Agronomie (1990) 10, 847-855

Plant pathology

Effect of nitrate on acetylene reduction and the activities of some enzymes of nitrogen and carbon metabolism, involved in nitrogen fixation in the root nodules of soybean

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(Received 12 February 1990; accepted 9 October 1990)

Summary — Soybean plants (*Glycine max* (L) Merr) cv Maple arrow were nodulated either by *Bradyrhizobium japonicum* (USDA 311 b 138 or *B japonicum* CB 1809 and grown on low-N solution (20 μM nitrate). When the seedlings were 40 days old, treatments with 5 mM nitrate for 1-4 d were used in order to allow effects on acetylene reduction, bacteroid nitrate reductase (EC 1.6.6.1), nodule cytosol nitrate reductase, glutamine synthetase (EC 6.3.1.2) and phosphoenolpyruvate carboxylase (EC 4.1.1.31) to be studied in the absence of nodule senescence. Significant alterations of enzyme activities were only observed in nodules infected by *B japonicum* USDA 311 b 138. Thus infecting soybean plants with either *B japonicum* USDA 311 b 138 of *B japonicum* CB 1809 was a good tool for investigating the effects associated with N_2 fixation inhibition by comparing a high nitrate-sensitive and a low nitrate-sensitive symbiosis.

Phosphoenolpyruvate carboxylase and acetylene reduction activities were rapidly lowered while the bacteroid inducible nitrate reductase increased. In contrast, the nodule cytosolic glutamine synthetase and nitrate reductase activities slowly increased after the ARA had declined. These data suggest that the nodule cytosol phosphoenolpyruvate carboxylase and the bacteroid nitrogenase are the 2 main regulatory enzymes controlling C and N metabolism respectively, in symbiotic nodules. On the other hand, parallelism of nodule cytosol nitrate reductase and glutamine synthetase suggest that in these soybean nodules, the cytosolic glutamine synthetase was devoted to assimilation of the NH_4^+ product of nitrate assimilation.

***Bradyrhizobium japonicum* / cytosol / glutamine synthetase / nitrate reductase / phosphoenolpyruvate carboxylase**

Résumé — Rôle du nitrate sur la réduction de l'acétylène et les activités de quelques enzymes des métabolismes carboné et azoté impliqués dans la fixation de l'azote dans les nodules racinaires de soja. *Il est bien connu que l'application de nitrates à des cultures de soja en symbiose avec* Bradyrhizobium japonicum *altère l'activité nitrogénase responsable de la fixation de l'azote moléculaire, avec vieillissement prématuré des nodosités. La nutrition azotée des plantes passe alors de la symbiose à l'autotrophie. L'objet des recherches décrites dans le présent article est d'étudier les effets des nitrates comparativement sur la nitrogénase (mesurée par l'activité de réduction de l'acétylène ou ARA) et sur 2 enzymes qui lui sont associées dans les premières étapes de l'assimilation de l'azote, la phosphoénolpyruvate carboxylase (EC 4.1.1.31) et la glutamine synthétase (EC 6.3.1.2), toutes 2 localisées dans le cytosol du tissu végétal des nodosités. Les activités de la nitrate réductase des bactéroïdes et de la nitrate réductase (EC 1.6.6.1) du tissu végétal des nodosités sont également mesurées. Des plantes de soja (*Glycine max *L Merr) cv Maple arrow sont inoculées soit par* Bradyrhizobium japonicum *USDA 311 b 138, soit par* B japonicum *CB 1809. Ces 2 souches ont été choisies car, ayant des sensibilités différentes aux nitrates, elles permettent la comparaison de systèmes symbiotiques de niveaux de résistance distincts. Les plantes inoculées sont cultivées sur solution minérale sans N, mais contenant cependant les traces de nitrate de l'eau (20 μmol•l^{-1}). Le développement des plantes des des nodosités, l'activité fixatrice d'azote, la teneur en hémoglobine sont identiques, dans les 2 types d'association symbio-*

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Abbreviations: ARA, acetylene reduction activity; FAD, Flavin Adenine Dinucleotide (USDA 311 b 138) nodules, nodules infected by *Bradyrhizobium japonicum* USDA 311 b 138; (CB 1809) nodules, nodules infected by *Bradyrhizobium japonicum* CB 1809; GS, glutamine synthetase; NR, nitrate reductase; PEPcase, phosphoenolpyruvate carboxylase.

