NITRATE REDUCTASE ACTIVITIES OF RHIZOBIA, THE CORRELATION BETWEEN NITRATE REDUCTION AND NITROGEN FIXATION, AND THE NITRATE EFFECT ON THE NITROGEN FIXATION ACTIVITY OF LEGUME ROOT NODULES

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INTRODUCTION

The research described herein can be divided into two sections. The first involved a systematic survey to determine nitrate reductase activities in various species of Rhizobium grown under different conditions. Data collected were then used to elucidate the correlation between nitrate reductase activity and nitrogen fixation (acetylene reduction) activity as reported by Cheniae and Evans (2). This research is contained in the paper entitled "Nitrate Reductase Activities of Rhizobia and the Correlation between Nitrate Reduction and Nitrogen Fixation", which has been written in a form suitable for publication in Canadian Journal of Microbiology.

Information obtained from the survey allowed the testing of the effects of nitrate on the nitrogen fixation activities of cowpea and lupine nodules induced by strains of Rhizobium that express and do not express nitrate reductase activity in the bacteroids. This research is contained in the paper entitled "Nitrate Effect on Nitrogen Fixation (Acetylene Reduction) Activities of Legume Root Nodules Induced by Rhizobia with Varied Nitrate Reductase Activities." This paper has been written in a form suitable for publication in Plant Physiology.

LITERATURE REVIEW

The presence of an active nitrate reductase in Rhizobium japonicum bacteroids has been well documented (2,3,4,6,7,8,9). Daniel and Gray (4) have shown that the synthesis of nitrate reductase in R. japonicum bacteroids is derepressed by low oxygen tension and not induced by nitrate. Lowe and Evans (9) solubilized a nitrate reductase from rhizobial membranes. Kennedy et al. (8) have reported its purification and characterization. More recently Zablotowicz et al. (16) reported that various strains of R. japonicum and R. sp. have dissimilatory nitrate reductase activity when grown under anaerobic conditions, but R. trifolii, R. phaseoli, and R. leguminosarum showed no dissimilatory nitrate reductase when grown under similar conditions. However, to date, there has not been a survey to determine the nitrate reductase activity of all species of Rhizobium grown under different conditions.

The inhibitory effect of nitrate on the legume-Rhizobium symbiosis can be measured morphologically (10), anatomically (5), physiologically (1,12), and biochemically (12) from nodule initiation to nodule functioning in nitrogen fixation (11). Although nitrate effects on symbiotic nitrogen fixation have been studied more than four decades (15), the mechanism of the inhibition is still unclear. Wilson (15) observed that the inhibitory effects of nitrate were reduced by adding sugars to the legume growth medium or by increasing photosynthesis with additional light or carbon dioxide. Wilson (15) proposed that the internal carbohydrate-nitrogen (C:N) ratio governs nodule formation and nitrogen fixation. The low C:N ratio in the presence of nitrate reduces both nodule formation and

nitrogen fixation. Adding sugars or increasing photosynthesis increases the C:N ration, thus improving both nodulation and nitrogen fixation. Oghoghorie and Pate (11) proposed a similar hypothesis by attributing the decrease in nitrogen fixation activity to a diminished supply of photosynthate to the nodules caused by nitrate assimilation.

Nitrate added to the legume growth medium could be reduced to nitrite by Rhizobium bacteroids. Nitrite could have several inhibitory effects. It could chemically oxidize indole-3-acetic acid, which is necessary for nodule formation (12,13). It could also inhibit nitrogenase activity directly (8), or it could form a nitro-compound with leghemoglobin (14) and, thus, prevent leghemoglobin from binding oxygen, which could interfere with the nitrogen-fixing process.

Nitrate reductase is present in some Rhizobium bacteroids (2,3,4,6,7,8,9). So if nitrate can be absorbed by or transported to the nodules where nitrate is reduced to nitrite by the bacteroid nitrate reductase, nitrite then could inhibit nitrogen fixation activity. However, Gibson and Pagan (7) have shown that the nitrate effect on the nitrogen fixation activity of cowpea plants inoculated with nitrate reductase deficient R. sp. 32H1 mutants is similar to the effect of nitrate on plants inoculated with the wild type. Their findings indicate that bacteroid nitrate reductase has no role in the inhibition of nitrogen fixation by nitrate.

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Paper: NITRATE REDUCTASE ACTIVITIES OF RHIZOBIA AND THE CORRELATION BETWEEN NITRATE REDUCTION AND NITROGEN FIXATION

ABSTRACT

All species of Rhizobium except R. lupini had nitrate reductase activity. Only R. lupini was incapable of growth with nitrate as sole source of nitrogen. However, the conditions necessary for the induction of nitrate reductase varied among species of Rhizobium. R. japonicum and some R. species of the cowpea strains expressed nitrate reductase activities both in the root nodules of appropriate leguminous hosts and when grown in the presence of nitrate. R. trifolii, R. phaseoli, and R. leguminosarum did not express nitrate reductase activities in the root nodules, but they did express when grown in the presence of nitrate. In bacteroids of R. japonicum and some strains of cowpea Rhizobium, high N_2 fixation activities were accompained by high nitrate reductase activities. In bacteroids of R. trifolii, R. leguminosarum, and R. phaseoli, high N_2 fixation activities were not accompanied by high nitrate reductase activities.

INTRODUCTION

Cheniae and Evans (1960) reported a direct correlation between nitrate reduction and N_2 fixation activities of legume root nodules. Based on this study and those of Nason et al. (1971), Evans and Russell (1971) proposed that rhizobial nitrate reductase and nitrogenase may share a common component. Kondorosi et al. (1973) reported that some nitrate reductase-deficient Rhizobium meliloti mutants did lose their N_2 fixation capacity. However, Pagan et al. (1977) isolated nitrate reductase-deficient mutants of R. sp. 32H1 which had N_2 fixation activity comparable to that of the wild type.

Recently, Chen and Phillips (1977) reported that, unlike R. japonicum bacteroids (Cheniae and Evans, 1960; Daniel and Gray, 1976; Kennedy et al., 1975), R. leguminosarum bacteroids had no nitrate reductase. Zablotowicz et al. (1978) reported dissimilatory nitrate reductase in various strains of R. japonicum and R. sp., but not in R. trifolii, R. phaseoli, and R. leguminosarum. They also reported that some strains of R. japonicum and R. sp. are denitrifiers. We report here the results of a series of experiments which assayed for the nitrate reductase activity of all species of Rhizobium grown under different conditions. We also report results which elucidate the interrelationship between nitrate reduction and N_2 fixation activities of legume root nodules.

MATERIALS AND METHODS

Rhizobial Strains and Growth Conditions

R. trifolii ATCC 14480, R. lupini ATCC 10318, R. meliloti ATCC 10312, and R. sp. ATCC 10244 were purchased from the American Type Culture Collection, Rockville, Maryland. All other strains of various Rhizobium species were generous gifts of Dr. J. C. Burton, the Nitragin Co., Milwaukee, Wisconsin.

