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Possible causes of the physiological decline in soybean nitrogen fixation in the presence of nitrate

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Abstract

Nodulated soybean plants (*Glycine max* (L.) Merr. cv. Clarke) were supplied with 10 mol m^{-3} nitrate at the vegetative stage. This treatment caused a rapid decline in nitrogen fixation (acetylene reduction) activity and a consequent decline in ureides in the xylem sap. However, there was virtually no effect on the nitrogenase complex, according to Western blots against components 1 and 2. The effect on nitrogen fixation was matched by a decrease in nitrogenase-linked respiration and increases in nodule oxygen diffusion resistance and the carbon cost of nitrogen fixation. The addition of nitrate had little effect on protein content from either nodule plant or bacteroid fractions. Activities of nitrate reductase (NR) and nitrite reductase (NiR) from either the plant fraction or the bacteroids were affected in different ways during 8 d of NO_3^- supply. Nodule plant NR and bacteroid NiR were not affected. However, nodule plant NiR increased 5-fold within 2 d of supplying NO_3^-. Bacteroid NR only increased after 6 d. These results could be interpreted in terms of a restricted nitrate access into the infected region of nodules. However, denitrification was detected within 2 d of nitrate supply in soybean nodules. The results are discussed in relation to possible causes of the nitrate-induced decline in nitrogenase activity.

Key words: *Glycine max*, nitrate, nitrogen fixation, nodules.

Introduction

The inhibition of nitrogen fixation in legume nodules by applied NO_3^- has been studied for many years without any consensus being reached as to the mechanism of the inhibition. The role of NO_3^- metabolism in this inhibition is highly controversial. The hypothesis of inhibition and/or damage due to NO_2^- accumulation (Rigaud, 1976) is challenged by studies using bacterial or plant mutants deficient in nitrate reductase (Streeter, 1988), by studies showing low levels of NO_2^- accumulation (Becana *et al.*, 1989) and, more importantly, by studies which indicate that NO_3^- entry into the bacteroid region can be restricted (Sprent *et al.*, 1987; Giannakis *et al.*, 1988; Becana *et al.*, 1989). However, several other studies have suggested that NO_3^- can reach the bacteroid region and can be metabolized (see reviews by Becana and Sprent, 1987; Streeter, 1988; Drevon *et al.*, 1988).

Other reports have indicated an indirect effect of NO_3^- via an increase in the resistance to oxygen diffusion across the nodule cortex resulting in a reduced availability of oxygen to the bacteroids (Minchin *et al.*, 1986; Carroll *et al.*, 1987; Schuller *et al.*, 1988; Vessey *et al.*, 1988). In an attempt to reconcile these hypotheses, Minchin *et al.* (1989) proposed that NO_3^- inhibition of nitrogen fixation occurs in two stages: (i) an initial increase in oxygen diffusion resistance, followed by (ii) the entry of NO_3^- into the bacteroid region with inhibition and/or damage resulting from its metabolism.

Evidence in support of this two-stage hypothesis has been provided for lucerne (Arrese-Igor *et al.*, 1990, 1992), but these studies lacked any direct measurement of oxygen diffusion resistance. In the work described here, this hypothesis was tested by investigating the effect of NO_3^- on parameters of nodule oxygen diffusion, on the stability of the two nitrogenase components and on the induction of nodule host plant and bacteroid NR and NiR activities.

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Materials and methods

Growth conditions

Seeds of soybean (*Glycine max* (L.) Merr. cv. Clarke) were inoculated at sowing, and then after 7 d, with a suspension of *Bradyrhizobium japonicum* strain RCR 3407. Plants were grown in 800 cm^3 volume pots of vermiculite in a growth cabinet providing 15 h light (600 μmol m^{-2} s^{-1}) set at 25/20 °C and 70/80% relative humidity (light/dark regime) and were watered daily with half-strength N-free nutrient solution (Ryle *et al.*, 1978). Treatments were started 42 d after sowing when plants were at the late vegetative stage. For nitrate treatments, the same nutrient solution was used supplemented with 10 mol m^{-3} KNO_3. Control plants received no nitrate. In some experiments, another set of plants were supplied with N-free nutrient solution supplemented with 10 mol m^{-3} KCl to account for osmotic effects. Nitrate treated plants were harvested at 0, 2, 4, 6, and 8 d, but in some experiments NO_3^- application continued for up to 24 d. For plant growth analyses, dry weights were obtained after drying in an oven at 85 °C for 48 h.

Acetylene reduction activity (ARA) and nodulated root respiration measurements

ARA and root respiration of intact roots were measured using a flow-through gas system (Minchin *et al.*, 1983) housed in a Fisons cabinet. Root systems were sealed into the growth pots, allowed to stabilize for 18–21 h in a gas stream of air enriched to 500 cm^3 m^{-3} CO_2, 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 by infra-red gas analysis and C_2H_4 production was measured by gas chromatography using a flame ionizer detector. Maximum rates of *ARA* were determined from the maximum rate of C_2H_4 production prior to the C_2H_2-induced decline which is shown by this soybean symbiosis (Minchin *et al.*, 1986). The extent of this decline was reduced with increasing periods of exposure to nitrate, as found with other species (Minchin *et al.*, 1989), but it was always possible to determine a distinct maximum for C_2H_4 production.

