchin et al., 1983). Root systems were sealed in the growth pots, allowed to stabilize for 18 to 21 h in a gas 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. Respiration was measured as CO_2 production using IR gas analysis, and N_2ase activity was measured as C_2H_4 production from C_2H_2 using a gas chromatograph fitted with a flame ionization detector. After steady-state conditions had been reached following exposure to C_2H_2 and 21% O_2 (within 40–50 min), the O_2 concentration in the gas stream was increased over the range 21 to 60% (8.55–24.54 mmol O_2 L^{-1}). Carbon costs of N_2ase activity and N_2ase-linked respiration were calculated from the linear relationship between changes in respiration and C_2H_4 production (Witty et al., 1983).

Calculations of O_2 diffusion resistance were performed according to Minchin et al. (1992). In essence, this involved a curve-fitting routine for N_2ase-linked respiration against external O_2 concentration, using a modified equation for Fick's first law of diffusion, with the inclusion of an additional respiration factor that, when added to N_2ase-linked respiration, produced the total flux of O_2 across the diffusion barrier. Other parameters gave a characterization of total diffusion resistance (R) into a minimum diffusion resistance (R_{min}), which would occur at 0% O_2, and a factor for resistance adjustment (RA) in response to changes in O_2. Total diffusion was calculated using a simplification of Fick's law to R = total flux of O_2 across the diffusion barrier/external O_2 concentration.

Plant Extracts and Enzyme Assays

Other determinations were made on detached nodules that were immediately frozen in liquid N_2 and stored at −80°C until used. Except where otherwise indicated, all enzymes and metabolites were extracted using an ice-cold mortar and pestle, and the homogenate was filtered through one layer of Miracloth (Calbiochem) and centrifuged at 15,000*g* for 20 min.

Enzymes were extracted from 0.5-g nodules in optimized medium as indicated previously (Gogorcena et al., 1995), and initial rates of enzymatic activities were measured at 25°C with a Lambda16 UVDM spectrophotometer (Perkin-Elmer Cetus). Catalase (EC 1.11.1.6) activity was assessed by following the decomposition of H_2O_2 at 240 nm (Aebi, 1984). ASC peroxidase (EC 1.11.1.11), MDHA reductase (EC 1.6.5.4), GSSG reductase (EC 1.6.4.2), and DHA reductase (EC 1.8.5.1) activities were assayed according to the procedures of Asada (1984), Dalton et al. (1992), Dalton et al. (1986), and Nakano and Asada (1981), respectively. Where appropriate, controls were run for correcting nonenzymatic rates, and Chelex-treated buffers and reagents were used to avoid contamination by trace amounts of transition metals.

For determination of SOD (EC 1.15.1.1) activity, extracts were depleted of low-molecular-mass compounds by passage through a PD-10 mini-column (Pharmacia) eluted with 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA. Total SOD activity was assayed by its ability to inhibit ferric Cyt *c* reduction by a constant flux of O_2^- generated by the xanthine-xanthine oxidase system. The reaction mixture contained 10 μM KCN to inhibit Cyt *c* oxidase without affecting Cu plus Zn-containing 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).

Low-Molecular-Mass Metabolites

ASC was extracted from nodules (0.5 g) with 5 mL of 5% (w/v) metaphosphoric acid and quantified by its ability to reduce Fe^{3+} at very low pH (Law et al., 1983). GSH and GSSG were extracted from nodules (0.5 g) with 5 mL of 5% (w/v) sulfosalicylic acid. The homogenate was filtered and centrifuged, and the concentrations of total glutathione

(GSH + GSSG) and GSSG were determined in two aliquots of the supernatant essentially by the method of Law et al. (1983). This method involves oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid), reduction of GSSG by GSSG reductase, and derivatization of GSH by 2-vinylpyridine at slightly acidic pH (Griffith, 1980).

Pyridine nucleotides were extracted from nodules in alkaline (NADH, NADPH) or acid (NAD^+, $NADP^+$) medium (Gogorcena et al., 1995) and quantified by an enzymatic method (Matsumura and Miyachi, 1980). Assessment of H_2O_2 concentration in nodules was done by coupling of H_2O_2 with aminoantipyrine and phenol in the presence of horseradish peroxidase (Frew et al., 1983), using catalase-treated samples as blanks (Gogorcena et al., 1995).