Rhizobia were grown in two different culture media. The yeast extract-mannitol medium consisted of the following (g/l): K_2HPO_4, 0.764; KH_2PO_4, 1.0; $MgSO_4$, 0.174; $CaSO_4 \cdot 2H_2O$, 0.13; yeast extract, 1.0; mannitol, 3.0; $FeCl_3 \cdot 6H_2O$, 0.004; $CoCl_2 \cdot 6H_2O$, 0.0001; and $Na_2MoO_4 \cdot 2H_2O$, 0.003. The defined medium had the following composition (g/l): K_2HPO_4, 0.764; KH_2PO_4, 1.0; KNO_3, 0.8; $MgSO_4$, 0.18; $CaSO_4 \cdot 2H_2O$, 0.13; mannitol, 3.0; arabinose, 3.0; and (mg/l) H_3BO_3, 1.45; $CuSO_4 \cdot 7H_2O$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.043; $ZnSO_4 \cdot 7H_2O$, 1.08; $Na_2MoO_4 \cdot 2H_2O$, 2.5; $CoCl_2 \cdot 6H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 4.0; $Na_2EDTA \cdot 2H_2O$, 5.5; riboflavin, 0.1; p-aminobenzoic acid, 0.1; nicotinic acid 0.1; biotin, 0.12; thiamine HCl, 0.4; pyridoxine HCl, 0.1; Ca panthenate, 0.5; inositol, 0.5; and vitamin B_{12}, 0.1. The pH of both media was adjusted to 6.8. The cultures were shaken at 200 rpm at 30C. At lage log phase, the cells were harvested by centrifugation and washed once with 0.05M potassium phosphate buffer, pH 7.0, and resuspended in the same buffer.

Legume Cultivars and Growth Conditions

White clover (Trifolium repens L., Cv. White Dutch) was given by Charles Lilly Co., Spokane, Washington. Pole beans (Phaseolus vulgaris L., Cv. Kentucky Wonder), lima beans (P. lunatus L., Cv. Henderson) and cowpeas (Vigna unguiculata, Cv. California Blackeye) were purchased from Burpee Seed Co., Clinton, Iowa. Blue lupin (Lupinus augustifolius, Cv. Frost) was obtained from Everett Seed Co., Atlanta, Georgia. Peas (Pisum sativum L., Cv. First and Best) was a gift of Dr. Fred Muehlbauer, Dept. of Agronomy, Washington State University, Pullman, Washington. Alfalfa (Medicago sativa L., Cv. Kanza) and soybeans (Glycine max L., Cv. Williams) were given by Dr. E. L. Sorensen and Dr. Cecil Nickel, Dept. of Agronomy, Kansas State University, respectively.

Legume seeds were surface sterilized by immersing in 75% (v/v) ethanol for 10 minutes, 20% (v/v) Clorox for another 10 minutes, and then washing thoroughly with sterile water. Seeds were sowed in sterile 6-inch pots containing an autoclaved mixture of 50% vericulite and 50% perlite. The pots were flushed with a nitrogen-free nutrient solution consisting of the following (g/l): KH_2PO_4, 0.272; K_2SO_4, 0.349; $MgSO_4 \cdot 7H_2O$, 0.247; H_3BO_3, 0.004; $CaSO_4 \cdot 2H_2O$, 1.034; and (mg/l) $MnCl_2 \cdot 4H_2O$, 0.990; $ZnSO_4 \cdot 7H_2O$, 0.575; $CuSO_4 \cdot 5H_2O$, 0.125; $Na_2MoO_4 \cdot 2H_2O$, 0.103; FeEDHA (sequestrene 138 Fe), 15.0; and $CoCl_2 \cdot$

$6H_2O$, 0.05. Then the pots were placed in a growth chamber (light intensity: 2200 ft.-candles, photoperiod: 16 hours light and 8 hours darkness, day temperatu 29C, night temperature: 22C for blue lupine, soybean and cowpea; for other legumes, day temperature: 25C, night temperature: 18C). Three days after being sown, legumes were inoculated with appropriate Rhizobium strains. All rhizobia were cultured in the yeast extract-mannitol medium to late log phase before being used as inoculants. The cells were harvested by centrifugation at 10,000 x g for 12 minutes. After washing once in the nitrogen-free legume nutrient solution, the cells were resuspended in the nutrient solution to give a suspension containing about 5×10^7 cells per ml. Each pot received approximately 5×10^9 cells. The plants were watered twice weekly, once with deionized water and once with the nitrogen-free nutrient solution.

Bacteroid Isolation

Nodules were removed from 30 to 35-day old plants and macerated with mortar and pestle in 0.05M potassium phosphate buffer at pH 7.0 (1 g nodule/5 ml buffer). The macerate was filtered through 4 layers of cheesecloth, and the filtrate was centrifuged at 500 x g for 3 minutes to remove plant debris. Bacteroids were collected by centrifuging the supernatant at 12,000 x g for 10 minutes. After washing once, bacteroids were resuspended in potassium phosphate buffer at 2 ml per g of nodules.

Nitrate Reductase Assay

The assay mixture consisted of 0.2ml of bacteroid or bacterial suspension, 0.1ml of 100mM KNO_3, 0.1ml of 50mM Na_2 succinate, and 0.1ml of 50mM glucose. All chemicals were dissolved in 0.05M potassium phosphate buffer, pH 7.0, and placed in 17-ml test tubes. The assay mixtures were incubated at 25C with shaking under either aerobic (in air) or anaerobic (under N_2) conditions. To achieve the anaerobic conditions, the assay tubes were flushed vigorously with a stream of 99.998% pure N_2 for 30 seconds. While still flushing, the tubes were capped with serum stopper. Bacteroid or bacterial suspension was added to start the reaction. An hour later, 0.2ml of 1M Zn acetate was added to each sample to stop the reaction, followed by 3 ml of 95% ethanol. The mixtures were then centrifuged at 3000 x g for 20 mins. The supernatant from each sample was assayed for nitrite, which was determined colorimetrically as diazo dye with sulfanilamide-naphthylethylenediamine reagent (Nicholas and Nason, 1957). Cell protein was determined by the Lowry method (1951), with bovine serum albumin as standard. Nitrate reductase activity was expressed as μmoles of nitrite produced per hour per g protein.

Data presented in all Tables are the average values of triplicate samples followed by the standard deviation from the mean. All experiments were repeated at least twice.

Nitrogenase Activity

Nitrogen fixation activity of root nodules was assayed by the acetylene reduction technique (Hardy et al., 1968). Sections of nodulated roots were incubated in 25ml serum bottles in air with 0.1 atmosphere of acetylene for one hour at 30C. Ethylene produced was measured by gas chromatography. After being assayed, nodules were detached from the roots and weighed. Activity was expressed as micromoles of ethylene produced per hour per g nodule.

Data presented in Tables 1 and 4 are the average values of triplicate samples followed by the standard deviation from the mean. All experiments were repeated at least twice.

RESULTS

Nitrate Reductase Activity of Bacteroids

From Tables 1 and 4, it is apparent that not all species of Rhizobium bacteroids have nitrate reductase activity. R. trifolii, R. phaseoli, R. leguminosarum, R. meliloti, and R. lupini bacteroids had no or very little nitrate reductase activity under either aerobic or anaerobic assay conditions. The rhizobia tested that showed substantial nitrate reductase activity were bacteroids of R. japonicum 61A133, R. sp. 32H1, R. sp. 127E15, and R. sp. ATCC 10244. All species tested were

highly capable of N_2 fixation as evidenced by acetylene reduction activities.

Nitrate Reductase Activity of Cells Grown in Yeast Extract-Mannitol Medium

Growing cells in yeast extract-mannitol medium seemed not to induce nitrate reductase enzyme (Table 2). None of the levels were very high relative to the activities found in most of the strains capable of growth in the defined medium containing nitrate as the sole nitrogen source (Table 3), or in the bacteroids which expressed nitrate reductase activities (Tables 1 and 4). This finding indicates that little nitrate reductase activity is expressed when the cells are grown in the presence of yeast extract under aerobic conditions.

Nitrate Reductase Activity of Cells Grown in the Defined Medium

Nitrate reductase activities of rhizobia grown in the defined medium varied widely (Table 3). None of the 7 strains of R. lupini tested was capable of growth in the defined medium with nitrate as sole nitrogen source. Generally, the strains that grew in the defined medium had high nitrate reductase activities, except for R. phaseoli 127K14, R. trifolii ATCC 14480, and R. meliloti ATCC 10312. R. leguminosarum 128C53 and R. phaseoli 127K12 expressed high activities, and the activities did not seem to be affected appreciably by

anaerobic assay conditions. R. japonicum 61A133, R. sp. 32H1, and R. sp. 127E15 expressed relatively high activities, and the activities were increased by anaerobic assay, especially those of R. sp. 32H1. All three strains had high nitrate reductase activities in the bacteroid form, and the nitrate reductase activities of the bacteroids were also increased by anaerobic assay (Tables 1 and 4).