After steady-state conditions had been reached following exposure to C_2H_2 in 21% O_2 (8.59 mol O_2 m^{-3}), the O_2 concentration in the gas stream was increased over the range 30–60% (12.27–24.54 mol O_2 m^{-3}), in steps of 10% O_2. Following each increase in O_2 concentration, there was a 25–30 min equilibration period to allow new steady-state conditions to be reached.

Carbon costs of nitrogenase activity and nitrogenase-linked respiration (*NLR*) were determined from the slope of the linear relationship between nodulated-root respiration and C_2H_2 reduction. This was based on changes which occurred in both parameters during the C_2H_2-induced decline and O_2 stepping (Witty *et al.*, 1983). These data were then used to calculate resistance values (*R*) using an exponential curve-fitting routine for *NLR* against external oxygen concentration which involves a modified equation for Fick's first law of diffusion (Minchin *et al.*, 1992). This allows for the calculation of an additional respiration factor which, when added to *NLR*, represents the total flux of oxygen across the diffusion barrier (*F*, see Minchin *et al.*, 1992, for full details). Root respiration data obtained immediately before exposure to C_2H_2 was used for calculation of the oxygen diffusion resistance in air.

For ureide analysis, xylem sap was collected from the tops of decapitated root systems over a period of 10–20 min. This procedure was followed every day at the middle of the photoperiod. Sap samples were frozen at −80 °C and analysed for ureides as the phenylhydrazone of glyoxylate (Vogels and van der Drift, 1970).

Nodule extraction for analysis of nitrogenase components

Nodules (*c.* 200 mg) were extracted in 2 × 0.5 cm^3 50 mol m^{-3} TRIS pH 7.5 containing 1% 2-mercaptoethanol, 0.29 mol m^{-3} phenylmethylsulphonylfluoride and 1 mol m^{-3} EDTA in a mortar and pestle at 2 °C. The total extract was centrifuged at 14 000 *g* at 5 °C in an Eppendorf microcentrifuge for 10 min and the supernatant collected.

The pellet was washed twice by resuspending in 0.5 cm^3 extraction buffer at 2 °C, centrifuging and discarding the supernatant. The pellet was then resuspended in 1 cm^3 buffer and sonicated (2 × 30 s) to disrupt the bacteroids. Bacteroids integrity was monitored by electron microscopy. Before sonication, more than 95% bacteroids were observed as intact, whilst after sonication they were totally disrupted. The supernatant was then collected after centrifugation (as above) and subsamples (100 mm^3) denatured by the addition of 25 mm^3 sample buffer (62.5 mol m^{-3} TRIS pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.125% bromophenol blue) and heating at 100 °C for 5 min.

Samples of the denatured extract (equal amounts of protein on each track) were separated by 12.5% SDS polyacrylamide gel electrophoresis (Gordon and Kessler, 1990).

Western immunoblotting

Electrophoretic transfer of proteins on to nitrocellulose membranes was carried out overnight (15 h) at a constant voltage of 60 V (2 °C). After washing and blocking, the membranes were incubated with either a monoclonal antibody to *Klebsiella pneumoniae* nitrogenase component 1 (Kp1) or a polyclonal antibody to nitrogenase component 2 (Kp2) for 1 h. After further washing, the membranes were treated with an appropriate second antibody conjugated with horse-radish peroxidase (Dako Ltd) and antigen bands visualized by incubating the membranes with 4-chloro-1-naphthol (50 μg 100 cm^{-3}) and 0.015% (v/v) H_2O_2 at room temperature.

Preparation of bacteroids and plant fractions

For the extraction of bacteroids, 1 g of nodules were rinsed thoroughly with distilled water and immediately ground in an ice-chilled mortar with 7 cm^3 of an extraction buffer consisting of 100 mol m^{-3} potassium phosphate buffer (pH 7.5) and 300 mol m^{-3} sucrose. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 500 *g* for 2 min to remove nodule debris. The resultant supernatant was centrifuged again at 12 000 *g* for 10 min at 2 °C and the pellet was washed twice with 100 mol m^{-3} potassium phosphate buffer (pH 7.5) and finally resuspended in the same medium. Bacteroids were disrupted in an MSE ultrasonicator at 0–4 °C for 3 min in 30 s pulses. The soluble fraction was obtained following centrifugation at 35 000 *g* for 20 min at 2 °C. The residual pellet was resuspended in 7 cm^3 of the same phosphate buffer.

For the extraction of the nodule plant fraction, 100 mg of nodules were ground as above, but with 2 cm^3 of an extraction buffer consisting of 100 mol m^{-3} potassium phosphate buffer (pH 7.5), 5 mol m^{-3} Na_2EDTA, 1 mol m^{-3} DTT, 0.01 mol m^{-3} FAD, and 1% casein. The homogenate was centrifuged at 35 000 *g* for 20 min. The resultant supernatant was used as the soluble plant fraction of nodules.