Oxidative Damage to Lipids and Proteins

Lipid peroxides and oxidized proteins were extracted from nodules (0.5 g) as described in detail earlier (Moran et al., 1994). Lipid peroxidation was estimated as TBARS content (Minotti and Aust, 1987) and as MDA content, after separation of the (thiobarbituric acid)$_2$-MDA complex by HPLC (Waters) on an analytical C_{18} column (Ultrasphere, Beckman). The solvent consisted of 15% acetonitrile, 0.6% tetrahydrofuran, and 5 mM potassium phosphate buffer (pH 7.0); the flow rate was 1 mL min^{-1}, and detection was at 532 nm (Draper et al., 1993). In both cases, 0.1% (v/v) butylated hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of TBARS during the acid-heating step of the assay (Minotti and Aust, 1987).

Protein oxidation was measured as the total content of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine after removal of possible contaminating nucleic acids with 1% (w/v) streptomycin sulfate (Levine et al., 1990).

Other Analyses

Nodules (0.2 g) to be used for quantification of Fe and free heme were ground with 6 mL of Chelex-treated 25 mM potassium phosphate buffer (pH 7.0) using an ice-cold mortar and pestle. The homogenate was centrifuged at 15,000*g* for 20 min, and the supernatant was filtered through Centricon (Amicon, Beverly, MA) membranes with a 3-kD nominal exclusion limit. The concentration of Fe in the low (<3 kD)- and high (>3 kD)-molecular-mass fractions was determined by atomic absorption spectrophotometry using a graphite furnace atomizer (AA-670G and GFA-4A, Shimadzu, Kyoto, Japan). The concentration of catalytic Fe in the <3-kD fraction was estimated as bleomycin-dependent DNA damage (Evans and Halliwell, 1994) using 40 μL of sample, incubation at 37°C for 30 min, and free Fe^{3+} as standard.

Free heme and Lb were separated by ultrafiltration as described above and were quantified by the fluorescence emitted by the tetrapyrrole group after removing the Fe with hot oxalic acid (LaRue and Child, 1979). The excitation was set at 403 nm (slit width, 5 nm) and the emission at 602 nm (slit width, 2.5 nm). Unheated samples were used as blanks, and myoglobin from horse skeletal muscle (Calbiochem) was used as the standard because both hemoproteins showed identical emission spectra.

Soluble protein was determined by the dye-binding microassay (Bio-Rad) using BSA as the standard. Total lipids were extracted as indicated by Folch et al. (1957). The organic phase (approximately 3.5 mL) was rinsed three times with 0.9% (w/v) NaCl and evaporated to dryness with N_2. The residual solvent was removed in vacuo (Savant Instruments, Farmingdale, NY), and total lipid was determined gravimetrically.

Statistical Treatment

All values reported in this work are means of at least six replicates corresponding to independent extracts. The exact number of replicates is stated in the figures and tables. Means were compared by one-way analysis of variance and Duncan's multiple range test at the 5% level of significance.

RESULTS

Nitrogen Fixation, Carbon Costs, and O_2 Diffusion Resistance

Exposure of pea plants to 10 mM nitrate for just 2 d caused an 86% decrease of N_2ase activity; a 98% decrease was seen after 4 d (Fig. 1). The 2-d decrease could be slightly reversed by increases in external O_2 concentration (a 59% increase, compared to a 15% increase for initial control plants), but such changes had no effect on N_2ase activity after 4 d or on the final control plants (Fig. 1). Also, the optimum external O_2 concentration following the C_2H_2-induced decline was reduced by nitrate, from 50 to 60% in control plants to 40 to 50% after 2 d or 25 to 30% after 4 d of nitrate treatment. There were no significant differences in N_2ase activity between the initial and final control plants.

