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Effects of Nitrate on Accumulation of Trehalose and other Carbohydrates and on Trehalase Activity in Soybean Root Nodules

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Summary

Soybean (*Glycine max* cv. Maple Arrow) plants were infected with *Bradyrhizobium japonicum* (strain 61-A-101), grown in sterilized Leonard jars, and exposed to various amounts of nitrate either from the beginning or after completion of nodulation. The presence of 5 mM and more nitrate during nodulation caused a considerable reduction of the number and biomass of nodules per plant, and of nitrogenase activity per nodule fresh weight. The carbohydrate content of nodules was determined on a dry weight basis. The level of the disaccharide trehalose, produced by the microsymbiont, was 50 % lower in nodules formed in the presence of 20 mM nitrate than in control nodules formed in its absence. With regard to the non-structural carbohydrates produced by the plant, nodules formed in the presence of high amounts of nitrate contained about 75 % less starch but three- to fourfold higher levels of sucrose and pinitol than control nodules. Sucrose was the most abundant non-structural carbohydrate in nodules formed in the presence of 20 mM nitrate, accounting for 4–5 % of the dry weight. When plants with fully established nodules, grown in the absence of nitrate, were shifted to 20 mM nitrate, the levels of trehalose and starch decreased over a period of 3 weeks while the level of sucrose increased, until the carbohydrate levels attained similar values as found in nodules established in the presence of nitrate. The activity of trehalase, an enzyme known to be induced in nodules, was about 75 % lower in nodules formed in the presence of nitrate than in control nodules. However, trehalase activity did not change in established nodules during a 3-week exposure to 20 mM nitrate. Similarly, the number of colony- forming bacteria recovered from the nodules and the activities of endochitinase and endoglucanase, two plant defense hydrolases, were not affected during a 3-week exposure to nitrate.

Key words: Bradyrhizobium, carbohydrate metabolism, nitrate inhibition, nodules, nodulins, soybean, symbiosis, trehalase.

Abbreviations: ARA = acetylene reduction acitivy (assay for nitrogenase); B. = Bradyrhizobium; CFU = colony forming units; dw = dry weight; fw = fresh weight; MES = 2-(N-morpholino)ethanesulfonic acid; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; TBS = Tris buffered saline; Tris = tris(hydroxymethyl)aminomethane.

Introduction

The depressive effect of nitrate on root nodule growth has been known for nearly a century (Fred and Graul, 1916). The mechanisms involved in the reduction of nodule growth and inhibition of nitrogenase activity are still unclear (Vessey and Waterer, 1992), although it has become apparent that a shoot-derived factor plays an important role in nitrate-mediated repression of nodule initiation (Gresshoff and Caetano-Anollès, 1992). Nitrate, as a major regulator of nodule

growth and nitrogenase function, may also influence carbohydrate partitioning in nodules.

Here, we present our investigations concerning some aspects of carbohydrate accumulation and metabolism in nodules exposed to nitrate. Special attention is paid to the non-reducing disaccharide trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside), a common sugar of procaryotes and of many heterotrophic eucaryotes, especially fungi and invertebrates (Elbein, 1974). Trehalose has been found in nitrogen-fixing nodules where it can be one of the major carbohydrates; it is primarily located in the bacteroids (Streeter, 1987). Interestingly, nodules contain large quantities of trehalase (Streeter, 1982; Kinnback and Werner, 1991). Upon nodule fractionation, this nodule-stimulated enzyme was found to be absent from the bacteroids (Mellor, 1988). Recently, we partially purified and characterized trehalase from soybean root nodules and found that it is a glycoprotein (Müller et al., 1992), which is in all likelihood formed by the plant.

We investigated the influence of nitrate on the pools of trehalose and other carbohydrates as well as on trehalase activity in soybean nodules. Moreover, we checked whether nitrate affects the survival of bacteroids after extraction from nodules and whether it has an influence on the activities of endochitinase and endoglucanase, two enzymes characteristic of the plant's defense response (Boller, 1988).