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Table 1. *Effect of nitrate on plant growth parameters*

Plants were supplied with N-free nutrient solution (control) or the same nutrient solution supplemented either with KCl or KNO_3 (10 mol m^{-3}) for 8 d. Results are means ±SE. Values in the same row containing the same letter do not differ statistically at 95% in Fisher's (protected) LSD tests ($n=6$).

Parameter	Initial value	Control	KNO_3	KCl
Plant DW (g)	7.08±0.43 b	10.63±0.70 a	9.77±0.70 a	8.68±0.82 ab
Shoot DW (g)	4.74±0.30 c	7.70±0.51 a	6.29±0.41 b	6.58±0.69 ab
Root DW (g)	1.84±0.14 b	2.13±0.16 b	3.06±0.30 a	1.44±0.07 b
Nodule DW (g)	0.50±0.04 bc	0.80±0.05 a	0.41±0.07 c	0.66±0.09 ab
Nodule numbers	247±24 b	353±37 a	211±12 b	265±30 ab
Single nodule volume (mm^3)	10.40±0.80 a	11.43±1.55 a	10.30±1.20 a	12.28±0.76 a

Leghaemoglobin and protein determination

Leghaemoglobin (Lb) was determined by the pyridine-haemochrome method (Appleby and Bergersen, 1980) after centrifugation at 35000 *g* for 20 min to pellet out the haem-containing bacteroids and membrane-bound proteins. Soluble protein from the plant fraction and bacteroid extracts was determined by the Lowry method, after trichloroacetic acid precipitation, according to Bensadoun and Weinstein (1976). Membrane-bound bacteroid protein was determined according to Wang and Smith (1975). In all cases, bovine serum albumin (Sigma, fraction V) was used as the standard.

Enzyme assays

Nitrate reductase (NR) activity from the nodule plant fraction extracts was assayed by measuring the amount of nitrite formed according to Wallace (1986). In a total volume of 1 cm^3, the reaction mixture contained 50 mol m^{-3} potassium phosphate buffer (pH 7.5), 5 mol m^{-3} KNO_3, 0.2 mol m^{-3} NADH (freshly prepared in 25 mol m^{-3} potassium phosphate buffer, pH 7.5), and 0.2 cm^3 extract. After incubation for 15 min at 25 °C, the reaction was stopped by adding the reagents for NO_2^- determination and shaking vigourously to ensure complete oxidation of residual NADH. The reagents for nitrite determination consisted of 1 cm^3 of 1% sulphanilamide in 1500 mol m^{-3} HCl and 1 cm^3 of 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Nicholas and Nason, 1957).

NR from bacteroids was assayed in the above-mentioned conditions, but using a dithionite-methyl viologen electron donor system as reported by Kennedy *et al.* (1975), as this enzyme is unable to use NAD(P)H as an electron donor (Heckmann and Drevon, 1987).

Nitrite reductase (NiR) activity from both sources was determined according to Hucklesby *et al.* (1972) by the disappearance of nitrite from the assay medium, using a dithionite-methyl viologen electron donor system. The reaction medium, in a total volume of 3 cm^3, consisted of 100 mol m^{-3} potassium phosphate buffer (pH 6.8), 0.167 mol m^{-3} KNO_2, 0.25 mol m^{-3} methyl viologen, 0.4 cm^3 extract, and 0.3 cm^3 sodium dithionite solution (7.2 g per dm^3 of 50 mol m^{-3} potassium phosphate buffer) to initiate the reaction. After incubation at 30 °C for 20 min, the tubes were vigorously shaken until the electron donor system was completely oxidized. Aliquots of 0.25 cm^3 were then removed, adjusted to 2 cm^3 with deionized distilled water and NO_2^- was determined as before. A control without enzyme was run in parallel to correct for the non-enzymatic reduction of nitrite by dithionite. In all cases, zero time controls were achieved by stopping the reactions immediately before the incubation period.

Denitrification assays

Nodules were assayed for nitrous oxide evolution in a final volume of 5 cm^3 of potassium phosphate buffer 50 mol m^{-3} (pH 7.5) containing 10 mol m^{-3} glucose, 10 mol m^{-3} sodium succinate, 100 g dm^{-3} chloramphenicol, and 20 mol m^{-3} KNO_3 (when added) in 50 cm^3 test tubes with Suba-seal stoppers (Arrese-Igor *et al.*, 1992). Acetylene 10% (v/v) was present to inhibit nitrous oxide reductase (Yoshinari and Knowles, 1976). Rates of nitrous oxide evolution in the absence of acetylene were less than 4.5% of those in the presence of acetylene. No effect of oxygen concentration upon the denitrification activity of whole nodules could be detected.

Aliquots (0.5 cm^3) of the gas phase were removed every hour, after swirling, and analysed for N_2O in a Philips gas chromatograph equipped with an electron capture detector and a Porapak Q column. Values were corrected to account for the dissolved N_2O in the liquid phase (Moraghan and Buresh, 1977).

Statistical analyses

Results were examined by one-way analysis of variance. All effects discussed in this study were significant at $P<0.05$ in Fisher's (protected) least significant difference (LSD) tests between means.