The 86% decrease in N_2ase activity after 2 d was accompanied by a 2-fold increase in the carbon costs of the enzyme activity (i.e. a 50% decrease in carbon use efficiency) and by a 3.2-fold increase in O_2 diffusion resistance

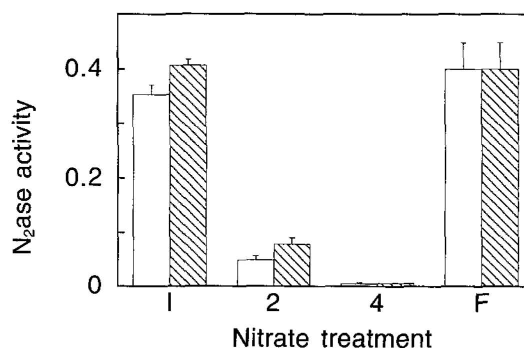

Figure 1. Effect of nitrate on maximum N_2ase activity (μmol C_2H_4 min^{-1} $plant^{-1}$) of pea nodules in 21% O_2 (□) or under optimum external O_2 concentration following the C_2H_2-induced decline (▧). This was 50 to 60% O_2 for treatments I and F, 40 to 50% for treatment 2, and 25 to 30% for treatment 4. Values are means ± SE (*n* = 6). Designation of treatments and statistical analysis are as for Table II.

Table I. *Effect of nitrate on parameters related to respiration, carbon costs of N_2ase activity, and O_2 diffusion resistance in pea nodules*

Treatments are designated as I, initial control; 2, nitrate treatment for 2 d; and F, final control. Data could not be determined for plants treated with nitrate for 4 d. For each parameter, means (n = 6) denoted by the same letter did not differ significantly at P < 0.05 according to Duncan's multiple range test.

Parameter[a]	Units	Treatment		
		I	2	F
TRR	μmol CO_2 plant^{-1} min^{-1}	2.00 ab	1.70 b	2.28 a
GMR	μmol CO_2 plant^{-1} min^{-1}	0.95 a	1.36 b	0.95 a
NLR	μmol CO_2 plant^{-1} min^{-1}	1.06 a	0.34 b	1.33 c
Carbon cost of N_2ase	mol CO_2 mol^{-1} C_2H_4	2.65 a	5.34 b	2.90 a
R ($-C_2H_2$)	s m^{-1}, $\times 10^{-6}$	1.19 a	3.75 b	1.30 a
R ($+C_2H_2$)	s m^{-1}, $\times 10^{-6}$	1.84 a	5.27 b	1.96 a
R_{min} ($+C_2H_2$)	s m^{-1}, $\times 10^{-6}$	1.33 a	4.39 b	1.36 a
RA ($+C_2H_2$)	s m^{-1}, $\times 10^{-6}$	0.036 a	0.023 b	0.039 a

[a] TRR, Total root respiration; GMR, nodulated root growth and maintenance respiration; NLR, N_2ase-linked respiration; R, O_2 diffusion resistance; R_{min}, minimum R; RA, adjustment of R to O_2 concentration. Resistances were calculated in an atmosphere containing 21% O_2 and 0 or 10% C_2H_2.

(i.e. a 70% decrease in O_2 supply to the bacteroids). After 4 d of nitrate treatment, N_2ase activity was too low to allow for accurate determination of these parameters (Table I).

Other Parameters Related to Nodule Senescence

Other parameters commonly considered as markers of nodule functioning, such as Lb and total soluble protein, significantly declined with 4 d of nitrate treatment (Table II). Relative to initial control plants, Lb content declined by 15% after 2 d of nitrate, but this can be ascribed for the most part to natural aging rather than to nitrate because final control plants, which were 4 d older, had 24% less Lb than initial control plants. The decrease in Lb after 4 d of nitrate exposure was more pronounced than that of total soluble protein (57 versus 32%). Thus, Lb accounted for approximately 20 and 12% of the total soluble protein in untreated and 4-d nitrate-treated nodules, respectively. The nitrate-induced decline in Lb (57%) was accompanied by a similar decrease (47%) in protein-bound Fe. This was estimated as the concentration of Fe in the high-molecular-mass fraction of the nodule cytosol and corresponds mainly to Fe of Lb heme. Contrary to what occurred with Lb and total soluble protein, the nodule contents of lipids and free heme did not vary with nitrate treatment (Table II).