Materials and Methods

Plant material

Seeds of soybean (*Glycine max* L. Merr.) cv. Maple Arrow were surface-sterilized by immersion in 30% H_2O_2 for 20 min followed by washing with sterile tap-water. They were grown for 4 days on plates containing 1% agar in half-concentrated nutrient solution (Werner et al., 1975). Then, the seedlings were grown in sterilized Leonard jars filled with perlite and nutrient solution (Werner et al., 1975) in a phytotron (14-h day at 300 μmol $m^{-2} s^{-1}$ and 26 °C, 10-h night at 20 °C). About 1 week after planting, the plantlets were infected with *Bradyrhizobium japonicum* 61-A-101, a strain yielding effective, nitrogen-fixing nodules (Stripf and Werner, 1978). The bacteria had been grown to stationary phase in 20E-medium (Werner et al., 1975). Nodules were harvested when indicated and used fresh or immediately frozen and stored at −20 °C.

Where indicated, the nutrient solution was amended with potassium nitrate. The nitrate remaining in the medium was assayed by spectroscopy at 230 nm.

Carbohydrate analysis

Carbohydrates were analyzed as described previously (Avigad, 1990; Schubert et al., 1992). Lyophilized aliquots of the harvested nodules (5–10 mg dw) were ground in methanol (80% v/v; 50 mL g^{-1} dw) containing 1% insoluble polyvinyl-pyrrolidone (Polyclar AT, SERVA, Heidelberg, Germany) and mannoheptulose (50 μg/sample) as internal standard. The homogenized samples were incubated at 60 °C during 10 min followed by centrifugation (13,000 × g, 10 min). The extraction was repeated three times. The supernatants were collected and vacuum-dried. The pellets were resuspended in 600 μL distilled water.

Charged compounds were removed with a mixed-bed ion-exchanger (Serdolit micro blue and red 2:1 (v/v), SERVA, Heidelberg, Germany). After adding 50 μL of the wet ion-exchange mix, the samples were vortexed and centrifuged (13,000 × g, 5 min). The supernatants were lyophilized, redissolved in methanol (50% v/v) and transferred to gas chromatography (GC) vials. The solvents were removed by vacuum-drying. Water was completely removed by adding twice pure methanol followed by vacuum-drying. The vials were tightly closed with a teflon-lined silicon septum (Varian, Sunnyvale, Cal., USA), and 50 μL pyridine containing 625 μg hydroxylamine and 50 μg phenyl-β-glucopyranoside (derivatization standard) were added to each sample by injection through the septum. The vials were incubated for 30 min at 80 °C. For the derivatization, 50 μL N-methyl-N-trimethylsilyl-heptafluoro-butyramide containing 1% (v/v) trimethyl-chlorsilane were added. The vials were incubated for 30 min at 80 °C.

Gas chromatography of the derivatized carbohydrates was performed on a gas chromatograph (GC-14A), Shimadzu, Kyoto, Japan) with a column (6 mm × 2 m) containing chromosorb W-HP coated with 5% OV-17 (Supelco, Gland, Switzer-land). The injections (4 μL) were done using an auto-injector and autosampler (models AOC 14 and AOC 1400, Shimadzu, Kyoto, Japan). The silylated carbohydrates were separated using a linear temperature gradient from 70 °C to 300 °C. One run was performed in 40 min. Carbohydrates were quantified using a flame ionization detector by comparison of the peak areas with those of standard carbohydrates and the internal standard.

Starch in the lyophilized nodules was solubilized with dimethylsulfoxide and quantified after digestion with glucoamylase (Boehringer, Mannheim, Germany) by measuring the glucose released, using the glucose-peroxidase method described below for trehalase measurements (Carpita and Kanabus, 1987).

Enzyme assays and protein determination

Crude enzyme extracts were obtained by grinding frozen soybean nodules in ice-cold 0.1 M MES (K^+), pH 6.3 (2 mL g^{-1} fw) containing 1 mM phenylmethyl-sulfonylfluoride, 2 mM ethylenediaminetetraacetic acid and insoluble polyvinylpyrrolidone (10 g kg^{-1} fw). The homogenate was centrifuged (13,000 × g, 10 min). The supernatant was used for the enzyme activity assays.