Results

The supply of 10 mol m^{-3} NO_3^- for 8 d did not show any benefit in terms of an increase in plant dry weight in comparison with control plants (Table 1). However, nitrate affected growth of shoots and roots in different ways. Thus, virtually all the growth of control plants was due to an increase in their shoot mass, leading to an increase in shoot/root ratio from 2.7 at the beginning of the experiment to 3.7 8 d later; whilst nitrate-fed plants increased both under- and above-ground mass in parallel. Conversely, nodule mass increased steadily in control plants, whilst in nitrate-fed plants the first effect was to stop nodule growth and the formation of new nodules, followed by a reduction in their mass after 4 d of treatment (data not shown). Treatment with KCl caused a slight decreased in nodule growth, as compared to control plants. Differences in nodule mass between control and KCl-treated plants was due to a decrease in the formation

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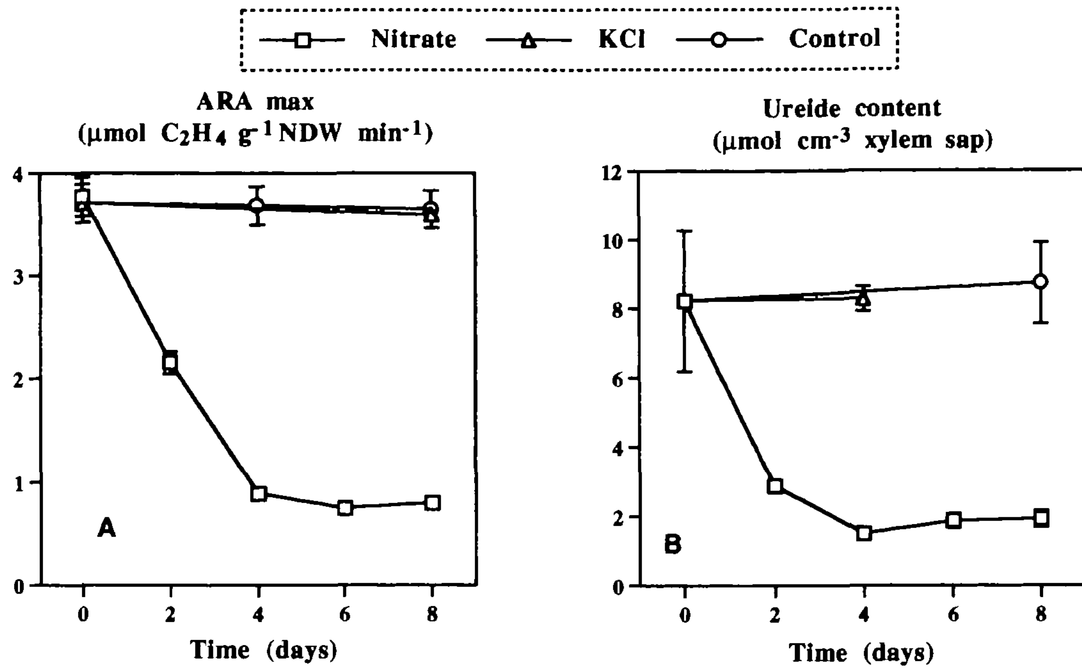

Fig. 1. Effect of 10 mol m^{-3} nitrate on acetylene reduction activity (ARA) of soybean nodules expressed on a nodule dry weight basis (A) and ureide content of xylem sap (B). (□) Denotes nitrate-treated plants, (O) their corresponding controls and (△) plants supplied with 10 mol m^{-3} KCl. Results are mean ±SE ($n=6$).

of new nodules, rather than changes in the weight of individual nodules.

Nitrogen fixation (expressed on a nodule DW basis) remained constant for control plants over the study period and was unaffected by the addition of KCl (Fig. 1A). Conversely, the presence of nitrate caused activity to decline to 23% of controls after 4 d, after which no further reduction was observed. This effect together with the effect of nitrate on nodule mass (Table 1) induced a strong decline in nitrogen fixation activity per plant (data not shown), whilst nitrogen fixation of control plants increased over the 8 d period, due to an increase in nodule mass (Table 1). The decline in nitrogen fixation activity was parallelled by a decrease in ureide content of the xylem sap (Fig. 1B).

However, in contrast to these effects, there were no corresponding declines in components of the nitrogenase complex over this time scale. Thus, immunodetection of components 1 and 2 (Fig. 2) showed the same amounts of both proteins after 3 d of nitrate supply, while after 9 d only component 2 had declined slightly with respect to controls. Both components, although reduced slightly in content, were still clearly present even after 24 d in the presence of nitrate.

Nitrogenase-linked respiration (Fig. 3A) closely paralleled nitrogen fixation activity; showing a sharp decline during the first 4 d of nitrate supply, with no further decline thereafter. Moreover, the presence of nitrate caused a steady increase in the carbon cost of nitrogen fixation (Fig. 3B), which was not observed in the presence of KCl.

The presence of nitrate also caused the nodule oxygen diffusion resistance (*R*) to increase (Fig. 3C). A slight increase was observed within 2 d and a major increase by 4 d, with no further significant changes occurring thereafter.