Enzymes of the ASC-GSH Pathway and Related Metabolites

Nitrate application to plants for 4 d led to a general decline in the enzymatic activities of the ASC-GSH cycle in nodules (Table III). These enzymes are believed to act coordinately to reduce, ultimately, H_2O_2 to water at the expense of NAD(P)H. The extent of the nitrate-induced decreases was rather variable: 17% for MDHA reductase, 22% for GSSG reductase, 28% for DHA reductase, and 46% for ASC peroxidase. Nevertheless, treatment of plants for 2 d caused only a small decrease in DHA reductase activity and had no effect on the remaining enzymes (Table III). The effect of nitrate on the activities of two other antioxidant enzymes of nodules was also examined. Catalase activity decreased by only 16% after 4 d, whereas SOD activity remained unaffected. Likewise, catalase activity in nodules of final control plants was 17% lower than in nodules of the initial control plants, but both types of nodules showed similar SOD activities (Table III).

ASC and GSH are found at concentrations of 1 to 2 mM in nodules, where they may perform important functions other than the scavenging of activated O_2 (Dalton, 1995; Gogorcena et al., 1995). Both reductants were substantially affected by nitrate supply (Table IV). The ASC and GSH contents declined by 31 and 48%, respectively, following 4

Table II. *Effect of nitrate on some parameters related to growth and senescence of pea nodules*

Treatments are designated as I, initial control; 2, nitrate treatment for 2 d; 4, nitrate treatment for 4 d; and F, final control. For each parameter, means (n = 6) denoted by the same letter did not differ significantly at P < 0.05 according to Duncan's multiple range test.

Parameter	Units	Treatment			
		I	2	4	F
Nodule dry weight	mg plant^{-1}	114 a	101 a	81 b	129 c
Nodule number	plant^{-1}	360 a	333 a	355 a	444 b
Free heme	μg g^{-1} fresh wt	1.19 a	1.28 a	1.24 a	1.16 a
Lb	mg g^{-1} fresh wt	2.67 a	2.26 b	0.87 c	2.03 b
Total soluble protein	mg g^{-1} fresh wt	12.4 a	11.6 ab	7.2 c	10.6 b
Protein-bound Fe	μg g^{-1} fresh wt	25.4 a	19.3 b	11.7 c	22.0 ab
Total lipids	mg g^{-1} fresh wt	12.9 a	16.1 a	15.9 a	14.5 a

Table III. *Effect of nitrate on the activities of antioxidant enzymes in pea nodules*

Except for SOD activity, which is expressed in SOD units g^{-1} fresh weight (×1000), enzyme activities are expressed in units min^{-1} g^{-1} fresh weight. For each parameter, means (n = 6) were compared as indicated in Table II. Treatments were as defined in Table II.

Enzyme	Units	Treatment			
		I	2	4	F
ASC peroxidase	μmol ASC	21.9 a	19.0 a	9.9 b	18.4 a
DHA reductase	μmol ASC	0.45 ab	0.40 c	0.34 d	0.47 a
GSSG reductase	μmol NADPH	0.62 a	0.68 a	0.52 b	0.67 a
MDHA reductase	μmol NADH	2.47 ab	2.62 a	1.94 c	2.33 b
Catalase	mmol H_2O_2	0.23 a	0.21 ab	0.16 c	0.19 b
SOD	SOD units	0.58 a	0.62 a	0.56 a	0.56 a

d of treatment. After 2 d, the ASC content had already decreased, but the decline is in part attributable to nodule aging (Table IV). The decrease in GSH content did not translate into an increase in GSSG, which remained at a low and constant level (<13% of total glutathione) over the nitrate treatment. Consequently, the GSH:GSSG ratio declined from approximately 11 to 8 with nitrate treatment (Table IV).

The contents of pyridine nucleotides in nodules were also determined because H_2O_2 scavenging through the enzymes MDHA reductase and GSSG reductase requires NADH and NADPH, respectively (Dalton, 1995). Nitrate application for 4 d caused large decreases in all four nucleotide forms: 44% for $NADP^+$ and 63 to 76% for the other nucleotides (Fig. 2). Indeed, the contents of NAD^+ and NADPH had already decreased by approximately 30% following treatment with nitrate for 2 d. At that time NADH was not affected, whereas the level of $NADP^+$ was similar to that of the initial controls and 18% lower than that of the final controls (Fig. 2).