Trehalase activity was determined according to Müller et al. (1992). The reaction mixture contained 10 mM trehalose and 50 mM MES (K^+), pH 6.3, and was incubated at 37 °C for 30 min. The reaction was stopped by boiling for 2 min, and the glucose released was measured with the glucose oxidase-peroxidase method, using a test kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

Endochitinase was assayed radiometrically at pH 5 (50 mM acetate (Na^+)) using [^{3}H]chitin as substrate (Boller et al., 1983).

Endoglucanase assays were performed essentially according to Mauch et al. (1984). The reaction mixture contained 0.2% (w/v) laminarin reduced with borohydride and 50 mM acetate (Na^+), pH 5. After incubation for 20 min at 37 °C, the reducing oligosaccharides were assayed with the neocuproin method (Mauch et al., 1984).

To estimate nitrogenase activity, the acetylene reduction activity (ARA) of nodulated roots was determined. Nodulated roots were put into a flask of known volume containing an atmosphere of 10% v/v acetylene. Gas samples (1 mL) were taken at different time points and their ethylene content was assayed using a gas chromatograph (a model GC-MINI 3, Shimadzu, Kyoto, Japan) according to standard procedures (Turner and Gibson, 1980). The ARA was expressed in terms of nodule fresh weight, determined by harvesting and weighing all nodules at the end of the experiment.

Protein was assayed according to Bradford (1976).

Table 1: Nodulation of soybean plants in Leonard Jars containing various amounts of nitrate. The nodules were harvested 4 weeks after infecting the plants with *B. japonicum*. The mean values and standard deviation are given for three (six in the case of 0 and 20 mM nitrate) independent replicates.

Nitrate in the medium (mM)	Nodule number per plant	Nodule biomass per plant (mg fw)
0	124±31	670±216
1	80±42	523±230
3	118±14	533±240
5	72±30	380± 76
10	46±30	120± 76
20	21±10	21± 15

Preparation of antiserum specific for soluble plant nodulins

An antiserum specific for nodule-specific, soluble proteins was prepared according to published procedures (Legocki and Verma, 1980). Briefly, nodules were obtained as described above and harvested 4 weeks after infection. They were pulverized with liquid nitrogen and transferred to an ice-cold buffer containing 50 mM Tris (Cl^-), pH 8.7, 20 mM KCl and 10 mM $MgCl_2$. Following removal of cell debris and bacteroids by centrifugation at 20,000 × g for 20 min, the supernatant was centrifuged at 105,000 × g for 2 h. The remaining supernatant was used to immunize a rabbit according to the schedule of Legocki and Verma (1980), using 1 mg of soluble nodule proteins per injection followed by bleeding 2 weeks later.

Antiserum specific for soluble nodule proteins was prepared by incubating the serum with increasing amounts of cytoplasmic proteins from uninfected roots at 28 °C for 30 min, followed by 4 °C overnight and centrifugation (13,000 × g, 15 min, 4 °C) to remove precipitates. The reaction was performed with 200 µL aliquots of the serum, and the following amounts of concentrated soluble root proteins (80 g L^{-1}) were added in sequence: 4, 8, 15, 30, 80, and 200 µL in the antiserum/antigen range of 50:1, 25:1, 15:1, 7:1, 3:1 and 1:1. In order to remove antibodies directed against bacteria and to render the serum specific for soluble nodulins produced by the plants, the antiserum prepared as described above (200 µL) was incubated with acetone powder of *B. japonicum* 61-A-101 (10 mg dw, prepared by mixing a stationary culture of the bacterium with four volumes acetone, followed by centrifugation and drying of the resulting pellet).

Immune blots

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % gels according to Laemmli (1970) and transferred electrophoretically onto nitrocellulose membranes (Towbin et al., 1979). The membranes were processed for immune blots as previously described (Staehelin et al., 1992). Briefly, they were blocked with 5 % (w/v) nonfat dry milk in TBS, incubated overnight with the substracted antiserum in TBS containing 5 % (w/v) dry milk (1:200 dilution), washed repeatedly, and then incubated with a goat-anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Biorad, Richmond, Ca., USA). The blots were developed with 4-chloro-1-naphthol and H_2O_2 as specified by the supplier (Biorad).