Nodule protein was not affected by the presence of nitrate. Thus, plant fraction protein and Lb content was not significantly lower than for controls (Table 2). This

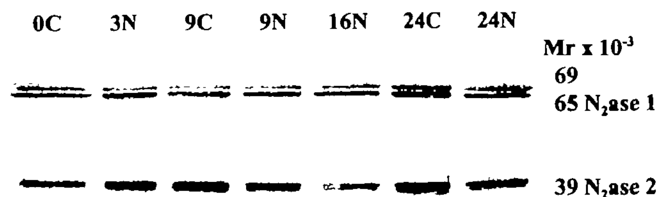

Fig. 2. Western blots of equal amount of protein extracted from nodules of soybean plants treated with nitrate (10 mol m^{-3}) for 3, 9, 16, and 24 d (N). The corresponding controls for days 0, 9 and 24 have been also added (C). Blots were challenged with antibodies to component 1 and component 2 of the nitrogenase complex.

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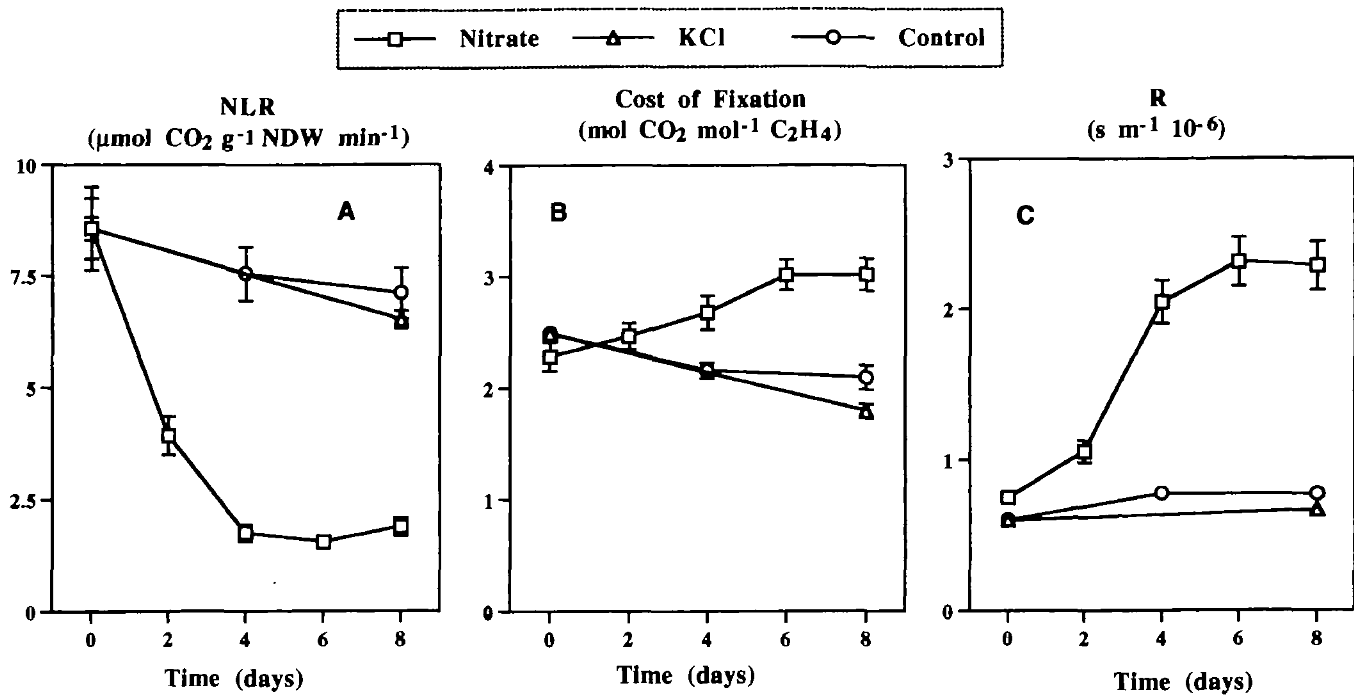

Fig. 3. Effect of nitrate on CO_2 evolution from nodules and resistance parameters of soybean nodules. (A) nitrogenase-linked respiration, (B) the carbon costs of nitrogen fixation and (C) oxygen diffusion resistance. (□) Denotes nitrate-treated plants and (O) their corresponding controls and (△) denotes plants supplied with 10 mol m^{-3} KCl. Error bars denote ±SE ($n=6$), when larger than the symbol itself. LSD value for carbon costs is 0.339.

Table 2. *Protein content of nodules as affected by nitrate*

Plants were fed 10 mol m^{-3} KNO_3 for 8 d. Data are expressed as mg protein g^{-1} nodule FW. Results are means±SE ($n=3$).

	Plant fraction		Bacteroids	
	Total protein	Lb	Soluble	Membrane-bound
Control	20.6±1.2	4.6±0.3	5.7±0.5	8.4±0.5
Nitrate-treated	17.0±2.0	4.0±0.6	5.1±0.5	8.5±0.9

was further confirmed by Western blots against Lb, in which no significant changes were detected even after 24 d of nitrate supply (data not shown). Also, both the soluble and the membrane-bound protein fractions of bacteroids remained virtually insensitive to nitrate supply.