tique (tableau I). Les traitements par le nitrate (5 mmol·l^{-1}) sont appliqués pendant 1–4 j afin de permettre l'étude de leurs effets sur la réduction de l'acétylène, et les activités enzymatiques citées plus haut, indépendamment des phénomènes liés à la sénescence des nodosités. Une partie des nitrates absorbés par les racines pénètre dans les nodosités via la sève du xylème (fig 1). Une diminution rapide et significative de l'ARA n'est observée que dans les nodosités infectées par B japonicum *USDA 311 b 138 (fig 2). Ainsi, l'inoculation du soja soit avec* B japonicum *USDA 311 b 138, soit avec* B japonicum *CB 1809 est un bon outil pour l'étude des effets associés à l'inhibition de la fixation de N_2 avec comparaison de symbioses ayant différents degrés de sensibilité au nitrate. La diminution rapide de l'ARA est accompagnée chez* B japonicum *USDA 311 b 138 d'une augmentation de l'activité nitrate réductase des bactéroïdes et de l'activité phosphoénolpyruvate carboxylase du cytosol. Au contraire, la glutamine synthétase et la nitrate réductase du tissu végétal des nodosités n'augmentent que très lentement après le déclin de l'activité de réduction de l'acétylène (fig 3). Dans les nodosités inoculées par* B japonicum *CB 1809, aucune modification d'importance des activités enzymatiques étudiées, y compris l'ARA, ne se remarque (fig 4). Ces données suggèrent que la phosphoénolpyruvate carboxylase du cytosol des nodosités ainsi que la nitrogénase des bactéroïdes sont 2 enzymes majeures de la régulation respectivement du métabolisme du carbone et du métabolisme de l'azote, dans les nodosités en symbiose. Le parallélisme de l'évolution de la glutamine synthétase et de la nitrate réductase cytosoliques suggère que dans les nodosités de soja, le rôle de la glutamine synthétase cytosolique serait essentiellement associé à l'assimilation de NH_4^+, produit de l'assimilation de NO_3^-.*

Bradyrhizobium japonicum / ***cytosol / glutamine synthétase / nitrate réductase / phosphoénolpyruvate carboxylase***

INTRODUCTION

It has repeatedly been shown that nitrate applied to legumes at moderate and high concentrations (> 5 mM) promotes nodule senescence and results in a shift of the legume N metabolism from symbiotic to autotrophic (Chen and Phillips, 1977; Bauer, 1981). Although the mechanism whereby nitrate exerts this effect is not yet fully understood, the nitrate-induced senescence of nodules is normally seen as a loss of acetylene reduction activity which reflects the decrease in nitrogenase activity (Becana and Sprent, 1987). In nodules fixing atmospheric nitrogen, the products of nitrogenase must be linked to an enzyme system for further assimilation of the products of N_2 reduction which would be regulated according to the physiological requirement of the plant (Ratajaczak *et al,* 1979). Studies of the effects of nitrate on this enzyme system, at concentrations which affect nitrogenase, may throw some light on the interrelationship between the enzymes of symbiotic assimilation of dinitrogen and the regulation of the shift from atmospheric N-nutrition to soil fixed N-nutrition in roots of legumes.

This paper concerns 2 enzymes that may function in the initial step of assimilation in soybean root nodules: *ie* glutamine synthetase (GS, EC, 6.3.1.2.), the primary enzyme of the reduced N assimilation pathway (Meeks *et al,* 1978) and phosphoenolpyruvate carboxylase (PEPcase, EC 4.1.1.31), an enzyme of carbon metabolism which catalyzes the supply of carbon and energy to bacteroids (Deroche and Carrayol, 1988). Both enzymes are located in the cytosol of the plant part of the nodules.

It was shown in a preceding paper that the decrease in N_2 fixation activity caused by nitrate was sharper when inoculation of the soybean seedlings was with *Bradyrhizobium japonicum* USDA 311 b 138 than when it was with *Bradyrhizobium japonicum* CB 1809 (Champigny *et al,* 1985). The inoculation of soybean cv Maple arrow with either of the above cited *B japonicum* strains was used as a tool for the preparation of symbiotic associations of different sensitivity to the presence of nitrate in the nutrient solution. In addition to glutamine synthetase (GS) and phosphoenolpyruvate carboxylase (PEPcase), the activities of acetylene reduction (ARA), bacteroid nitrate reductase (EC 1.6.6.1.) (bacteroid NR), and plant nodule tissue nitrate reductase (cytosolic NR), were followed for 1 to 4 d of a 5 mM nitrate treatment of symbiotic plants previously developed for 40 d on low-N nutrient solution (20 μM nitrate).