Quantitation of living bacteria in nodules

Freshly harvested nodules were surface-sterilized by immersion in 70 % (v/v) ethanol for 5 min. They were washed twice in sterile TBS buffer containing 0.2 M mannitol and homogenized in the same buffer. After convenient dilution of the resulting suspension, aliquots were streaked out on agar plates containing 2 % agar and a modified 20E-medium containing no glycerol, but 4 g L^{-1} yeast extract (instead of 2 g L^{-1}) and 20 mM mannitol (Vincent, 1970). As the strain *B. japonicum* 61-A-101 was highly resistant against tetracyclin and ampicillin (minimal inhibitory concentration ≫ 100 ppm) and weakly resistant against nalidixic acid (minimal inhibitory concentration > 50 ppm), the medium was supplied with tetracyclin, ampicillin (50 ppm each), and nalidixic acid (10 ppm). Cycloheximide (50 ppm) was added to inhibit fungal growth.

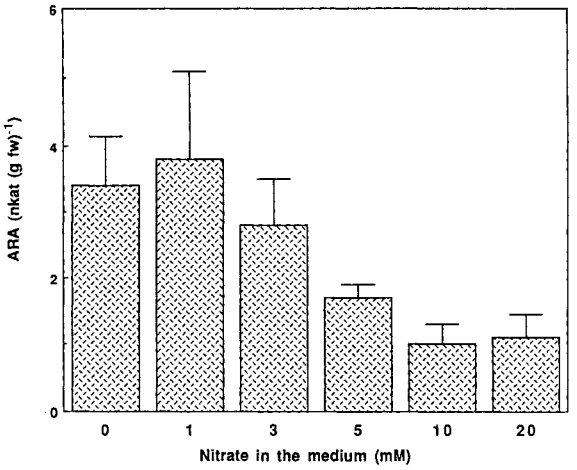

Fig. 1: Nitrogenase activity in whole soybean nodules formed in the presence of increasing concentrations of nitrate. The mean values and standard deviations are given for three or six (in the case of 0 and 20 mM nitrate) independent replicates. The nitrogenase activity was determined as acetylene reduction activity (ARA).

Results

Influence of nitrate on nodule formation

Soybean (*G. max* cv. Maple Arrow) plants were exposed to *B. japonicum* 61-A-101 and nodulated in Leonard jars containing a nutrient solution without nitrate or with nitrate concentrations ranging between 1 and 20 mM. Nodules were harvested about 1 month after infection. The presence of nitrate affected biomass and number of nodules per plant at concentrations of 5 mM or higher (Table 1). On the basis of nodule fresh weight, nitrogenase activity was lower in nodules formed in the presence of nitrate at 5 mM or higher, compared with control nodules formed in its absence. Nodules formed in the presence of 20 mM nitrate had still about 30 % of the nitrogenase activity (assayed as ARA) found in nodules grown without nitrate (Fig. 1). The nitrate concentration of the medium at the end of the experiment was very close to the initial concentration.

Influence of nitrate on carbohydrate pools and trehalase activity

Soluble carbohydrates extracted from nodules grown in the presence of various amounts of nitrate in the medium were analyzed by gas chromatography (Fig. 2). The amount of the bacterial disaccharide trehalose was about 1.6 % of the