Correlation Between Nitrate Reduction and N_2 Fixation

Results presented in Tables 1 and 4 demonstrate the relationship between nitrogenase and nitrate reductase activities. As shown in Table 1, nodules of clover, pole bean, pea, alfalfa, and lupine had high acetylene reduction activity but bacteroids isolated from these nodules had no or little nitrate reductase activity. Nodules of soybean and cowpea showed high acetylene reduction activity, and bacteroids isolated from these nodules also had high nitrate reductase activity (Table 1). Nitrate reductase activity-deficient strains of R. sp. and R. lupini can induce as effective nodules on cowpea and lupine as the strains of R. sp. with high nitrate reductase activity (Table 4). However, when R. sp. ATCC 10244, which induces effective nodule formation on lima bean and the bacteroids of which have high nitrate reductase activity, was used to inoculate pole bean, the bacteroids

isolated from the partially effective pole bean nodules had low nitrate reductase activity (Table 4).

DISCUSSION

With the exception of R. lupini, all rhizobia are capable of growth in the defined medium with nitrate as sole nitrogen source. This finding indicates that all rhizobia, except R. lupini, have nitrate reductase. However, the conditions for induction of nitrate reductase in various species of Rhizobium are different.

Nitrate reductase activities of all rhizobia grown in yeast extract-mannitol medium were nil or very low, indicating rhizobia synthesize little nitrate reductase in the presence of yeast extract under aerobic growth conditions (Table 2). Kennedy et al. (1975) reported the presence of a very active nitrate reductase in R. japonicum CC705 (Wisconsin 505) grown in a similar yeast extract-mannitol medium. However, the growth conditions reported by Kennedy et al. (1975) may be partially anaerobic because they did not vigorously aerate their rhizobial culture. Murphy and Elkan (1965) and Daniel and Gray (1976) reported that R. japonicum strains synthesized nitrate reductase in the presence of combined nitrogen under partially anaerobic conditions.

R. japonicum 61A133, R. sp. 127E15, and R. sp. 32H1 showed high nitrate reductase activities in root nodules of legumes growing in a nitrogen-free nutrient solution (Tables 1 and 4). The induction of nitrate reductase in these rhizobia does not require the presence of nitrate. Since oxygen concentrations in the nodules are very low (Appleby, 1962, 1969), induction of nitrate reductase activities in the bacteroids of R. japonicum 61A133, R. sp. 127E15, and R. sp. 32H1, may be a response to the low O_2 potential in the nodules.

Nitrate reductase activities were detected in R. trifolii, R. phaseoli, R. leguminosarum, and R. sp. 127E14 when these rhizobia were grown in the defined medium (Table 3). However, nitrate reductase activities could not be detected in the bacteroids (Tables 1 and 4). The induction of nitrate reductase in these rhizobia may require the presence of nitrate. Low O_2 potential probably does not induce nitrate reductase in these rhizobia.

The induction of nitrate reductase activities in Rhizobium bacteroids is not host dependent. As shown in Tables 1 and 4, R. sp. 32H1 and R. sp. 127E15 bacteroids isolated from cowpea nodules had high nitrate reductase activity, but R. sp. 127E14 bacteroids isolated from nodules of the same host had no nitrate reductase activity. All strains of R. lupini bacteroids isolated from lupine nodules had very low nitrate reductase

activity (Table 1). However, R. sp. 127E15 bacteroids isolated from lupine nodules had high nitrate reductase activity (Table 4).

The inability of all strains of R. lupini to grow in the defined medium may be considered a taxonomic characteristic. We can consider R. lupini as rhizobia strains which are incapable of using nitrate as sole source of nitrogen, but are capable of forming effective nodules on lupines. R. sp. 127E15 was originally isolated from effective lima bean nodules (personal communication from J. C. Burton). Nevertheless, it is also capable of forming effective nodules on lupines (Table 4). However, because R. sp. 127E15 is capable of using nitrate as sole source of nitrogen, we cannot consider it as a strain of R. lupini.

R. sp. 127E14 and R. sp. 127E15 were originally isolated from lima bean nodules and are promiscuous in that R. sp. 127E14 can also induce nodule formation on cowpea and that R. sp. 127E15 can induce nodule formation on both cowpea and lupine (Table 4). Another lima bean Rhizobium, R. sp. ATCC 10244, can also induce partially effective nodules on pole bean (Table 4). Furthermore, R. sp. 127E15 bacteroids isolated from cowpea nodules had high nitrate reductase activities, whereas R. sp. 127E14 bacteroids did not (Table 4). We used those findings to

study the correlation between nitrogenase and nitrate reductase activities.

The correlation between these two enzyme activities varies, depending on the legume-Rhizobium combinations. If a Rhizobium bacteroid normally has high nitrate reductase activity in effective nodules, then there is a positive correlation between these two enzyme activities. R. sp. 127E15 elicited cowpea nodules had higher acetylene reduction activity than that of R. sp. 127E15-induced lupine nodules (Table 4). R. sp. 127E15 bacteroid isolated from cowpea nodules also had higher nitrate reductase activity than that of R. sp. 127E15 bacteroids isolated from lupine nodules. Similarly, R. sp. ATCC 10244 elicited effective nodules on lima bean, and the bacteroids also exhibited high nitrate reductase activity. R. sp. ATCC 10244 induced partially effective nodules on pole bean, and the bacteroids also showed low nitrate reductase activity (Table 4).

In contrast, R. sp. 127E14 bacteroids isolated from cowpea nodules had no nitrate reductase activity, but the acetylene reduction activity of these nodules was higher than that of cowpea nodules induced by R. sp. 127E15, which had high nitrate reductase activity in the bacteroid form (Table 4). Similar results were obtained with lupines inoculated with R. sp. 127E15 and R. lupini ATCC 10318 (Table 4). In the cowpea and

lupine nodules, there is no correlation between the two enzyme activities. In addition, bacteroids of R. trifolii, R. phaseoli, R. leguminosarum, R. meliloti, and R. lupini had no or very little nitrate reductase activity, but all the nodules had high acetylene reduction activity (Table 1). In these cases, there is again no correlation.

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Table 1. Nitrate reductase and acetylene reduction activities of Rhizobium bacteroids

Rhizobium	Legume Host	µmoles NO_2^- formed / hr g protein		µmoles C_2H_4 formed / hr g nodule
		aerobic assay	anaerobic assay	
R. trifolii ATCC 14480	White Clover	0 ± 0	0 ± 0	7.585 ± 0.036
R. phaseoli 127K12	Pole Bean	0 ± 0	0 ± 0	11.731 ± 2.224
R. phaseoli 127K14	Pole Bean	0 ± 0	0 ± 0	8.394 ± 3.425
R. leguminosarum 128C53	Pea	0 ± 0	0 ± 0	11.788 ± 2.367
R. leguminosarum 128C76	Pea	0 ± 0	0 ± 0	13.354 ± 2.628
R. leguminosarum 128C78	Pea	0 ± 0	0 ± 0	12.230 ± 2.034
R. leguminosarum 92A3	Pea	0 ± 0	0 ± 0	11.542 ± 2.341
R. leguminosarum 175P1	Pea	0 ± 0	0 ± 0	10.982 ± 1.978
R. meliloti ATCC 10312	Alfalfa	0 ± 0	17 ± 0	12.744 ± 2.152
R. lupini ATCC 10318	Lupine	0 ± 0	0 ± 0	3.761 ± 0.069
R. lupini 96E4	Lupine	2.6 ± 0.2	3.1 ± 0	1.331 ± 0.867