MATERIAL AND METHODS

Biological material

Bradyrhizobium japonicum USDA 311 b 138 obtained from INRA (Dijon) and *Bradyrhizobium japonicum* CB 1809 obtained from La Faculté des Sciences et Techniques de Nice, were maintained at 4°C on slants of yeast extract–mannitol–agar (Robertson and Taylor, 1973). Before inoculation of soybean, the bradyrhizo-

bia were grown for 3 d at 30°C in a broth medium containing per l: 0.5 g K_2HPO_4, 0.2 g $MgSO_4$ 7 H_2O, 0.2 g NaCl, 0.1 g $CaCO_3$, 10 g mannitol, 500 mg yeast extract (Institut Pasteur). The pH was adjusted to 6.8 with KOH.

Seeds of soybean cv Maple arrow were obtained from McMaster University at Hamilton (Canada). After 2 d germination on moist filter paper, the root system of seedlings was 5-7 cm long. It was entirely soaked for 30 min in a fresh late-log-phase culture (200 ml at approximately 109 cells/ml) of either of the 2 *B japonicum* suspensions. Thereafter, the seedlings were planted in vermiculite and growth was in a greenhouse for 4–5 wk with watering every other day with water deionized by electro-dialysis (Millipore milli-Q reagent water), and still containing traces of NO_3^- (20 mM), followed by growth in hydroponic culture (2 seedlings for 3 l of solution). The low-N aerated nutrient solution contained in deionized water (NO_3^- = 20 µM): 5 mM K_2SO_4, 0.75 mM KH_2PO_4, 0.25 mM K_2HPO_4, 0.2 mM NaCl, 2.5 mM $CaCl_2$, 2.5 mM $MgSO_4$, 10 mg l^{-1} Fe-chelate and micronutrients. Solution pH was controlled at 6.3. The solution was changed every other day. Plants were given a 16 h photoperiod. Natural light was supplemented with light from HQI-TS 400 W bulbs (Osram) giving a total irradiance of 550 µmol PAR•m^{-2}•s^{-1}.

Treatment with nitrate, harvesting of plant material

Treatment with nitrate was performed in the greenhouse by replacing the low-N solution by a 5 mM NO_3^- solution (2.5 mM $Ca(NO_3)_2$ in place of 2.5 mM $CaCl_2$ of the low-N solution) at 9 am. Treatment was for 1 to 4 d under the greenhouse regime of light and temperature.

At intervals, acetylene reduction activity (ARA) was assayed on the intact root systems. Nodules were then picked from the roots, weighed and immediately processed for isolation of the bacteroids or stored at –70°C for later preparation of nodule tissue extracts in view of nitrate assay and enzyme analysis.

Acetylene reduction activity

Nitrogenase activity was estimated by the acetylene reduction technique (Hardy *et al,* 1968). The entire root system of intact plants was placed into gas tight tubes, fitted with a Terostat stopper. Acetylene was injected in amounts equivalent to 10% net volume of the tubes. After 30 min, 3 samples of 1 ml were withdrawn from each tube and immediately analyzed for ethylene with a gas chromatograph (Infotronics Corp, model 2400, series Graphmatic) using a Porapak N column. Mean values of 3 replicates are reported. ARA was calculated in µmol ethylene produced• h^{-1}•g nodule fresh wt^{-1}. It is known that ARA varies during the *in vitro* assay (Kalia and Drevon, 1985). In view of this drawback, studies were carried out to check that the lag period was very short and that ARA was already at its maximum less than 5 min after the beginning of incubation.

Preparation of bacteroids and nodule cytosol

Homogenates were obtained by grinding the nodules at 4°C under nitrogen in 100 mM phosphate (pH 7.5) buffer containing 0.3 M sucrose, 5 mM dithionite, 2% (w/v) polyvinylpyrrolidone 40, 1 mM cysteine, 20 µM FAD, 1 µM Na_2MoO_4, 10 µM EDTA, 10 µM leupeptin, 250 µM paramethylsulfonylfluoride. The brei was centrifuged at 2 000 x *g* for 2 min. The red supernatant after sedimentation of the bacteroids was used as the nodule cytosol preparation for nitrate reductase assays. After 2 washings in 100 µM phosphate (pH 6.8), 0.3 M sucrose buffer, the pellet was used as the bacteroid preparation (Stephens and Neyra, 1983).

Nodule tissue extracts for enzyme analysis

Nodule tissue extracts were prepared by grinding weighed nodules in a prechilled mortar in the presence of the following buffer solutions.