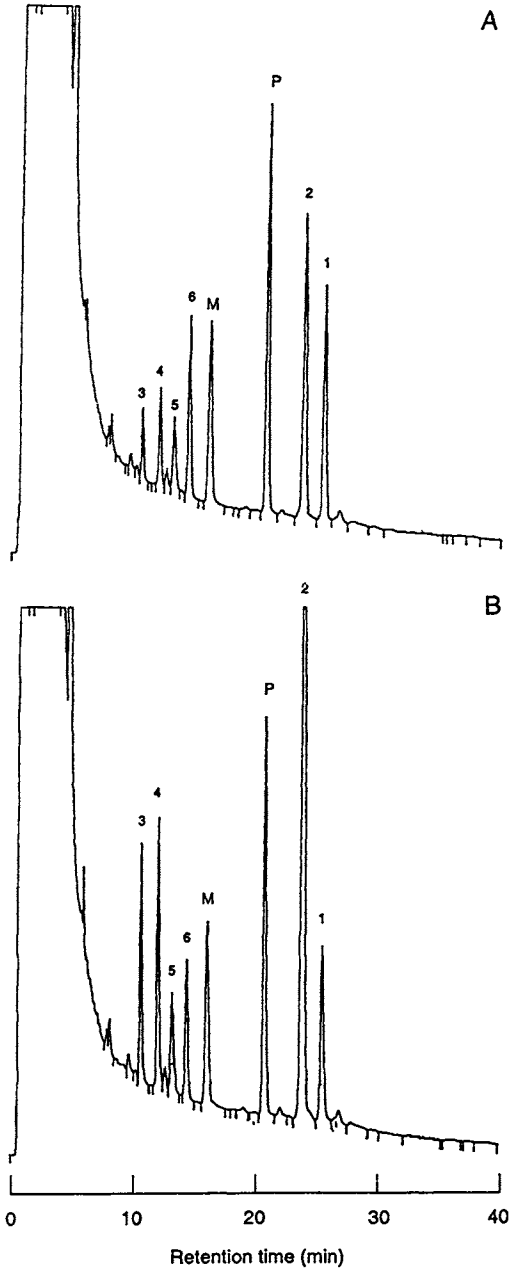

Fig. 2: Typical gas chromatograms of the silylated derivatives of soluble, neutral carbohydrates extracted from soybean nodules 4 weeks after infecting the plants with *B. japonicum* 61-A-101. A. Carbohydrates from nodules (6.6 mg dw) formed on a medium without nitrate. B. Carbohydrates from nodules (7.8 mg dw) formed on a medium containing 20 mM nitrate. M = mannoheptulose (internal standard), P = phenyl-glucopyranoside (derivatization standard), 1 = trehalose, 2 = sucrose, 3 = pinitol, 4 = fructose, 5 = glucose, 6 = inositol.

nodule dry weight in nodules grown without nitrate in the medium. It was only slightly lower in nodules grown in the presence of nitrate. At the highest nitrate concentration tested, namely 20 mM, it was about 50 % of the control level (Fig. 3). The pools of the plant-borne carbohydrates starch, sucrose and pinitol were more affected by nitrate than trehalose. In nodules formed in the presence of 20 mM nitrate, starch was 75 % lower than in control nodules, whereas sucrose and pinitol were more than threefold higher. Glucose, fructose and inositol had pool sizes smaller than 10 mg $(g\,dw)^{-1}$ in all nodules. Glucose was not affected by nitrate. Inositol was lower and fructose was higher in nodules formed in the presence of nitrate than in control nodules (Fig. 4). Nitrate affected the level of trehalase activity in nodules when supplied at concentrations of 10 mM and higher. Nodules formed in the presence of 20 mM nitrate had about 25 % of the trehalase activity of control nodules (Fig. 3).

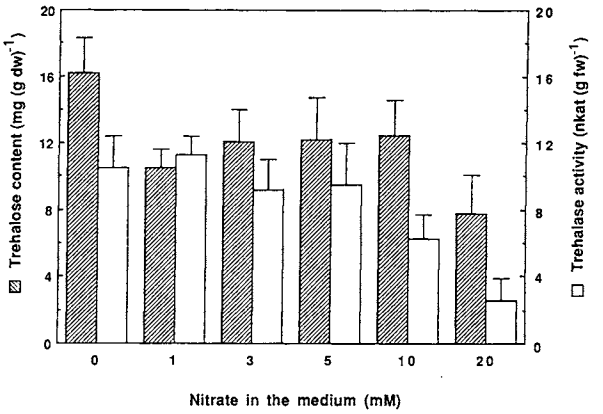

Fig. 3: Trehalose content and trehalase activity of soybean root nodules formed in the presence of various amounts of nitrate. The trehalase assay was performed at 37 °C (pH 6.3). The mean values and standard deviations are given for three or six (in the case of 0 and 20 mM nitrate) independent replicates.