Table 1 (Continued)

Rhizobium	Legume Host	μmoles NO_2^- formed / hr g protein		μmoles C_2H_4 formed / hr g nodules
		aerobic assay	anaerobic assay	
R. lupini 96E6	Lupine	5.4 ± 0.9	3.4 ± 0.2	2.415 ± 1.311
R. lupini 96E9	Lupine	5.5 ± 0	6.5 ± 0	2.117 ± 0.791
R. lupini 96B6	Lupine	8.0 ± 1.8	16.0 ± 2.6	4.038 ± 1.241
R. lupini 96B9	Lupine	6.2 ± 0.8	7.2 ± 4.4	1.277 ± 0.675
R. lupini 96B10	Lupine	2.7 ± 0.1	2.9 ± 0.2	3.725 ± 1.143
R. japonicum 61A133	Soybean	116.9 ± 19.4	208.3 ± 31.6	4.447 ± 1.474
R. sp. 32H1	Cowpea	993.0 ± 95.0	2800.0 ± 283.0	8.520 ± 0.756

Table 2. Nitrate reductase activity of rhizobia grown in yeast extract-mannitol medium

Rhizobium	Growth[1]	µmoles NO_2^- formed / hr g protein	
		aerobic assay	anaerobic assay
R. trifolii ATCC 14480	+	0 ± 0	0 ± 0
R. phaseoli 127K12	+	7.4 ± 2.9	4.9 ± 0
R. phaseoli 127K14	+	10.7 ± 0	5.1 ± 0.7
R. legumino-sarum 128C53	+	0 ± 0	0 ± 0
R. meliloti ATCC 10312	+	5.5 ± 1.2	7.3 ± 1.3
R. lupini ATCC 10318	+	0 ± 0	0 ± 0
R. japonicum 61A133	+	4.4 ± 0.9	6.7 ± 1.8
R. sp. ATCC 10244	+	0 ± 0	0 ± 0
R. sp. 127E14	+	0 ± 0	0 ± 0
R. sp. 127E15	+	19.6 ± 2.4	29.7 ± 1.9
R. sp. 32H1	+	0 ± 0	0 ± 0

[1] The occurrence of growth was determined by optical densities at 620 nm. + = good growth.

Table 3. Nitrate reductase activity of rhizobia grown in defined medium

Rhizobium	Growth[1]	µmoles NO_2^- formed / hr g protein	
		aerobic assay	anaerobic assay
R. trifolii ATCC 14480	+	6.0 ± 0.3	5.0 ± 1.1
R. phaseoli 127K12	+	340.0 ± 12.7	326.0 ± 6.3
R. phaseoli 127K14	+	5.5 ± 0.7	4.0 ± 0
R. leguminosarum 128C53	+	1310.0 ± 76.1	1418.0 ± 50.8
R meliloti ATCC 10312	+	17.0 ± 2.8	5.7 ± 1.8
R. lupini ATCC 10318	-	---	---
R. lupini 96E4	-	---	---
R. lupini 96E6	-	---	---
R. lupini 96E9	-	---	---
R. lupini 96B6	-	---	---
R. lupini 96B9	-	---	---
R. lupini 96B10	-	---	---
R. japonicum 61A133	+	41.3 ± 2.8	96.0 ± 13.2
R. sp. ATCC 10244	+	59.6 ± 11.3	22.6 ± 0.40
R. sp. 127E14	+	34.6 ± 11.3	45.2 ± 8.8
R. sp. 127E15	+	104.9 ± 17.4	163.4 ± 89.5
R. sp. 32H1	+	12.7 ± 7.7	520.0 ± 166.1

[1] The occurrence of growth was determined by opitcal densities at 620 nm. + = good growth and - = no growth.

Table 4. Correlation between nitrate reductase and nitrogenase activities

Rhizobium	Legume	μmoles NO_2^- formed / hr g protein						μmoles C_2H_4 formed / hr g nodules		
		aerobic assay			anaerobic assay					
R. sp. 127E14	cowpea	0	±	0	0	±	0	9.970	±	1.870
R. sp. 127E15	cowpea	304.3	±	50.8	330.6	±	27.0	6.501	±	1.549
R. sp. 127E15	lupine	35.8	±	1.8	178.8	±	21.7	1.356	±	0.612
R. lupini ATCC 10318	lupine	0	±	0	0	±	0	3.761	±	0.069
R. sp. ATCC 10244	pole bean	44.1	±	6.1	206.6	±	43.1	0.027	±	0
R. sp. ATCC 10244	lima	815.0	±	185.8	1620.0	±	353.6	7.654	±	1.437

Paper: NITRATE EFFECT ON NITROGEN FIXATION (ACETYLENE REDUCTION) ACTIVITIES OF LEGUME ROOT NODULES INDUCED BY RHIZOBIA WITH VARIED NITRATE REDUCTASE ACTIVITIES

ABSTRACT

Nitrate reductase of Rhizobium bacteroids in the nodules of cowpea and lupine reduced NO_3^- to NO_2^-. Both cowpea and lupine nodules accumulated NO_2^- when grown in the presence of 15 mM NO_3^- and induced by Rhizobium strains which express NO_3^- reductase activity (R. sp. 32H1 and R. sp. 127E15). The N_2 fixation (acetylene reduction) activities of cowpea and lupine nodules were inhibited by NO_3^- whether the nodules were induced by Rhizobium strains that express (R. sp. 32H1 and R. sp. 127E15) or do not express (R. sp. 127E14 and R. lupini ATCC 10318) NO_3^- reductase activity. These findings indicate that NO_2^-, the product of bacteroid NO_3^- reductase, plays no role in the inhibitory effect of NO_3^- on the N_2 fixation activities of legume root nodules. However, the degree of inhibition on the fixation activity by NO_3^- varied in different legume-Rhizobium combinations.

The inhibitory effect of NO_3^- on the N_2 fixation activity of legume root nodules has been under serious investigation for some time (14). Munns (8) and Gibson (2) have recently published thorough reviews on the subject. There are two hypotheses as to the cause of this inhibition. One has been termed the photosynthate deprivation hypothesis which attributes the decrease in N_2 fixation activity to a diminished supply of photosynthate to the nodules caused by NO_3^- reduction in the shoots (10). The other hypothesis involves a more direct effect and attributes the inhibition to the formation of NO_2^- in the nodules by bacteroid NO_3^- reductase (2). Nitrate itself seems not to affect N_2 fixation activity in cultures of Rhizobium sp. 32H1 (11), but NO_2^- inhibits fixation in cultures of R. sp. 32H1 (11), R. japonicum bacteroid suspensions (12), and crude R. japonicum bacteroid nitrogenase extracts (5). In addition, NO_2^- may form a NO-compound with leghemoglobin (13) and, thus, prevent leghemoglobin from binding O_2, which could interfere with the N_2-fixing process.

The presence of an active NO_3^- reductase in some Rhizobium bacteroids has been well documented (1,5-7). So if NO_3^- can be absorbed by or transported to the nodules where NO_3^- is reduced to NO_2^- by the bacteroid NO_3^- reductase, NO_2^- then could inhibit N_2 fixation activity. However, Gibson and Pagan (3) have shown that the NO_3^- effect on N_2 fixation activity of cowpea plants inoculated with NO_3^- reductase-deficient R. sp. 32H1 mutants is similar to the effect of NO_3^- on plants inoculated with the wild type. Their finding indicates that the bacteroid NO_3^- reductase has no role in inhibiting N_2 fixation by NO_3^-.