Nitrate reductase

100 mM phosphate (pH 7.5), 10 mM EDTA, 20 µM FAD, 1 µM Na_2MoO_4, 1 mM cysteine, 1 mM dithiothreitol, 10 µM leupeptin, 250 µM paramethylsulfonylfluoride, 1% bovine serum albumin.

Glutamine synthetase

Twenty-five mM imidazol (pH 7.6), 1 mM $MgCl_2$, 10 mM glutamate, 1 mM dithiothreitol, 1 mM EDTA, 10% (w/v) insoluble polyvinylpyrrolidone.

Phosphoenolpyruvate carboxylase

One hundred mM Tris–HCl (pH 8.25), 10 mM $NaHCO_3$, 2 mM mercaptoethanol, 1% (w/v) insoluble polyvinylpyrrolidone, 20% glycerol (v/v). The homogenates were filtered, centrifuged at 10 000 *g* for 20 min and supernatants used as tissue extracts.

Enzyme assays

Enzyme assays were all carried out at 30°C.

Glutamine synthetase

The activity was determined by a transferase assay as described by O'Neal and Joy (1973) and modified by Guiz *et al* (1979). The reaction mixture contained 50 mM Tris–HCl (pH 7.7), 2 mM $MgSO_4$, 8 mM L-[glutamate, 0.6 mM hydroxylamine, 0.4 mM $(NH_4)_2SO_4$, 0.4 mM EDTA, 0.8 mM ATP, and aliquots (100–200 µl) of the nodule tissue extract. γ-Glutamylhydroxamate formed was assayed colorimetrically at 540 nm. Enzyme activity was expressed in nmol γ-glutamylhydroxamate formed•min^{-1}•g fresh wt^{-1}.

Phosphoenolpyruvate carboxylase

The PEPcase activities of nodule tissue extracts were measured by following the oxidation of NADH at 340 nm in the presence of malate dehydrogenase (Lane *et al*, 1969). The reaction mixture contained 100 mM Tris–HCl (pH 8.3), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM phosphoenolpyruvate, 1 mM NADH, 6 U per ml of malate dehydrogenase (Sigma) and aliquots (20–100 µl) of nodule tissue extract. Enzyme activity was expressed in nmol NADH oxidized•min^{-1}•g fresh wt^{-1}.

Nitrate reductase

Nitrate reductase activities of nodule cytosol and bacteroid preparations were assayed as described by Streeter (1982). The assay mixture contained 50 mM phosphate buffer (pH 7.5), 10 µmM KNO_3, 0.5 mM EDTA. The reductant was either 1.4 µM NADH (nodule cytosol) or 10 mM succinate (bacteroid suspension) and aliquots (20–100 µl) of nodule cytosol or bacteroid preparation. Nitrite formed within 20 min was assayed colorimetrically at 540 nm (Hageman and Hucklesby, 1971). Enzyme activity was expressed in nmol NO_2^- formed• min^{-1}•g^{-1} fresh wt.

Each enzyme assay was performed at least in triplicate on preparations from separate seedlings. The enzyme activity tests were performed at least twice for each preparation.

Leghaemoglobin

Extracts prepared for enzyme assays were analyzed for leghaemoglobin by the cyanomethaemoglobin method (Wilson and Reisenauer, 1963), as modified by Schiffman and Löbel (1970).

Plant total N determination

The organic N contents in leaves, stems, roots and nodules were determined by the Nessler colorimetric assay as described by Umbreit *et al* (1964) after sulfuric digestion of the tissues, with ammonium oxalate as standard (0.36–2.86 µmol).

Nitrate assay

Nitrate was assayed in 0.1 ml of tissue extracts by nitration of salicylic acid, according to the colorimetric assay method reported by Cataldo *et al* (1975).

RESULTS

Nodule growth and symbiosis

The efficiency of the symbiosis resulting from infection of soybean cv Maple arrow by *B japonicum* USDA 311 b 138 or *B japonicum* CB 1809 were compared by the analysis of nodule growth and activity characteristics of nodules sampled from 40-d-old plants before addition of nitrate into the nutrient solution.

Nodule growth, measured by fresh weight, and number per plant at 40 d was greater when infected by *B japonicum* CB 1809 than by *B japonicum* USDA 311 b 138 (table I). Two features

Table I. Nodule development (number and mass per plant), leghaemoglobin content, acetylene reduction activity and total nitrogen fixed per plant during 40 days growth of nodules on soybean *(Glycine max)* cv Maple arrow, infected with *B japonicum* USDA 311 b 138 or *B japonicum* CB 1809.