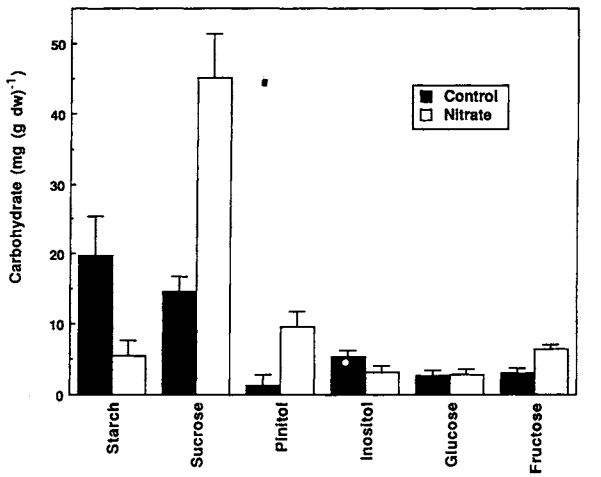

Fig. 4: Starch and soluble carbohydrates in soybean root nodules formed in the absence or presence of nitrate. The culture medium contained no nitrate (control) or 20 mM KNO_3 (nitrate). The mean values and standard deviations are given for six independent replicates.

Carbohydrate pools and trehalase activity in established nodules exposed to nitrate

To treat well-established nodules with nitrate, soybean plants were nodulated and grown for 4 weeks in the absence

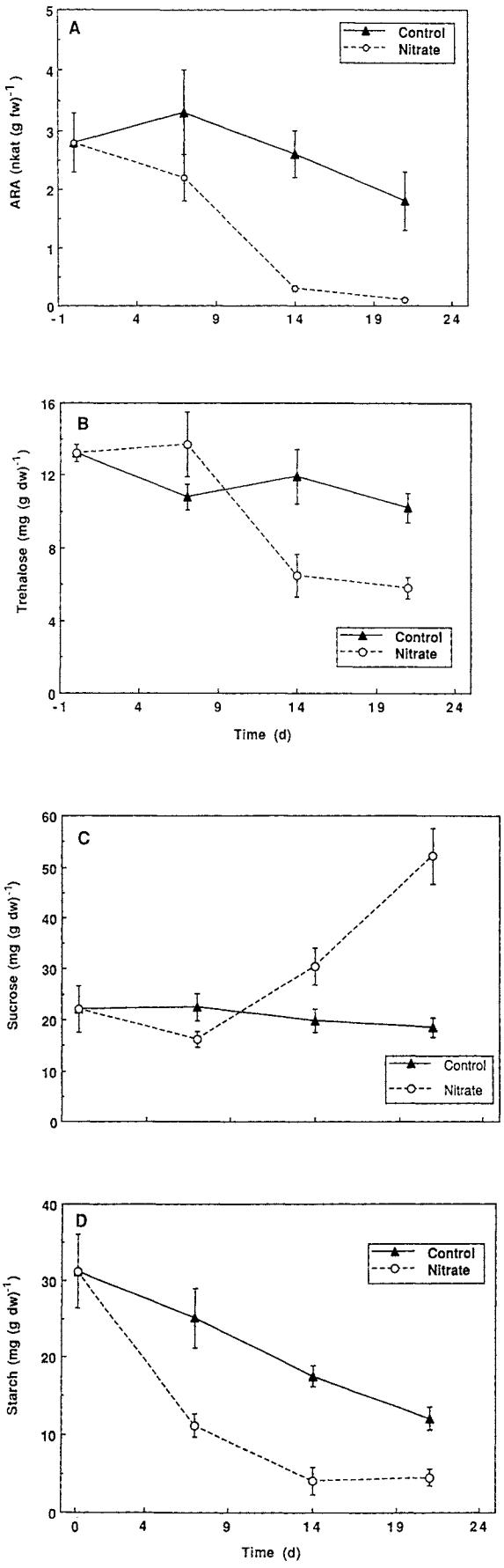

Fig. 5: Nitrogenase activity and major carbohydrates in established soybean root nodules during incubation of the plants in the absence (control) or presence of nitrate (nitrate). Soybean plants were infected with *B. japonicum* and grown for 4 weeks in the absence of nitrate. Then the plants, having well-established nodules, were shifted to fresh medium with 20 mM KCl (control) or 20 mM KNO_3 (nitrate). Nodules were harvested after various times of incubation. Where indicated, the standard deviation corresponds to three independent treatments. A. Nitrogenase activity, estimated as acetylene reduction activity (ARA). B. Trehalose, assayed by gas chromatography. C. Sucrose, assayed by gas chromatography. D. Starch, measured enzymatically.