In our earlier studies (6,7), we found that R. lupini ATCC 10318 and R. sp. 127E14 bacteroids isolated from lupine and cowpea nodules, respectively,

did not express NO_3^- reductase activity, but R. sp. 32H1 and R. sp. 127E15 bacteroids from cowpea nodules did. Furthermore, we found that R. sp. 127E14 could induce effective nodules on cowpea and R. sp. 127E15 on both cowpea and lupine. We used those findings to test the involvement of bacteroid NO_3^- reductase in the NO_3^- effect on N_2 fixation activity. Cowpea plants were nodulated with two NO_3^- reductase-expressing strains (R. sp. 32H1 and R. sp. 127E15) and a nonexpressing strain (R. sp. 127E14). Lupine plants were nodulated with a NO_3^- reductase-expressing strain (R. sp. 127E15) and a nonexpressing strain (R. lupini ATCC 10318). We studied the effect of NO_3^- on N_2 fixation activities of those legume-Rhizobium combinations. The results of these experiments are reported in this paper.

MATERIALS AND METHODS

Legume Cultivars and Growth Conditions. Cowpea (Vigna unguiculata Cv. California Blackeye) was purchased from Burpee Seed Co., Clinton, Iowa, and lupine (Lupinus augustifolius Cv. Frost) from Everett Seed Co., Atlanta, Georgia. Cowpea and lupine seeds were surface sterilized by immersing in 75% ethanol for 10 minutes, 20% clorox for another 10 minutes, and then washing thoroughly with sterile water. Seeds were sowed in sterile 6-inch pots containing an autoclaved mixture of 50% vermiculite and 50% perlite. The pots were flushed with either a N-free nutrient solution (7) or a solution with chemical composition identical to the N-free solution except for the added 15 mM NO_3^- as 5 mM $NaNO_3$ and 5 mM $CaNO_3 \cdot 2H_2O$ (NO_3^--solution). The pots were placed in growth chambers (light intensity: 2200 ft-candles; photoperiod: 16 hours light and 8 hours darkness; day temperature: 29°, night temperature: 22°; relative

humidity: 60%). Four days after being sown, cowpea plants were inoculated with R. sp. 127E14, R. sp. 127E15, or R. sp. 32H1. Lupine plants were inoculated with either R. lupini ATCC 10318 or R. sp. 127E15. All Rhizobium strains were cultured in a yeast extract-mannitol medium (7) to late log phase before being used as inoculants. Each pot received approximately 5 x 10^9 Rhizobium cells. All Rhizobium strains except R. lupini ATCC 10318 were generous gifts from Dr. J. C. Burton, The Nitragin Co., Milwaukee, Wisconsin. R. lupini ATCC 10318 was purchased from the American Type Culture Collection, Rockville, Maryland.

The pots were thinned to 4 plants per pot one week after seeds were planted. Thereafter, pots that had been flushed with NO_3^--solution received 200 ml of the same solution every other day throughout the experiment. Pots that had been flushed with N-free solution were divided into two groups. In one group, each pot received 200 ml of N-free solution every other day throughout the experiment. In the other group, each pot received, every other day, 200 ml of N-free solution until the 27th day after planting for cowpea and 30th day for lupine. Then each pot was first eluted with NO_3^--solution and subsequently given 200 ml of the NO_3^--solution every two days.

Assays. Nitrogen fixation activities of root nodules were assayed by the acetylene reduction technique (4). Sections of nodulated roots were incubated in 25-ml serum bottles in air with 0.1 atmosphere of acetylene for one hour at 30°. Ethylene produced was measured by gas chromatography. After being assayed, nodules were detached from the root and weighed. Acetylene reduction activity was calculated as μmoles of ethylene produced per hour per g nodules. The data presented in Figs. 1 to 5 are expressed as % activity of the nodules of the N-free grown

plants. Each point in Figs. 1 to 5 represents the mean and standard deviation of five replicates.

To determine NO_2^- content of nodules, 0.5 g of nodules were macerated in 1 ml of 1 M zinc acetate with mortar and pestle. After maceration, the total volume was adjusted to 5 ml with deionized water, and cellular debris was removed by centrifugation at 12,000 x g for 10 minutes. One ml of the supernatant was combined with 1.7 ml of 1 M zinc acetate and 1 ml of 95% ethanol and centrifuged at 3,000 x g for 20 minutes to remove precipitated proteins. The supernatant from the second centrifugation was assayed quantitatively for NO_2^- by a colorimetric method (9). Nitrite content was expressed as nmoles of NO_2^- per g nodule fresh weight. Each point in Fig. 6 represents the mean and standard deviation of three replicates.

RESULTS

Nitrate Effect on Acetylene Reduction Activities of Cowpea Nodules. R. sp. 127E14 bacteroids isolated from effective cowpea nodules showed no NO_3^- reductase activity (7). The acetylene reduction activity of the R. sp. 127E14-induced nodules from cowpea plants grown continuously in 15 mM NO_3^- (the NO_3^--grown plants) was about 7 to 15% of the acetylene reduction activity of the nodules from plants grown continuously in the N-free solution (the control plants) (Fig. 1). When N-free grown plants were given NO_3^- the 27th day after planting, the acetylene reduction activity of the nodules decreased quickly to that of nodules from NO_3^--grown plants (Fig. 1).

R. sp. 32H1 and R. sp. 127E15 bacteroids exhibit active NO_3^- reductase activities (7). As shown in Fig. 2, the acetylene reduction activity of R. sp. 32H1-induced nodules from NO_3^--grown plants was 27 to 35% of the activity of nodules from control plants. When N-free grown plants were given NO_3^- on the 27th day, the acetylene reduction activity decreased precipitously to approximately the same level as that of nodules from NO_3^--grown plants (Fig. 2). Nitrate also drastically inhibited the acetylene reduction activity of nodules induced by R. sp. 127E15 (Fig. 3). The acetylene reduction activity of nodules from NO_3^--grown plants was only about 4 to 13% of the activity of the control plants (Fig. 3). However, the inhibitory effect of NO_3^- on the R. sp. 127E15-induced nodules of plants given NO_3^- on the 27th day was less pronounced than the inhibitory effect on nodules induced by the other two strains of Rhizobium. In this case, the acetylene reduction activity ranged from 30 to 50% of the activity of nodules from control plants (Fig. 3). The activity did not decrease to that of the nodules from NO_3^--grown plants as did the activities of nodules induced by the other two strains (Figs. 1-3).

Nitrate Effect on Acetylene Reduction Activities of Lupine Nodules. The acetylene reduction activity of R. lupini ATCC 10318-induced nodules from the NO_3^--grown lupine plants was 33 to 70% of the activity of nodules from control plants (Fig. 4). Adding NO_3^- to the N-free grown plants the 30th day after planting decreased the activity to that of nodules from the NO_3^--grown plants (Fig. 4).

The effect of NO_3^- on R. sp. 127E15-induced lupine nodules was surprising in that the acetylene reduction activities of nodules from NO_3^--grown plants and the plants given NO_3^- on the 30th day were higher than the activity of nodules from control plants (Fig. 5).

Nitrate Effect on Nodule Fresh Weight of Cowpea and Lupine Plants. Nitrate-grown cowpeas had about 34% of nodule mass of the control plants when inoculated with R. sp. 127E14 or R. sp. 32H1 (Table 1). When inoculated with R. sp. 127E15, NO_3^--grown cowpeas had only about 23% as much nodule mass as the control plants. When grown in the presence of NO_3^- and inoculated with R. lupini ATCC 10318, lupine plants had only 37% of nodule mass of the control plants, but when inoculated with R. sp. 127E15, the NO_3^--grown lupines had similar nodule mass as the control plants (Table 1).

Nitrite Contents of Cowpea and Lupine Nodules. Cowpea nodules induced by a Rhizobium strain that has no bacteroid NO_3^- reductase activity (R. sp. 127E14) had no NO_2^- whether the nodules were harvested from NO_3^--grown plants or plants that received NO_3^- on the 27th day. Similarly, lupine nodules induced by R. lupini ATCC 10318 contained no NO_2^- even when the nodules were harvested from NO_3^--grown plants or plants that received NO_3^- on the 30th day.