B japonicum	*Nodules (No per plant)*	*Nodules (Fwt, mg per plant)*	*Leghaemoglobin (mg per g nodule Fwt)*	*ARA (µmol C_2H_4•h^{-1} per g nod Fwt)*	*Fixed (dinitrogen mg per plant)*
USDA 311 b 138	17	79.6	12.4	26.4	22.0
CB 1809	64	121.2	15.0	21	22.9

Fwt = fresh weight.

linked with nitrogen fixation, *ie* leghaemoglobin content and ARA per g fresh weight of nodule tissue were almost identical. Total nitrogen fixed per plant during 40 days of culture, calculated by substraction of the seed nitrogen content from the total seedling organic N showed no difference between plants inoculated with either *B japonicum* (table I). However, the capacity for N_2 fixation was higher in the plants infected with *B japonicum* CB 1809 which had reached the highest nodule development. A possible explanation for this deficient expression of N_2 fixation capacity could be the presence of a hydrogenase activity in *B japonicum* CB 1809 (Drevon *et al,* 1987).

Effects of 5 mM nitrate treatment

Nitrate content of nodules

The nitrate present in nodules (and also in root tissue; data not shown) of plants grown on 20 µM nitrate solution was ascribed to the high capacity of the roots to absorb nitrate, even when it was present at trace level in the medium, and to concentrate it in the root system tissues. Experiments indicate that NO_3^- experiences difficulty in access to the central part of the nodules (Becana *et al,* 1989). Nevertheless, the results presented here show that some of the NO_3^- absorbed after application of 5 mM nitrate was translocated to the root nodules (fig 1). Although similar amounts of nitrate were taken up by soybean inoculated by either strain of *B japonicum* (data not shown), less NO_3^- accumulated in the nodules infected with *B japonicum* USDA 311 b 138 (fig 1).

ARA

Decrease in ARA after one day of 5 mM nitrate application to well nodulated soyean plants was higher when inoculation was with *B japonicum* USDA 311 b 138 than with *B japonicum* CB 1809 (fig 2).

Bacteroid and nodule cytosolic nitrate reductase activities

Low nitrate reductase activities were observed before the nitrate application, probably due to the nitrate absorbed from the 20 µM nitrate solution (figs 3 and 4). Nitrate (5mM) increased the nitrate reductase activity of the bacteroids USDA 311 b 138 by a factor of 10 within the first 2 h of its application (fig 3A). The enhancement was 400% at midday, the time of the highest activity of the day (Hardy *et al,* 1968). A significant increase in the cytosol nitrate reductase activity of the same nodules appeared after only 24 h of the nitrate treatment (fig 3B).

Nitrate reductase of the bacteroids CB 1809 remained low throughout the experiment and was not affected by the presence of nitrate in the nodule tissue (fig 4A). The nitrate reductase of the corresponding nodule cytosol was significantly stimulated by nitrate after only 8 h of treatment (fig 4B).

Glutamine synthetase activity

During the 48 h experiment, the GS activity of non treated nodules slowly increased whether

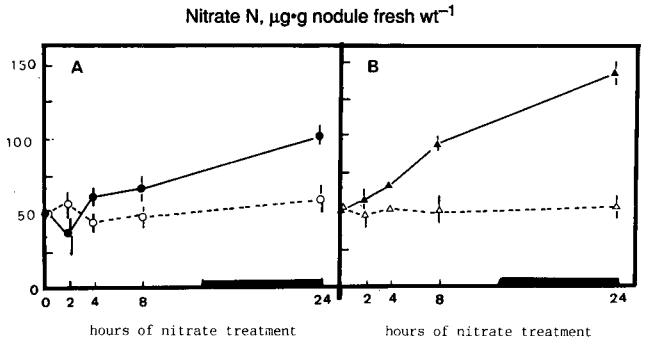

Fig 1. Nitrate content in nodule tissue of soybean cv Maple arrow inoculated with (A) *B japonicum* USDA 311 b 138, (B) *B japonicum* CB 1809 and grown on 20 µM nitrate. Effects of 24 h treatment with 5 mM nitrate. ——— 5 mM NO_3^-; – – – – 20 µM NO_3^-. Vertical bars represent the standard error of the mean of 3 replicates. Darkness and light are shown on the abscissa as black and white bars.