Table 2: Trehalase activity in established soybean root nodules during incubation of the plants in the absence or presence of nitrate (20 mM). Growth conditions as in the experiments described in Fig. 6. Where indicated, the standard deviation corresponds to three independent treatments.

Time (d)	Trehalase (nkat mg prot.$^{-1}$)	
	control	nitrate
0	0.9	
7	0.8	0.9±0.1
14	0.8	1.0±0.1
21	0.9	1.0±0.1

of nitrate in the nutrient solution. Then, the nutrient solution was replaced by a fresh one containing 20 mM KNO_3. Controls received a fresh medium with 20 mM KCl instead. At various times after addition of the fresh medium, nodules were harvested and analyzed.

The nitrogenase activity remained at high levels 7 days after addition of nitrate but decreased to background values after more than 2 weeks on nitrate (Fig. 5 A). In the control nodules, the nitrogenase activity remained stable (Fig. 5 A). The amount of starch in the nodules treated with nitrate was strongly reduced already after the first week, whereas it decreased only slightly in control nodules (Fig. 5 D). In parallel to the decay of nitrogenase activity, the amount of sucrose increased (Fig. 5 C) and the amount of trehalose decreased (Fig. 5) in the nodules exposed to nitrate starting 1 week after treatment. The respective pool sizes were close to those found after continuous application of nitrate (Figs. 3 and 4).

In contrast to the results obtained with nodules continuously grown on nitrate, trehalase activity was not affected by treating established nodules with nitrate (Table 2). The late nodulin leghemoglobin did not disappear either, as shown by an immune blot with antiserum specific for soluble nodule proteins (Fig. 6).

Activities of chitinase and glucanase and content of living bacteria in nodules treated with nitrate

Nitrate treatment might induce some kind of defense reactions analogous to that observed in nodules colonized by some ineffective strains (Staehelin et al., 1992). To check this hypothesis, we measured the activities of two defense-related

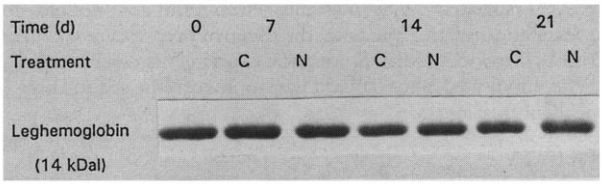

Fig. 6: Leghemoglobin in established soybean root nodules during incubation of the plants in the absence (control, C) or presence of nitrate (nitrate, N). Growth conditions as in the experiments described in Fig. 6. Nodules were harvested at the times indicated and extracted, and 20 µg total protein were loaded per lane on the SDS-PAGE gel. The proteins were blotted on a nitrocellulose membrane and treated with an antiserum specific for soluble plant nodulins. The figure represents the 14 kD band of the immune blot, representing leghemoglobin.

Table 3: Endochitinase and endoglucanase activities and number of living bacteria (as CFU) in soybean root nodules during incubation of the plants in the absence or presence of nitrate (20 mM). Growth conditions as in the experiment described in Fig. 6. Where indicated, the standard deviation corresponds to three independent treatments (n.d., not determined).

Time (d)	Endochitinase (nkat mg prot.$^{-1}$)		Endoglucanase (nkat mg prot.$^{-1}$)		Living bacteria (10^9 CFU g fw^{-1})	
	control	nitrate	control	nitrate	control	nitrate
0	0.13		1.8		n.d.	
7	0.21	0.20±0.06	1.0	1.3±0.3	5.8	3.0±1.0
14	0.16	0.15±0.01	1.3	1.6±0.1	2.0	2.5±0.5
21	0.21	0.22±0.07	1.2	1.3±0.1	6.6	8.5±2.0

proteins and the content of living bacteria in the nodules, using colony formation on a selective medium as an assay. Neither endochitinase nor endoglucanase activity was stimulated after nitrate treatment. Interestingly, the amount of living microsymbionts extracted from the nodules was not affected at all. Nodules from plants exposed for 3 weeks to 20 mM nitrate contained even slightly more colony-forming units than control nodules (Table 3).