When cowpea nodules were induced by Rhizobium strains that have bacteroid NO_3^- reductase activity (R. sp. 32H1 and R. sp. 127E15), NO_2^- was detected in the nodules from both the NO_3^--grown plants and plants that received NO_3^- on the 27th day (Fig. 6). However, the NO_2^- content was higher in nodules from the NO_3^--grown plants than in nodules from plants that received NO_3^- on the 27th day. The amount of NO_2^- in all nodules increased with time (Fig. 6).

When lupine plants were nodulated by a NO_3^- reductase-expressing Rhizobium strain (R. sp. 127E15), NO_2^- was detected in the nodules from the NO_3^--grown plants and the plants treated with NO_3^- on the 30th day (Fig. 6). As with cowpea nodules, lupine nodules from the NO_3^--grown

plants contained more NO_2^- than plants treated with NO_3^- on the 30th day. Again the quantity of NO_2^- accumulated gradually increased with time (Fig. 6).

DISCUSSION

Our results confirmed Gibson and Pagan's findings (3) that NO_2^-, the product of bacteroid NO_3^- reductase, plays no role in the inhibitory effect of NO_3^- on acetylene reduction activity. This is evidenced by comparing the effect of NO_3^- on nodules induced by Rhizobium strains having bacteroid NO_3^- reductase activity with the effect on nodules induced by strains lacking the activity. R. sp. 127E14 bacteroids do not express NO_3^- reductase activity (7), and the cowpea nodules induced by this Rhizobium strain contained no NO_2^-. However, these nodules are more susceptible to NO_3^- inhibition than cowpea nodules induced by R. sp. 32H1 and R. sp. 127E15 (Figs. 1-3), both of which express NO_3^- reductase activity (7), and nodules induced by either of the strains contained NO_2^- (Fig. 6). The same holds true for lupine nodules induced by R. lupini ATCC 10318 and R. sp. 127E15 (Figs. 4-6).

The strain of Rhizobium and the species of legume both may have an effect on the degree of NO_3^- inhibition of acetylene reduction activity. R. sp. 127E15-induced cowpea nodules were inhibited more than R. sp. 127E15-induced lupine nodules were (Figs. 3,5), so lupine nodules are more resistant to NO_3^- inhibition. R. sp. 127E15-induced lupine nodules are more resistant to NO_3^- inhibition than nodules induced by R. lupini ATCC 10318 (Figs. 4,5). Moreover, although the acetylene reduction activity of NO_3^--grown cowpea nodules induced by R. sp. 127E15 was as

drastically inhibited as other NO_3^--grown cowpea nodules, the inhibition by NO_3^- added on the 27th day was significantly less in nodules induced by R. sp. 127E15 than in other nodules (Figs. 1-3). These findings indicate that Rhizobium strains differ in their reactions to NO_3^-.

When lupine plants were nodulated by R. sp. 127E15, the presence of 15 mM NO_3^- in the nutrient solution actually stimulated acetylene reduction activity (Fig. 5). This experiment demonstrates that when a legume which is more resistant to the NO_3^- effect is nodulated by a Rhizobium strain which is also more resistant, the resulting legume-Rhizobium combination has additive resistance to NO_3^- inhibition. This additive effect is also evident in nodule mass of NO_3^--grown lupines nodulated with R. sp. 127E15 (Table 1).

Nitrite accumulated in the nodules is the product of NO_3^- reduction in the bacteroids because in both cowpea and lupine nodules NO_2^- was detected only when the nodules were induced by Rhizobium strains with NO_3^- reductase activity and only when the plants were grown in the presence of NO_3^- (Fig. 6). R. phaseoli 127K12 and R. leguminosarum 128C53 bacteroids isolated from pole bean and pea nodules, respectively, had no NO_3^- reductase (7). Nodules from pea and pole bean plants grown in NO_3^- also contained no NO_2^- (Manhart and Wong, unpublished results). Furthermore, the amount of NO_2^- accumulated in nodules is positively correlated with NO_3^- reductase activity of the bacteroids. As shown in our earlier report (7), R. sp. 32H1 bacteroids isolated from cowpea nodules had NO_3^- reductase specific activity (μmoles of NO_2^- formed/g protein/hr) of 993, whereas R. sp. 127E15 bacteroids had only 304. Nitrite content of R. sp. 32H1 induced cowpea nodules was much higher than that of nodules induced with R. sp. 127E15 (Fig. 6).

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Table 1. Effect of NO_3^- on nodule fresh weight of cowpea and lupine plants.

The plants were grown either with N-free nutrient solution (control plants) or with nutrient solution containing 15 mM NO_3^- (NO_3^--grown plants) throughout the experiment. Nodules were detached from 34-day old cowpeas or 38-day old lupines and washed in cold deionized water. The washed nodules were blotted dry with cheesecloth and weighed. Data presented for each legume-*Rhizobium* combination represent the mean and standard deviation of 12 plants.

Legume-*Rhizobium* combination	Nodule fresh weight (mg/plant)		
	Control	NO_3^--grown	% of control
Cowpea-127E14	692 ± 248	237 ± 50	34.2 ± 7.2
Cowpea-32H1	380 ± 76	129 ± 50	33.9 ± 13.4
Cowpea-127E15	427 ± 64	97 ± 42	22.7 ± 8.7
Lupine-10318	812 ± 242	301 ± 127	37.1 ± 15.6
Lupine-127E15	292 ± 24	301 ± 124	103.1 ± 42.4

Legend for Figures

Fig. 1. Nitrate effect on acetylene reduction activity of cowpea nodules induced by R. sp. 127E14, a strain with no NO_3^- reductase activity. Control plants were grown in N-free nutrient solution throughout the experiment. Plants were treated with 15 mM NO_3^- either throughout the experiment or beginning the 27th day after planting as described under "Materials and Methods." The actual specific acetylene reduction activity (micromoles of C_2H_4 formed/g nodule/hr) of nodules from the control plants taken at the various dates as indicated was 4.32± .65.

Fig. 2. Nitrate effect on acetylene reduction activity of cowpea nodules induced by R. sp. 32H1, a strain with NO_3^- reductase activity. The actual specific acetylene reduction activity of nodules from the control plants taken at the various dates as indicated was 7.67 ± 2.09. Growth conditions of control plants, NO_3^- treatment of plants, and definition of specific activity are described in legend for Fig. 1.

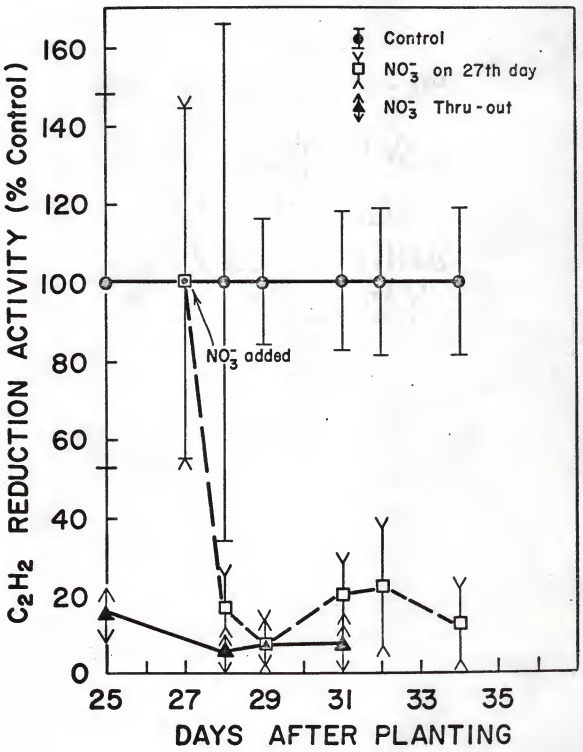
Fig. 3. Nitrate effect on acetylene reduction activity of cowpea nodules induced by R. sp. 127E15, a strain with NO_3^- reductase activity. The actual specific acetylene reduction activity of nodules from the control plants taken at the various dates as indicated was 2.16 ± .46. Growth conditions of control plants, NO_3^- treatment of plants, and definition of specific activity are described in legend for Fig. 1.