Enzymes of inorganic nitrogen metabolism were detected in both the plant and the bacteroid fractions. NADH-dependent NR of the nodule plant fraction was constitutive, but nitrate treatments did not significantly increase its activity (Fig. 4A). Very low bacteroid NR activity could be detected in the membrane-bound fraction of bacteroids extracted from plants not treated with nitrate (15% of the total nodule NR activity, Fig. 4C) and significant changes were found only after 6 d of nitrate supply. Even in this case, bacteroid NR activity represented less than 50% of the total nodule NR activity. However, between 6 and 8 d of nitrate treatment, bacteroid NR increased by a factor of 4. No soluble NR activity was detected in these bacteroids.

Plant NiR activity was always in excess of the corresponding NR activity (Fig. 4B) and showed a rapid enhancement within 2 d of nitrate supply, but no further increases occurred thereafter. Bacteroid NiR activity was also in excess of the corresponding bacteroid NR activity and was located in the soluble fraction (Fig. 4D), and its activity was not significantly enhanced in plants supplied with nitrate.

Whole detached soybean nodules were assayed for denitrification activity as N_2O evolution. With nitrate in the assay medium, denitrification could be observed within 2 d of nitrate supply, with further increases in subsequent days (Fig. 5A). Also, when no nitrate was added to the assay medium (Fig. 5B), and denitrification rates depended on the availability of nitrate within the nodules, N_2O evolution was observed within 2 d of nitrate supply with a similar pattern to that of nodules assayed with nitrate. However, rates were 10- to 20-fold lower than in the $+NO_3^-$ assays, suggesting that, under physiological conditions, nitrate availability may constrain denitrification activity.

Discussion

Nitrate has been described as having pleiotropic effects on symbiotic nitrogen fixation, with dramatic effects on

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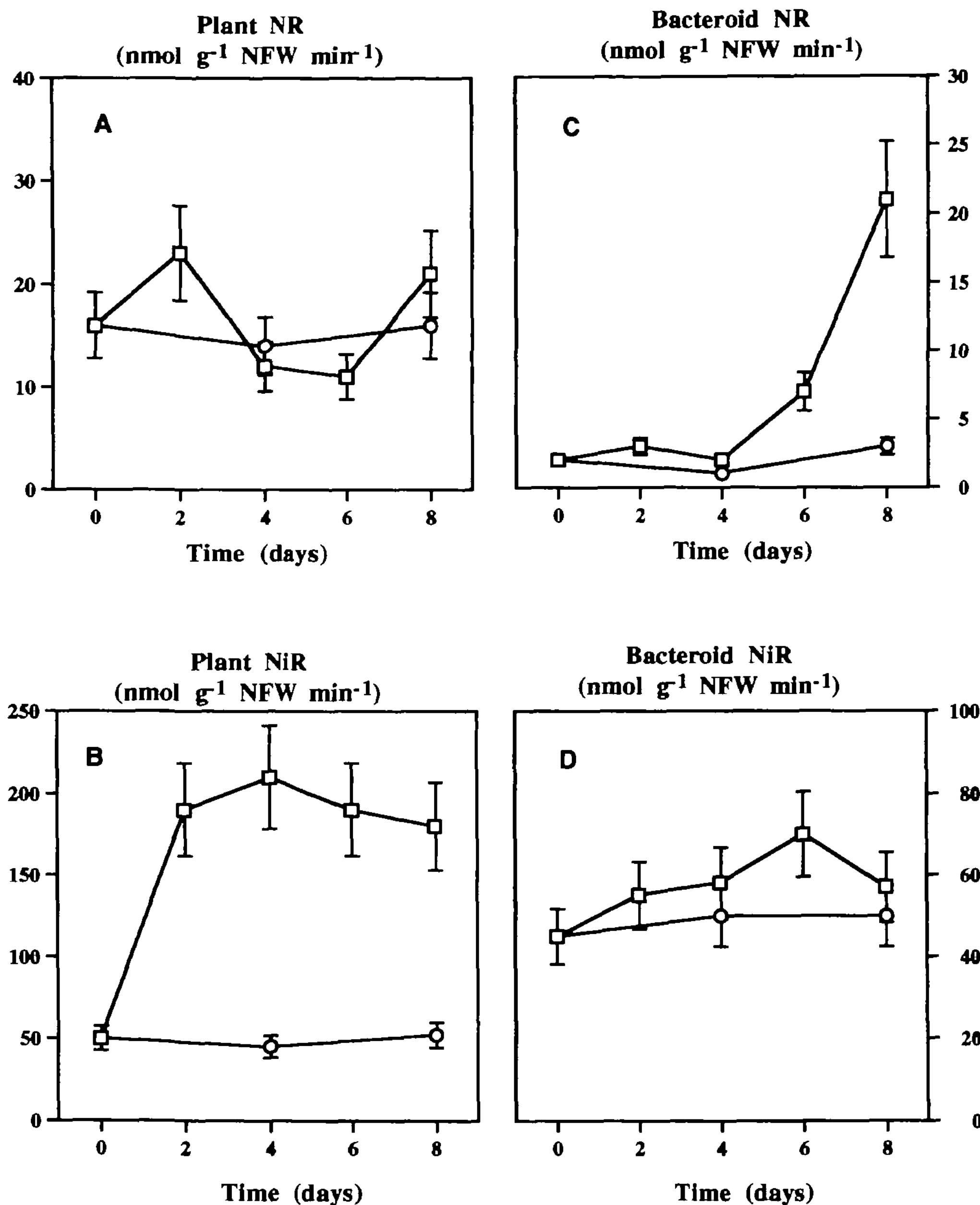