Catalytic Fe and Oxidative Damage to Biomolecules

The Fe present in the low-molecular-mass fraction (<3 kD) of the nodule cytosol was separated from the protein-bound Fe by ultrafiltration under carefully controlled conditions. Low-molecular-mass Fe was quantified by atomic absorption and it was shown to catalyze the generation of free radicals by the bleomycin assay (Evans and Halliwell, 1994). The concentration of Fe in the <3-kD fraction of nodules, whether determined by atomic absorption or by the bleomycin assay, responded similarly to nitrate. Thus, there was an increase of 45 to 53% after 4 d of nitrate treatment, although there were no changes after 2 d (Fig. 3).

Table IV. *Effect of nitrate on the content of low-molecular-mass antioxidants in pea nodules*

For each parameter, means (n = 6) were compared as indicated in Table II. Treatments were as defined in Table II.

Antioxidant	Treatment			
	I	2	4	F
	μmol g^{-1} fresh wt of nodules			
ASC	0.41 a	0.32 b	0.24 c	0.35 b
GSH	0.75 a	0.76 a	0.40 b	0.77 a
GSSG	0.07 a	0.09 a	0.05 a	0.06 a

Another potential oxidant in nodules, H_2O_2, apparently did not vary with nitrate treatment, with values being in the range of 0.1 to 0.3 μmol g^{-1} fresh weight (see discussion in Gogorcena et al. [1995] for further comments on these measurements).

The nodule content of oxidatively modified proteins increased by 37% after 4 d of nitrate treatment but did not change after 2 d (Table V). In contrast, the content of lipid peroxides, measured as nmol TBARS (MDA equivalents) mg^{-1} lipid, decreased by 21% after 4 d (Table V), although the decrease was not statistically significant if expressed per gram fresh weight. This rather unexpected result was further investigated by the specific and sensitive determination of MDA by HPLC. This showed that MDA content per milligram lipid (Table V) or per gram fresh weight (not shown) remained constant with nitrate treatment.

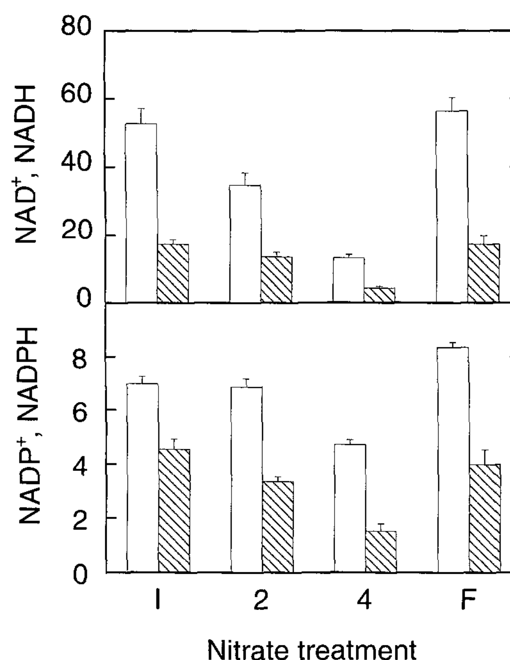

Figure 2. Effect of nitrate on pyridine nucleotide content (nmol g^{-1} fresh weight) of pea nodules. Values are means ± SE (n = 8). Designation of treatments and statistical analysis are as for Table II. □, NAD^+ and $NADP^+$; ▨, NADH and NADPH.

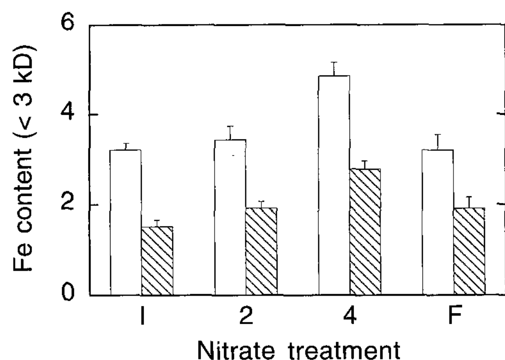

Figure 3. Effect of nitrate on Fe content (μg g^{-1} fresh weight) in the <3-kD fraction of pea nodule cytosol. Fe was quantified by atomic absorption spectrophotometry (□) or by the bleomycin assay (▨). This assay provides an estimate of the content of catalytic Fe and is dependent on the experimental conditions, which in our case are indicated in "Materials and Methods." Values are means ± SE (n = 6–8). Designation of treatments and statistical analysis are as for Table II.