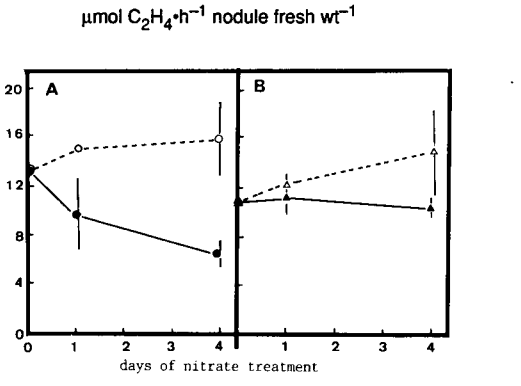

Fig 2. Acetylene reduction activity of soybean cv Maple arrow inoculated with (A) *B japonicum* USDA 311 b 138, (B) *B japonicum* CB 1809 grown on 20 µM nitrate. Effect of 1 and 4-d treatments with 5 mM nitrate. ——— 5 mM NO_3^-; – – – – 20 µM NO_3^-. Vertical bars represent the standard error of the mean of 3 replicates.

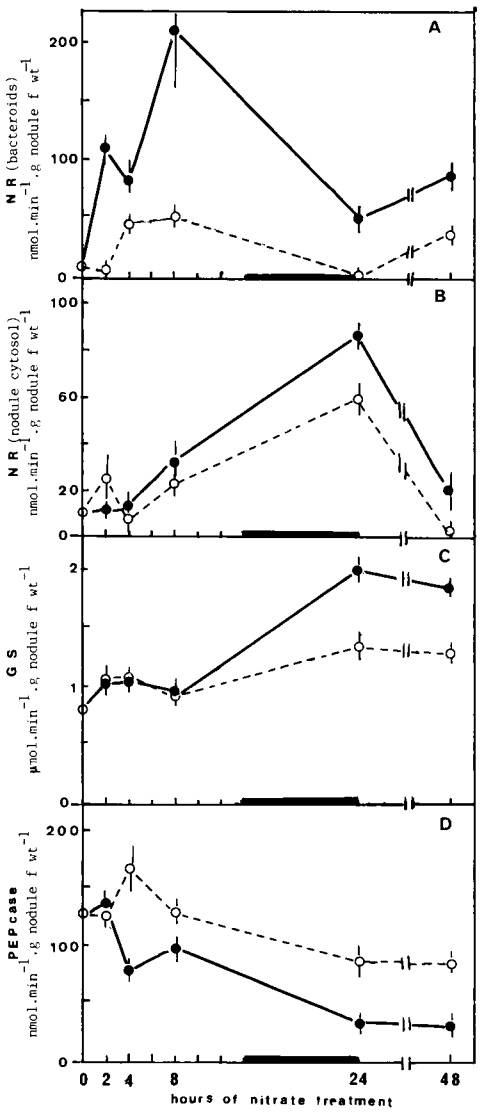

Fig 3. Soybean cv Maple arrow inoculated with *B japonicum* USDA 311 b 138 and grown on 20 µM nitrate. Effect of a 48-h treatment with 5 mM nitrate on (A) nitrate reductase activity of bacteroids, (B) nitrate reductase activity of nodule cytosol, (C) glutamine synthetase of nodule tissue, (D) phosphoenolpyruvate carboxylase of nodule tissue. Vertical bars represent the standard error of the mean of 3 replicates. ——— 5 mM NO_3^-; — — — 20 µM NO_3^-. Darkness and light are shown on the abscissa as black and white bars. Vertical bars represent the standard error of the mean of 3 replicates.