Fig. 4. Nitrate effects on inorganic nitrogen metabolism enzymes of soybean nodules. (A) plant NR, (B) plant NiR, (C) bacteroid NR, (D) bacteroid NiR. Activities are expressed on a nodule fresh weight basis. (□) Denotes nitrate-treated plants and (O) their corresponding controls. Results are mean ±SE ($n=3$).

this activity when expressed on a whole plant basis (Streeter, 1988). In our experimental conditions, nitrate decreased nodule mass (Table 1) which, therefore, contributed to a decrease in nitrogen fixation rates per plant. This result contrasted not only with KCl effects on nodules, suggesting that the regulatory mechanism is other than an osmotically-related response, but also to the overall nitrate effect on the plants. Thus, nitrate supply had a substantial effect on carbohydrate partitioning leading to enhanced root development providing an increased absorption surface. However, in the short-term, this had no beneficial effect on the shoot and whole plant biomass in this symbiosis (Table 1). However, caution should be used in the interpretation of this result, as growth will depend on the effectiveness of the symbiosis, the genetic capability of the plant in terms of nitrate reduction and the extent of the adverse effect of nitrate on nitrogen fixation.

To eliminate these nitrate effects on nodule development and to account only for the effects on nitrogen fixation, this was expressed on a nodule DW basis (Fig. 1A). Nitrogen fixation by control and KCl-treated nodules

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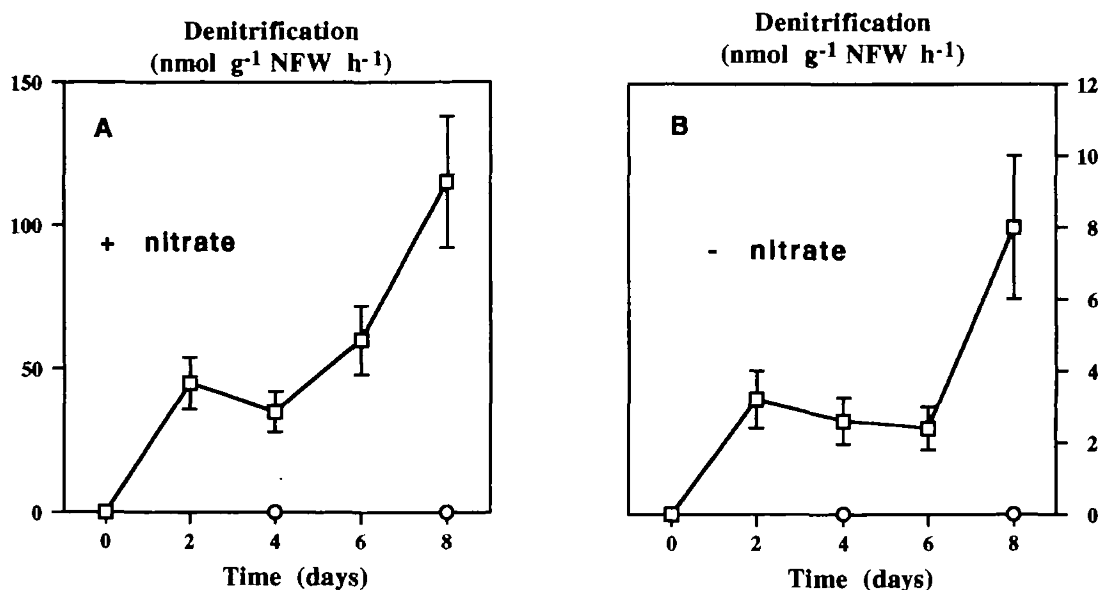

Fig. 5. Denitrification activity by whole soybean nodules. (A) Maximum potential for denitrification and (B) denitrification rates by nodules dependent on internal nitrate concentrations. (□) Denotes nitrate-treated plants and (O) their corresponding controls. Results are mean ±SE (n =3).

was constant throughout the 8 d study period, showing that any osmotic effects, at this osmoticum concentration, are mainly on nodule formation and development rather than on nodule functioning. Conversely, nitrate sharply affected nitrogen fixation when measured as acetylene reduction. This was further confirmed by the reduced ureide levels of xylem sap (Fig. 1B) which is considered to be a reliable index of nitrogen fixation in those species transporting allantoin and allantoic acid in the xylem (Peoples *et al.*, 1985; Sprent and Sprent, 1990).