Discussion

In the soybean cultivar Maple Arrow, nitrate reduced nodulation and nitrogen fixation at concentrations of 5 mM or higher. Results of similar experiments with the cultivar Bragg were very similar in our assay system (data not shown). The reduction of nodule number and biomass was lower than in a study published earlier where 5 mM were sufficient to cause 80 % inhibition of nodulation (Carrol et al., 1985). The discrepancy may be due to different experimental approaches. Another possibility is that the microsymbiont strains also play their role in nitrate tolerance. This aspect has been completely disregarded so far (Vessey and Waterer, 1992).

The bacterial disaccharide trehalose was a major carbohydrate in whole nodules. High amounts of trehalose may not only have a function as reserve carbohydrate, but also as protectant of enzymes and membranes (Crowe et al., 1984; Wiemken, 1990). As it has been found that 66 % of the nodule trehalose are located in the bacteroid (Streeter, 1987), the concentration in the bacterial cytosol must be very high. The decrease in the trehalose pool in the experiments reported above was quite parallel to the decay of nitrogenase activity. In all likelihood, it reflects a decrease of assimilate import in bacteroids (Quispel et al., 1985). This decrease can not be due to a general lack of assimilates in the nodule. In fact, nitrate causes an increase of the sucrose pool. This sucrose may come from the shoot or, more probably, from the mobilization of starch in nodules exposed to nitrate (also reported by Vessey et al., 1988). The decrease of respiration in nitrate treated nodules also plays in favour of an increase of the sucrose pool (Hansen et al., 1992).

On the bacterial side, the nitrate supplied may induce a bacterial nitrate reductase (Manhart and Wong, 1979; Becana and Sprent, 1987). As nitrate reduction consumes nearly as much energy as nitrogenase, the metabolic status of the bacteroids would not favour the accumulation of storage compounds.

From nodules exposed to nitrate, nearly as much colony-forming units of the microsymbionts could be isolated as from control nodules. Therefore, in all likelihood, the changes of metabolic activities in the symbiont upon nitrate treatment, i.e. breakdown of nitrogenase and the decrease in the trehalose pool, were not due to death and lysis of the bacteroids. Of course, the number of colony forming units may not match exactly the number of bacteroids inside the nodule. Some authors distinguish different bacteroid populations based on the change in buoyant density of bacteroids that occurs due to differences in the accumulation of polyhydroxy-butyrate granules (e.g. Ching et al., 1977). Analysis of gene expression in these subpopulations, however, revealed no significant differences (Adams and Chelm, 1988).

Recent data obtained with alfalfa suggest that ethylene may play a role in the nitrate inhibition of nodulation (Ligero et al., 1991). We examined the possibility that nitrate could induce the expression of defense-related proteins such as endochitinase or endoglucanase, which are often induced by ethylene (Boller, 1988). In fact, these enzymes are stimulated in soybean nodules colonized by some ineffective *B. japonicum* strains (Staehelin et al., 1992 and unpublished data). However, we found that the levels of these two enzymes were not affected after a treatment with nitrate.

Interestingly, when nitrate was continuously present during nodule development, the trehalase activity and the size of the trehalose pool were similarly reduced in comparison to control nodules developed in the absence of nitrate. This might suggest the hypothesis that trehalase levels are directly regulated by the availability of trehalose inside the nodule (Mellor, 1992). However, when nitrate was added to well-nodulated plants, the correlation between the two parameters was broken. The regulation of nodule trehalase activity may therefore depend on other factors than the mere amount of trehalose. In any case, the trehalase activity in nodules follows a pattern resembling that of common «late» nodulins like leghemoglobin or uricase (Kouchi et al., 1989). If developed nodules are exposed to nitrate, the amount of leghemoglobin as monitored by immune blots and of trehalase activity is not affected. This observation can be ex-

plained by two ways: (i) Nitrate may have no effect on the expression of late nodulins and nodule-stimulated proteins, and (ii) Nitrate, besides its other effects inhibits the expression of nodulins and nodule-stimulated proteins, but their turnover is so slow that their amount does not change even in nodules treated with nitrate for a long period. Further studies are needed to elucidate these points.

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