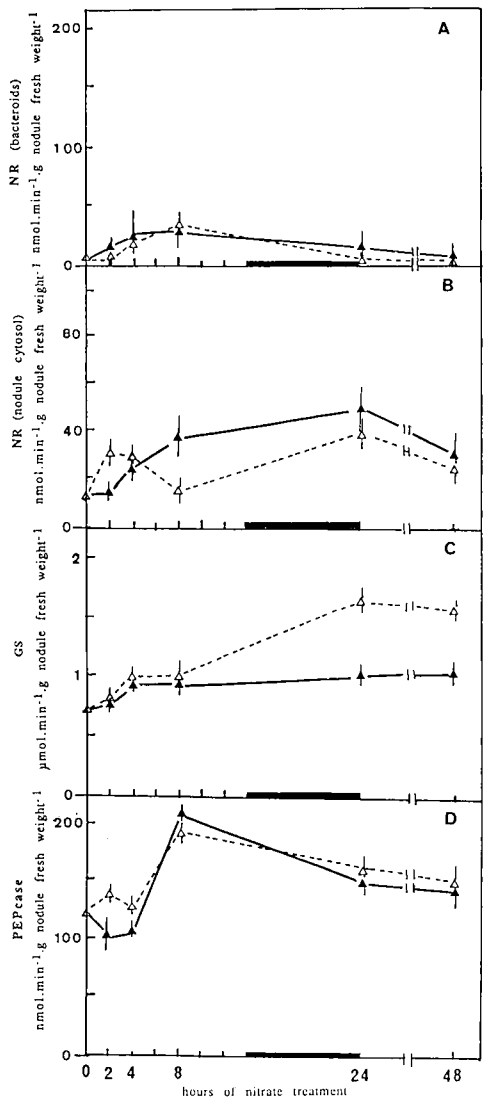

Fig 4. Soybean cv Maple arrow inoculated with *B japonicum* CB 1809 and grown on 20 µM nitrate. Effect of a 48-h treatment with 5 mM nitrate on (A) nitrate reductase activity of bacteroids, (B) nitrate reductase activity of nodule cytosol, (C) glutamine synthetase of nodule tissue, (D) phosphoenolpyruvate carboxylase of nodule tissue. ——— 5 mM NO_3^-; — — — 20 µM NO_3^-. Darkness and light are shown on the abscissa as black and white bars. Vertical bars represent the standard error of the mean of 3 replicates.

nodules were infected with (USDA 311 b 138) (fig 3C) or (CB 1809) *B japonicum* (fig 4C). The responses to NO_3^- treatment were opposite: the increase in GS activity was abolished in (CB 1809) nodules, while it was stimulated in (USDA 311 b 138) nodules, after addition of 5 mM NO_3^-. As with the cytosolic nitrate reductase, the enhancement of GS in (USDA 311 b 138) nodules was evidenced after only a 24-h period of NO_3^- treatment.

Phosphoenolpyruvate carboxylase activity

The 5 mM NO_3^- treatment lowered the PEPcase activity in the (USDA 311 b 138) nodules (fig 3D) but did not affect the PEPcase activity in the (CB 1809) nodules (fig 4D).

DISCUSSION

As nitrate was applied after nodulation, the results reported here were independent of the ef-

fect of NO_3^- on infection. At the time of the nitrate treatment the developmental stage of the plants was similar and the nodule tissue NR, GS, PEPcase and ARA activities as well as the bacteroid NR activity were similar, whether the plants were infected by *B japonicum* USDA 311 b 138 or CB 1809. Similar amounts of dinitrogen were fixed by *B japonicum* USDA 311 b 138 and *B japonicum* CB 1809 and assimilated in plants during the symbiotic period which preceded the nitrate treatment. Any dependency of the responses to nitrate on the previous history of the symbiotic system can thus be eliminated. During the treatment, only the lower parts of the roots were exposed to the nutrient solution and nodules were not directly in contact with the nitrate solution. The observed changes in enzyme activities can thus be interpreted as following nitrate uptake by the roots.

Significant alterations in ARA activity were only observed in nodules infected with *B japonicum* USDA 311 b 138. Therefore, inoculation of soybean with either *B japonicum* USDA 311 b 138 or *B japonicum* CB 1809 represented a good tool for the investigation of effects associated with the inhibition of ARA and N_2 fixation by comparing a high nitrate-sensitive and a low nitrate-sensitive symbiosis. The apparent discrepancy between the results presented here and those of other reports which point to the conclusion that nodules initiated with (USDA 311 b 138) or (CB 1809) *B japonicum* are equally susceptible to inhibition by NO_3^- (Manhart and Wong, 1980; Streeter, 1985) can be explained by the fact that, in contrast to other reports, our results were obtained by short-term (less than 4 d) treatments with low dose (5 mM) of nitrate. The fact that the observed changes are distinct from nodule senescence, is shown by the constancy of the leghaemoglobin content in nodule tissues.

The ARA is the expression of the total electron flow associated with the 2 activities of the enzyme nitrogenase; that is N_2 reduction (nitrogenase *sensu stricto*) and H^+ reduction (hydrogenase activity of the nitrogenase) (Koch *et al,* 1967; Drevon and Salsac, 1984). It was not possible in the present study to determine the exact proportion of the dinitrogen reduction activity that the measured ARA represents, and the results concern the whole nitrogenase activity.