DISCUSSION

Nodule Function and Senescence

Short-term exposure (2–4 d) of pea plants to nitrate (10 mM) seriously impaired nodule function, as shown by the very large decreases in N_2ase activity (Fig. 1). Decreases of approximately 80 to 85% have previously been recorded for common bean, soybean, and white clover using flow-through gas systems, but only after 3 to 4 d of exposure to 10 mM nitrate (Minchin et al., 1989, 1992; Faurie and Soussana, 1993). Other authors have reported lower decreases after 4 to 5 d of nitrate exposure: 60% for white clover (Minchin et al., 1986) and 65% for soybean (Vessey et al., 1988). Likewise, in all previous cases, N_2ase activity was reported to be maintained at a low rate (approximately 15–25% of controls) during long-term exposure to nitrate. The almost complete cessation of N_2ase activity after 4 d is an unusual response that has been observed in repeated runs (Fig. 1). Taken together, these observations suggest that the pea symbiosis used in this study responds more rapidly and more strongly to nitrate than any other legume symbiosis so far investigated.

The 2-d response to nitrate of our pea symbiosis can be accounted for by a 70% decline in O_2 supply to the bacteroids and a 50% decrease in carbon-use efficiency in N_2ase activity (Table I). The O_2 limitation could be partially overcome by increasing external O_2 concentration (Fig. 1). However, N_2ase activity started to decline again at 50 to 60% O_2 (data not shown). This rather low tolerance to O_2, given the marked increase in diffusion resistance, may reflect the low carbon-use efficiency of the bacteroids. The 4-d response to nitrate seems to involve a small loss of nodule mass and a very large, apparently irreversible, loss of N_2ase activity. Unfortunately, it proved impossible to accurately relate this to changes in either carbon costs or O_2 diffusion resistance.

Other markers of nodule senescence were minimally affected by 2 d of nitrate exposure. Thus, total cytosol protein remained constant and Lb decreased at most by 15%, since there was a decline in Lb with plant aging overlapping the effect of nitrate itself (Table II). By this stage of nitrate application, however, N_2ase activity was very low and nodule O_2 diffusion resistance had increased severalfold (Fig. 1; Table I). This is consistent with the observations made in other legumes that treatment with 10 mM nitrate for up to 3 d had no effect on the concentration of functional Lb but decreased nodule O_2 permeability (Denison and Harter, 1995). On the other hand, the larger decrease of Lb relative to total soluble protein indicates that the hemoprotein is particularly sensitive to nitrate treatment. This was also found to be the case for alfalfa and clusterbean nodules (Becana et al., 1988; Swaraj et al., 1993).

In nitrate-treated nodules, Lb may be inactivated by oxidation of the heme Fe, formation of a complex with NO, or degradation of the globin or heme group (Becana et al., 1991; Becana and Klucas, 1992; Jun et al., 1994). Inactivation of Lb by formation of the nitrosyl complex is unlikely to occur in pea nodules because their bacteroids do not express nitrate reductase and hence probably do not generate NO (Becana et al., 1989). In contrast, the 57% drop in Lb following 4 d of nitrate treatment (Table II) may be ascribed to degradation of the hemoprotein by acidic proteases with high affinity for Lb (Pladys and Rigaud, 1985), a process that is favored by the low pH of nitrate-treated nodules (Pladys et al., 1988). The decrease in Lb and protein-bound Fe was not accompanied by an increase in free heme but by an increase in the concentration of Fe in the <3-kD fraction (Table II; Fig. 3). A logical explanation for this is that Lb degradation starts with the heme group and proceeds with the apoprotein once the Fe has been released from the heme. Support for this hypothesis comes from the finding of abundant modified forms of Lb in senescent soybean nodules. These proteins retain the globin moiety intact but cannot carry O_2 because their heme groups have been altered (Jun et al., 1994).

Antioxidants of Nodules

Except for SOD activity and GSSG content, nitrate application for 4 d caused an overall decrease in the antioxidant enzymes and metabolites of nodules. The inhibitory effect of nitrate ranged from approximately 16% for catalase and MDHA reductase to 45% for ASC peroxidase and GSH (Tables III and IV). Comparison of our results with earlier reports on the antioxidant capacity of nitrate-treated nodules (Becana et al., 1988; Swaraj et al., 1993; Lorenzo et al.,

Table V. *Effect of nitrate on the oxidative damage of lipids and proteins in pea nodules*

Oxidative damage is expressed in units mg^{-1} lipid or units mg^{-1} protein. For each parameter, means (n = 6) were compared as indicated in Table II. Treatments were as defined in Table II.