Fig. 4. Nitrate effect on acetylene reduction activity of lupine nodules induced by R. lupini ATCC 10318, a strain with no NO_3^- reductase activity. The actual specific acetylene reduction activity of nodules from the control plants taken at the various dates as indicated was $3.52 \pm .72$. Growth conditions of control plants, NO_3^- treatment of plants, and definition of specific activity are described in legend for Fig. 1.

Fig. 5. Nitrate effect on acetylene reduction activity of lupine nodules induced by R. sp. 127E15, a strain with NO_3^- reductase activity. The actual specific acetylene reduction activity of nodules from the control plants taken at the various dates as indicated was $4.45 \pm .92$. Growth conditions of control plants, NO_3^- treatment of plants, and definition of specific activity are described in legend for Fig. 1.

Fig. 6. Nitrite contents of cowpea and lupine nodules. Cowpea plants were inoculated with R. sp. 32H1 and R. sp. 127E15, both of which have NO_3^- reductase activity. Lupine plants were inoculated with R. sp. 127E15. The plants were treated with 15 mM NO_3^- either throughout the experiment or beginning the 27th day after planting for cowpea and the 30th day for lupine.

Figure 1


Control
NO_3^- on 27th day
NO_3^- Thru-out
NO_3^- added
C_2H_2 REDUCTION ACTIVITY (% Control)
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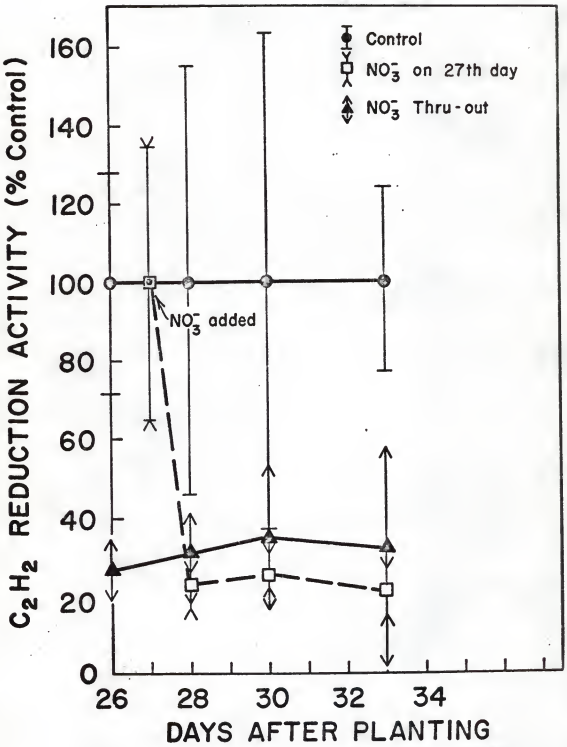
20
40
60
80
100
120
140
160
25
27
29
31
33
35
DAYS AFTER PLANTING

Figure 2


Control
NO_3^- on 27th day
NO_3^- Thru-out
NO_3^- added
C_2H_2 REDUCTION ACTIVITY (% Control)
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20
40
60
80
100
120
140
160
26
28
30
32
34
DAYS AFTER PLANTING

Figure 3

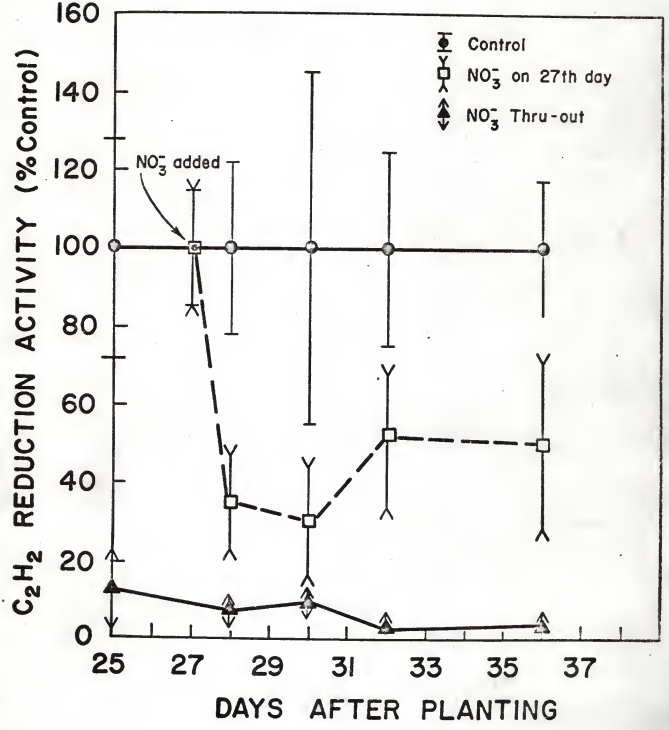

160
140
120
100
80
60
40
20
0
C_2H_2 REDUCTION ACTIVITY (% Control)
Control
NO_3^- on 27th day
NO_3^- Thru-out
NO_3^- added
25
27
29
31
33
35
37
DAYS AFTER PLANTING

Figure 4

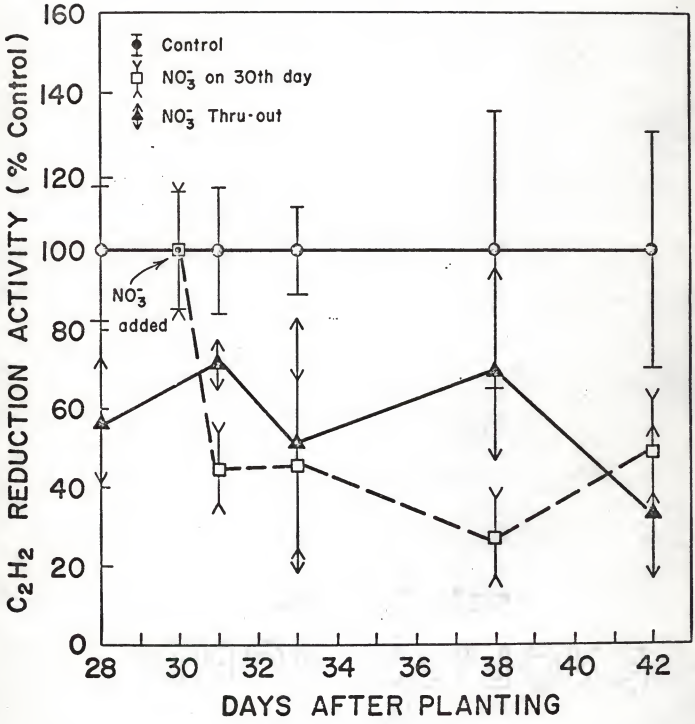

Control
NO3- on 30th day
NO3- Thru-out
NO3- added
C2H2 REDUCTION ACTIVITY (% Control)
DAYS AFTER PLANTING
0
20
40
60
80
100
120
140
160
28
30
32
34
36
38
40
42