This decrease in nitrogen fixation was matched by several changes in metabolic and gas permeability characteristics of these nodules. Thus, carbon costs of nitrogen fixation increased with nitrate supply (Fig. 3B). This event has been previously explained by a direct damage to the nitrogenase complex (Witty *et al.*, 1983). Indeed, in free-living nitrogen-fixing organisms the presence of nitrate causes a decay in nitrogenase mRNA within a few hours (Sprent and Sprent, 1990). However, Noel *et al.* (1982) reported that nitrogenase of soybean nodules was not degraded relative to other bacteroid proteins by up to 6 d exposure of plants to 5, 10 or 20 mol m^{-3} nitrate. In the present study, nitrogenase protein levels (Fig. 2) remained largely unchanged during the 8 d measurement period, and were still present after 24 d of nitrate supply. This strongly suggests that nitrate does not cause degradation of the nitrogenase components in the short term in soybean, and the observed decline in nitrogen fixation must be due to other mechanisms. Also, nitrate treatments had little effect on the content of other soybean nodule proteins, including Lb (Schuller *et al.*, 1986; Table 2 of this study). Indeed, from the present data, the nitrogen-fixation decline was closely correlated only with decreases in nitrogenase-linked respiration (Fig. 3A) and with increases in oxygen diffusion resistance (Fig. 3C) and carbon costs of nitrogen fixation (Fig. 3B).

In order to identify a metabolic pathway which could be involved in the decline of nitrogenase activity we looked at inorganic nitrogen metabolism in nodules. The role of this in the nitrate response has been highly controversial (Becana and Sprent, 1987; Streeter, 1988). Apart from some enhancement in plant NiR activity and a very slow response of bacteroid NR (Fig. 4), enzymes of inorganic nitrogen metabolism seemed to be very little influenced by nitrate supply. This lack of activity enhancement is in agreement with the hypothesis of a restricted access of nitrate to the infected region of determinate nodules (Sprent *et al.*, 1987; Giannakis *et al.*, 1988) as opposed to indeterminate nodules (Arrese-Igor *et al.*, 1992). However, this conclusion is not supported by the substantial bacteroid NiR detected in our experimental conditions. To reconcile these apparent contradictory results, obtained by *in vitro* assays, denitrification was monitored as an indicator of nitrate metabolism within the infected region of the nodules, as it has been shown that denitrification may be used for assessing dissimilatory nitrate metabolism in legume nodules (Arrese-Igor *et al.*, 1992). While no denitrification could be detected, either in the presence or absence of added nitrate, with nodules of control plants, denitrification was detected within 2 d of nitrate supply (Fig. 5A). This shows the presence of nitrate within the nodules and confirms nitrate metabolism in the infected region within 2 d of nitrate supply (Fig. 5B). These results have been further confirmed by direct nitrate measurements in the infected region (Arrese-Igor *et al.*, unpublished results). The low rates of nitrate

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metabolism in these nodules gives little support to the nitrite hypothesis of nitrogen fixation inhibition (Rigaud, 1976). In addition, the presence of NiR activity of *c*.10-fold greater rates than that of NR suggests that any nitrite produced in the infected region could be rapidly reduced. An alternative possibility is the proposal for a direct effect of nitrate on nitrogen fixation through membrane potential dissipation which could interfere with the transport of dicarboxylic acids across the peribacteroid membrane (Udvardi and Day, 1989; Vassileva and Ignatov, 1996). Another possibility could be a direct effect of nitrate on carbohydrate metabolism. A key role for sucrose synthase (SS) in nodule metabolism has recently been proposed (González *et al.*, 1995) and SS activity of the present soybean symbiosis is reduced following exposure to 10 mol m^{-3} nitrate (Gordon, unpublished data).

Minchin *et al.* (1989) hypothesized two stages for the nitrate effect; an initial stage where nitrate was more-or-less excluded from the infected region and induced indirect effects on nitrogenase activity and a second stage where nitrate entered the infected region and induced irreversible senescence through the production of nitrite. The present data do not support either possibility for soybean. Nitrate entered the nodules within 2 d, but appeared to have little direct effect on the biochemical machinery, in that the amounts of Lb and proteins (including nitrogenase) were largely unchanged for several days (Table 2; Fig. 2). However, nitrate itself could contribute to the decline in nitrogen fixation through other effects, as discussed above, and these effects could account for the apparent decrease in carbon use efficiency (increased carbon costs of nitrogen fixation) and the overall decrease in nitrogenase-linked respiration. In this sequence of events, changes in oxygen diffusion resistance could be a secondary response to metabolic changes, in a similar manner to that recently proposed for the effects of water stress and low temperature (Diaz del Castillo and Layzell, 1995; Kuzma *et al.*, 1995). It also appears from both the present data and previous publications (Streeter, 1985; Schuller *et al.*, 1986) that soybean nodules do not enter into a second stage of nitrate-induced senescence, in terms of irreversible protein breakdown; although Schuller *et al.* (1986) reported a loss of nitrogenase activity by isolated bacteroids after 7 d plant exposure to 10 mol m^{-3} nitrate. The mechanisms by which soybean nodules apparently avoid nitrate-induced senescence, in contrast to many other legume symbioses (Becana and Sprent, 1987), have yet to be determined.

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