Molecule	Units	Treatment			
		I	2	4	F
Lipids	nmol TBARS	0.84 a	0.67 bc	0.60 c	0.76 ab
Lipids	nmol MDA	0.27 a	0.21 a	0.19 a	0.21 a
Proteins	nmol C=O	8.8 a	9.2 a	11.1 b	8.1 a

1994) is hampered for several reasons. First, in such studies only a few enzymes and metabolites were investigated. Second, very high, nonphysiological nitrate concentrations were used. Third, initial control plants were not included to discern the effects of nodule aging from those of nitrate itself after medium-term exposures (>4 d) of plants to nitrate.

With these reservations in mind, some analogies can be drawn from the different studies. For example, catalase activity was inhibited by 20 to 30% after treatment of alfalfa nodules with 20 mM nitrate for 7 d (Becana et al., 1988), clusterbean nodules with 50 mM nitrate for 3 d (Swaraj et al., 1993), or lupin nodules with 20 mM nitrate for 4 to 6 d (Lorenzo et al., 1994). In the latter case, the nitrate-induced decline in catalase activity was associated with a decreased cytochemical staining of the enzyme in peroxisomes of infected cells (Lorenzo et al., 1990). In pea nodules exposed to a physiological "nitrate stress," the inhibition of catalase activity was rather small (Table III), which suggests that this enzyme is not involved in the nitrate-induced senescence of pea nodules. Furthermore, because catalase is confined to peroxisomes and the cytosol is an important source of activated O_2 within the nodules, enzymes should exist in the cytosol to scavenge H_2O_2 and to protect Lb. Lb is very sensitive to attack by activated O_2 and in turn may contribute to the generation of more oxidants by autoxidation (Puppo and Halliwell, 1988; Becana and Klucas, 1989; Dalton, 1995). Not surprisingly, the cytosol contains high levels of ASC peroxidase (Dalton, 1995) and probably of other enzymes of the ASC-GSH cycle. In our conditions, the activities of all four enzymes of the cycle were found to be substantially reduced after 4 d of treatment. In particular, almost 50% of the ASC peroxidase activity was lost (Table III). This is in contrast with earlier observations that treatment with 20 to 50 mM nitrate for 6 d was necessary to cause a decline of approximately 30% in lupin (Lorenzo et al., 1994) or in clusterbean (Swaraj et al., 1994) nodules.

The ASC peroxidase-catalyzed reaction yields MDHA and DHA, which have to be reduced back to ASC to avoid the collapse of the ASC-GSH cycle. Regeneration of ASC requires MDHA reductase or DHA reductase plus reduced pyridine nucleotides. After 4 d, nitrate caused a decline of 17 to 28% in MDHA and DHA reductase activities and of 63 to 74% in NADH and NADPH. In contrast, when nitrate was applied for 2 d, very small or no decreases were observed (Table III; Fig. 2). Similarly, the contents of ASC and GSH, which are required to preserve the activity of the H_2O_2 detoxification pathway, consistently decreased after 4 d but remained nearly the same after 2 d (Table IV). Collectively, these results strongly suggest that the operativity of the ASC-GSH cycle of pea nodules is considerably diminished between 2 and 4 d after nitrate treatment.

Oxidants and Oxidative Damage in Nodules

H_2O_2 and catalytic Fe are pro-oxidant factors that interact in vitro, and presumably in vivo, generating highly reactive O_2 species (Halliwell and Gutteridge, 1989; Becana and Klucas, 1992). The nodule content of H_2O_2, a common metabolite in plant cells, was not affected by nitrate, with values being similar to those reported earlier (Gogorcena et al., 1995). Two main sources of H_2O_2 exist in nodules: respiration in the mitochondria and bacteroids and Lb oxidation in the cytosol (Dalton, 1995). After 2 d of nitrate treatment, the O_2 flux to the infected region of nodules (based on N_2ase-linked respiration) was still 30% of controls, but Lb content had not changed; after 4 d, the O_2 flux was very low and Lb had decreased by 57% (Tables I and II). Therefore, the respiratory activity remaining after 2 d of nitrate and oxidative reactions accompanying Lb degradation after 4 d may account for continuing H_2O_2 generation in nitrate-treated nodules.