Figure 5

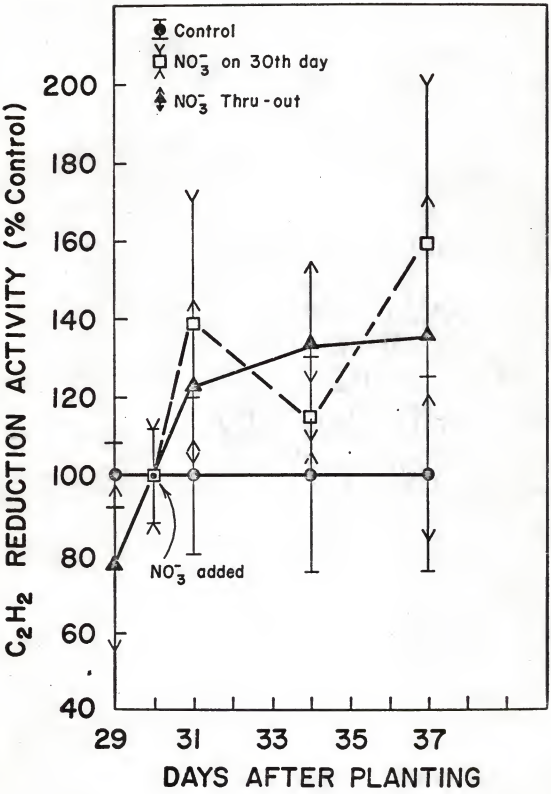
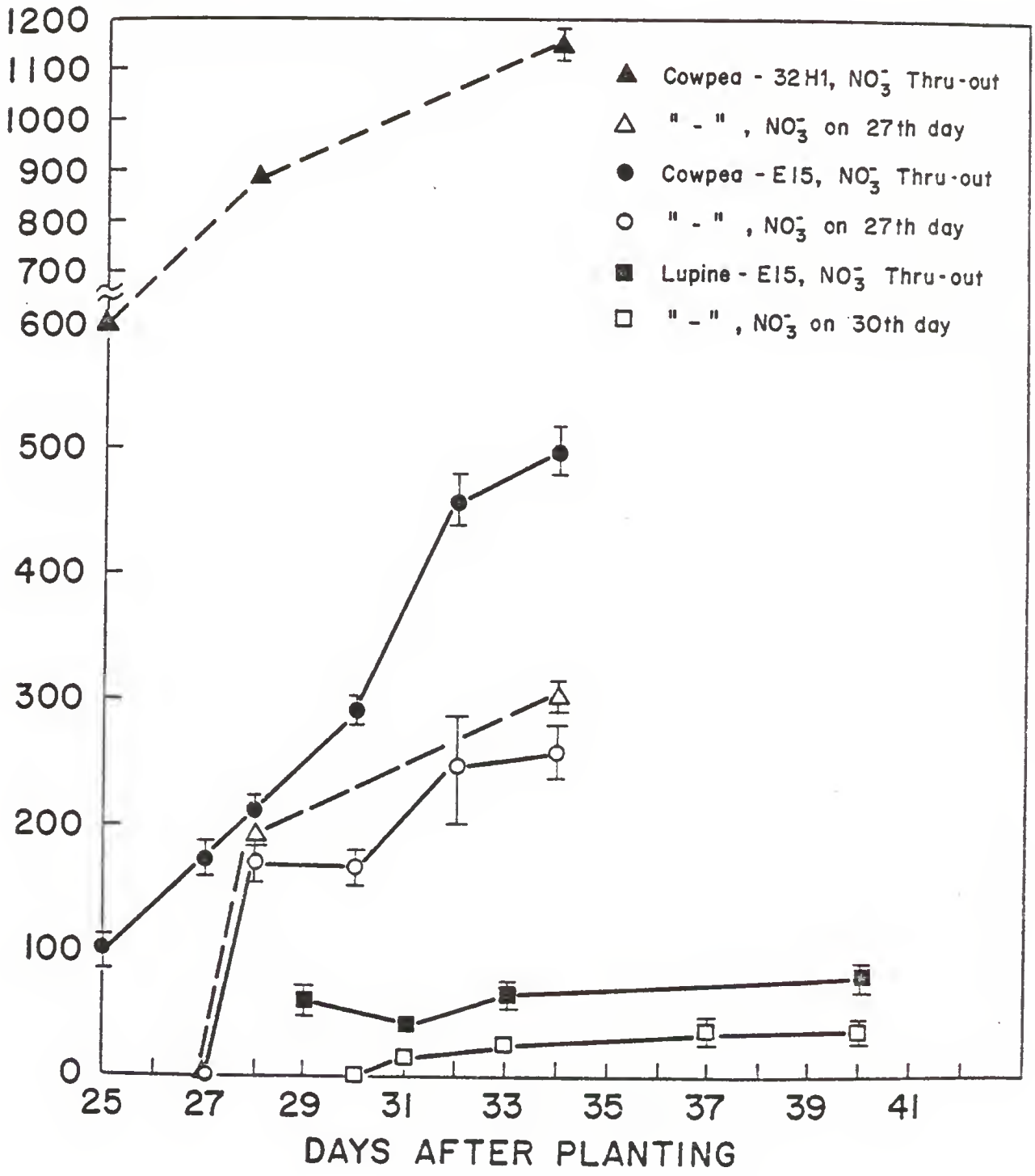

Control
NO3- on 30th day
NO3- Thru-out
200
180
160
140
120
100
80
60
40
C2H2 REDUCTION ACTIVITY (% Control)
NO3- added
29
31
33
35
37
DAYS AFTER PLANTING

Figure 6


1200
1100
1000
900
800
700
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500
400
300
200
100
0
25
27
29
31
33
35
37
39
41
DAYS AFTER PLANTING
Cowpea - 32H1, NO3- Thru-out
" - " , NO3- on 27th day
Cowpea - E15, NO3- Thru-out
" - " , NO3- on 27th day
Lupine - E15, NO3- Thru-out
" - " , NO3- on 30th day

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NITRATE REDUCTASE ACTIVITIES OF RHIZOBIA, THE CORRELATION BETWEEN NITRATE REDUCTION AND NITROGEN FIXATION, AND THE NITRATE EFFECT ON THE NITROGEN FIXATION ACTIVITY OF LEGUME ROOT NODULES

by

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All species of Rhizobium except R. lupini had nitrate reductase activity. Only R. lupini was incapable of growth with nitrate as sole source of nitrogen. However, the conditions necessary for the induction of nitrate reductase varied among species of Rhizobium. R. japonicum and some R. species of the cowpea strains expressed nitrate reductase activities both in the root nodules of appropriate leguminous hosts and when grown in the presence of nitrate. R. trifolii, R. phaseoli, and R. leguminosarum did not express nitrate reductase activities in the root nodules, but they did express when grown in the presence of nitrate. Rhizobia expressed little or nil nitrate reductase activity when grown aerobically in yeast extract-mannitol medium.

The correlation between nitrogenase activity and nitrate reductase activity in bacteroids of various species of Rhizobium was investigated. In bacteroids of R. japonicum and some strains of cowpea Rhizobium, high nitrogen fixation (acetylene reduction) activities were accompanied by high nitrate reductase activities. In bacteroids of R. trifolii, R. leguminosarum, and R. phaseoli, high nitrogen fixation activities were not accompanied by high nitrate reductase activities.

The nitrogen fixation activities of cowpea and lupine nodules were inhibited by nitrate whether the nodules were induced by Rhizobium strains that express (R. sp. 32H1 and R. sp. 127E15) or do not express (R. sp. 127E14 and R. lupini ATCC 10318) nitrate reductase activity. These findings indicate that nitrite, the product of bacteroid nitrate reductase, plays no role in the inhibitory effect of nitrate on the nitrogen fixation activities of legume root nodules. However, the degree of inhibition on the fixation

activity by nitrate varied in different legume-Rhizobium combinations.

Nitrate reductase of Rhizobium bacteroids in the nodules of cowpea and lupine reduced nitrate to nitrite. Both cowpea and lupine nodules accumulated nitrite when grown in the presence of 15mM nitrate and induced by Rhizobium strains which express nitrate reductase activity (R. sp. 32H1 and R. sp. 127E15).