The present study with inoculated Maple arrow soybeans confirms the sensitivity to nitrate of *B japonicum* USDA 311 b 138 which has an inducible nitrate reductase. In contrast, symbiosis of soybean inoculated with *B japonicum* CB 1809 which has no inducible nitrate reductase, was not affected by nitrate under similar treatment conditions. This is in agreement with the results of Stephens and Neyra (1983) who showed that addition of NO_3^- to bacteroids of a wild-type nitrate reductase expressing strain caused a decrease in nitrogenase activity while the nitrate reductase negative strains were insensitive to nitrate. The inducible nitrate reductase activity of *B japonicum* USDA 311 b 138 developed soon after the beginning of the nitrate treatment and reached a high *in vitro* level. That it was also active *in vivo* can be deduced from the observation that less NO_3^- was accumulated in the (USDA 311 b 138) nodules than in the (CB 1809) nodules. It has been shown that the dissimilatory nitrate reductase is generally widespread among bacteria and isolated bacteroids (O'Hara and Daniel, 1985) and that free-living rhizobia can reduce NO_3^- simultaneously to NH_4^+ (assimilation) and to N_2 (denitrification) Becana and Sprent,1987). A number of reports have attempted to explain the mechanism by which nitrate affects N_2 fixation. The rapidity and simultaneous nature of the decrease of ARA with the increase of the bacteroid NR reported here eliminate the possibility of a diversion of photosynthates with a decrease of carbohydrate content in the nodules (Small and Leonard, 1969; Streeter, 1986) and also the mediation of the adverse effect of NO_3^- by a decrease in O_2 available to the N_2-fixing region of the nodule (Minchin *et al,* 1986; Schuller *et al,* 1988). Rather, it supports the hypothesis of an effect of the respiratory nitrate reductase activity itself. Inhibition by nitrate could be due to competition for reducing power between nitrate reduction and bacteroid or mitochondrial respiration inside the nodule (Heckmann *et al,* 1989). A direct effect of NO_3^- *per se* has not been demonstrated (Trinchant and Rigaud, 1981). The hypothesis that NO_3^-, NO, N_2O, products of dissimilatory nitrate reduction, are inhibitory to nitrogenase is well documented (Trinchant and Rigaud, 1980; Caroll and Gresshoff, 1983; O'Hara and Daniel, 1985), but still highly controversial (Becana *et al,* 1989; Heckman *et al,* 1989).

The effect of nitrate uptake on the PEPcase activity was similar to that on the ARA. Like the bacteroid ARA, the cytosol PEPcase activity decreased only in the (USDA 311 b 138) nodules. However, the response of PEPcase was slightly delayed compared to that of the ARA. The nodule specific isoenzyme of PEP-carboxylase is regarded as a nodulin (Miller *et al,* 1987), localized in the cytoplasm of the host cells. Its multiple

functions have been reviewed by Deroche and Carrayol (1988). The PEP carboxylase mediated fixation of CO_2 provides both oxaloacetate for assimilation of ammonia excreted by the bacteroids into aspartate, after N_2 fixation by the nitrogenase, and also malate, an important source of energy for the N_2-fixing bacteria (Suganuma *et al,* 1987) which are known to rapidly metabolize organic acids (De Vries *et al,* 1980). A correlation between the nitrogenase and PEPcase activities was also found for the nodules of alfalfa and yellow lupin (Vance *et al,* 1983; Tomaszewska *et al,* 1988). A decrease in the PEP-carboxylase activity in the nodules of alfalfa plants treated with nitrate was observed by Deroche *et al* (1984).

The cytosol GS and NR of the (USDA 311 b 138) nodules were affected in parallel. They were both enhanced by the nitrate treatment after the ARA was reduced, which suggests that the increase in the cytosol GS was associated with the development of the nitrate assimilation pathway in the plant part of the nodules. Glutamine synthetase was reported to be induced in the nodule cytoplasm during nodule development (Robertson *et al,* 1975). This enzyme which is of plant origin, seems to play a central role in the immediate assimilation of NH_4^+ by the GS-GOGAT pathway (Bergersen, 1965; Kennedy, 1966; Dilworth, 1974). The observation that the levels of glutamine synthetase and glutamine varied according to the effectiveness of the strain of the *Bradyrhizobium* used, was interpreted by Kretovich *et al* (1969) as showing evidence that the rate of nitrogen fixation in legume nodules was directly related to the levels of these constituents. However, in the present study, the evolution of the nodule cytosol GS did not follow the ARA. This observation supports a possible functioning of a bacteroid GS-GOGAT system in assimilation of the ammonium product of N_2 fixation (Dunn and Klucas, 1973) while the GS located in the cytosol of the plant part of the nodule would participate in the nitrate assimilation pathway.

ACKNOWLEDGMENTS

We are grateful to A Oaks for providing the seeds of soybean, B Lagacherie and J Rigaud for providing *Bradyrhizobium japonicum*, JJ Drevon for carefully reviewing the manuscript and giving valuable advice, C Foyer and M Hodges for reading the English manuscript.

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