However, catalytic Fe is critical for Fenton reactions to proceed in vivo (Halliwell and Gutteridge, 1989), and we have advanced the hypothesis that the increase in catalytic Fe that occurs in drought-stressed leaves and nodules is a causative factor for the accumulation of damaged proteins and lipids (Moran et al., 1994; Gogorcena et al., 1995). A comparison between the effects of nitrate and drought on senescence markers, antioxidant capacity, and oxidative damage in pea nodules is shown in Table VI. Both types of

Table VI. *Comparative effects of nitrate and drought on antioxidant defenses and on markers of senescence and oxidative stress in pea nodules*

Values are percent changes relative to control plants. +, Increase; −, decrease; =, no change.

Parameter	Nitrate[a]	Drought[b]
Senescence markers		
N_2ase	−98	−n.d.[c]
Lb	−57	−33
Soluble protein	−32	−31
Total lipids	=0	−5
NAD^+	−76	−43
NADH	−74	−44
$NADP^+$	−44	−31
NADPH	−63	−17
Antioxidants		
ASC peroxidase	−46	−18
DHA reductase	−28	−15
MDHA reductase	−17	=0
GSSG reductase	−22	−31
Catalase	−16	−25
ASC	−31	−59
GSH	−48	−57
Pro-oxidants and oxidant damage		
Low-molecular-mass Fe	+53	+102
Catalytic Fe	+45	+n.d.[d]
Lipid peroxides	−21	+143
Oxidized proteins	+37	+40

[a] Nitrate was applied as 10 mM KNO_3 for 4 d (this work). [b] Drought was applied by withholding water until a leaf water potential of −1.15 MPa was reached (Gogorcena et al., 1995). [c] Not determined, but activity is inhibited according to the literature (e.g. Durand et al., 1987). [d] Not determined by the bleomycin assay, but shows increasing trend as estimated by the deoxyribose assay (Gogorcena et al., 1995).

stress are known to decrease N_2ase activity and nodule O_2 permeability (Durand et al., 1987; this work). They induced senescence and also caused a decrease in antioxidant defenses and related metabolites and an increase in catalytic Fe and oxidized proteins (Table VI). Therefore, the decrease in antioxidants is not a specific effect of nitrate but a feature that may be common to nodule senescence. However, there was an important difference between the effects of nitrate and drought. In the latter case the total content of lipids decreased by 55% and the lipid peroxide content was augmented by almost 1.5-fold, whereas nitrate did not affect the lipid content and even decreased that of lipid peroxides by 21% (Table VI). A logical explanation for this discrepancy is that drought triggers a process of enzymatic, Fe-independent lipid peroxidation in nodules, whereas this process may be slow or virtually nonexistent in nitrate-treated nodules. However, another explanation is that the increase in catalytic Fe, which occurs in nitrate-stressed nodules and which is markedly lower than that in drought-stressed nodules, is insufficient to trigger nonenzymatic, Fe-dependent lipid peroxidation. In any case, our results suggest that lipid peroxidation is not necessarily involved in plant tissue senescence.

CONCLUSIONS

The main conclusion of this work is that the loss of N_2 fixation by pea nodules following nitrate treatment can be dissected into two stages that are reminiscent of those proposed by Minchin et al. (1989). The first stage is characterized by a rapid fall in N_2ase activity and nodule O_2 permeability. During this stage there is no apparent oxidative damage and the decrease in N_2ase activity is, to some extent, reversible. In contrast, the second stage, which is initiated between the 2nd and 4th d of nitrate treatment, is characterized by an important lowering in antioxidant defenses and by a moderate accumulation of oxidized proteins.

The decrease in antioxidant content and the ensuing oxidative stress are not responsible for the initial decline of N_2ase activity, which is probably due to the limited O_2 supply to the bacteroids as a result of an increased resistance to O_2 diffusion. Rather, they may reflect a serious impairment of nodule metabolism related to senescence and are likely to mark the transition from the first (reversible) to the second (irreversible) stage in the nitrate-induced decline of nodule activity.

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