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Nitrate and Carbohydrate Effects on Nodulation and Nitrogen Fixation (Acetylene Reduction) Activity of Lentil (*Lens esculenta* Moench)[1]

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ABSTRACT

Lentils (*Lens esculenta* Moench, cv. Tekoas) grown in a nutrient solution containing 15 millimolar nitrate had 84% fewer nodules than lentils grown in nitrate-free nutrient solution. Nodules from the nitrate-grown plants weighed 71% less than nodules from the nitrate-free plants. Nitrate-grown plants also fixed much less nitrogen (measured by acetylene reduction) than the nitrate-free plants. When lentils were grown in a solution containing 15 millimolar nitrate and 75 millimolar fructose, glucose, or sucrose, however, the nitrogen fixation activity of their nodules was similar to that of nodules from nitrate-free plants. Leaves of lentils grown in the nitrate-sugar solutions had only about 7% as much nitrate reductase activity and accumulated only 10% as much nitrate as leaves from lentils grown in the nitrate solution alone. Roots of lentils grown in the nitrate-sugar solutions had similar nitrate reductase activity but accumulated only 17 to 25% as much nitrate as roots from lentils grown in the nitrate solution. The results indicate that the added sugars alleviated the inhibitory effects of nitrate on symbiotic nitrogen fixation not only by increasing the carbohydrate supply so lentils could support both nitrogen fixation and nitrate reduction but also by inhibiting the accumulation of nitrate and, hence, lowering nitrate reductase activity in the leaves.

The inhibitory effect of NO_3^- on legume *Rhizobium* symbiosis can be measured morphologically (11), anatomically (3), physiologically (1), and biochemically (16) from nodule initiation to nodule functioning in N_2 fixation (13). Although NO_3^- effects on symbiotic N_2 fixation have been studied more than four decades (20), mechanism of the inhibition is still unclear. Wilson and others (20) observed that the inhibitory effects of NO_3^- were reduced by adding sugars to the legume growth medium or by increasing photosynthesis with additional light or CO_2. Wilson (20) proposed that the internal carbohydrate to nitrogen (C/N) ratio governs nodule formation and N_2 fixation. The low C/N ratio in presence of NO_3^- reduces both nodule formation and N_2 fixation. Adding sugars or increasing photosynthesis increases the C/N ratio, thus improving both nodulation and N_2 fixation. Small and Leonard (15) and Oghoghorie and Pate (13) proposed a similar hypothesis by attributing decrease in N_2 fixation activity to a diminished supply of photosynthate to the nodules caused by NO_3^- assimilation.

Nitrate added to the legume growth medium could be reduced to NO_2^- by *Rhizobium* bacteroid NO_3^- reductase. Nitrite could have several inhibitory effects. It could inhibit nitrogenase activity directly (7), or it could form a NO compound with leghemoglobin (19), which could interfere with the N_2-fixing process. Gibson and Pagan (4) and Manhart and Wong (10), however, have demonstrated that bacteroid NO_3^- reductase plays no role in NO_3^- inhibition of nodulation and N_2 fixation.

Some quantitative measurements of NO_3^- effects on nodulation and N_2 fixation activity of lentil plants are reported here. In addition, the reason why adding sugars to the growth medium can alleviate some of the inhibitory effects of NO_3^- is discussed.

MATERIALS AND METHODS

Lentils (*Lens esculenta* Moench, cv. Tekoas) were obtained from Dr. Van Wilson, Department of Agronomy, Washington State University, Pullman, Washington. *Rhizobium leguminosarum* 128C53 was a gift of Dr. Joe Burton, The Nitragin Co., Milwaukee, Wisconsin.

Lentil Growth Conditions. Lentil seeds were surface-sterilized by immersing in 75% (v/v) ethanol for 10 min, 1% NaOCl (w/v) for another 10 min, and then washing thoroughly with sterile H_2O.

Wide mouth (6.5 cm diameter) 900-ml capacity bottles were used for lentil cultivation. Each bottle was filled to the neck with Vermiculite. The mouth of the bottle was covered with two layers of filter paper secured by a rubber band. Four holes (1.2 cm diameter) were made on the filter paper cover, and they were plugged with cotton. The entire bottle, covered with a paper bag, was then autoclaved for 2 h at 121 C. The sterilized bottles then received either 550 ml of an autoclaved nutrient solution free of combined nitrogen (N-free solution) (9) 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 $Ca(NO_3)_2$ (NO_3^- solution). When sugars were needed in the growth medium, sugar solutions, sterilized by filtration, were added to the N-free or NO_3^- solution to achieve the desired sugar concentration.

The sterilized seeds then were planted in the bottles (five seeds per bottle). Bottles were inoculated with approximately 1×10^9 *R. leguminosarum* 128C53 cells. *Rhizobium* was cultured in a yeast extract-mannitol medium (9) to late log phase and then harvested by centrifuging at 10,000*g* for 10 min. The cells were washed once with sterile N-free nutrient solution. Before being used as inoculant, the cells were resuspended in the N-free or NO_3^- solution to give a suspension containing approximately 5×10^7 cells/ml. The entire procedure of adding nutrient solutions, planting seeds, and inoculating was performed aseptically in a laminar flow hood. The bottles were placed in growth chambers (light intensity: 24,000 lux; photoperiod: 16/8-h light/dark cycle at 26/20 C, RH 70%). Five to 6 days after the planting, paper bags were removed from the bottles, and three of the four holes on the filter paper covers were unplugged. Seedlings were gently guided through the holes

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with a pair of forceps (one seedling per hole). Then the holes were replugged with seedlings protruding through the cotton plugs. The fourth hole remained plugged except when nutrient solutions were added. Starting 2 weeks after planting, each bottle received 150–200 ml of nutrient solution weekly until the experiments were terminated at 30 days after planting.

Bacteroid Isolation. Nodules were detached from 30-day-old plants grown in various nutrient solutions, and 2 g of nodules were macerated with mortar and pestle in 10 ml of 50 mM K-phosphate buffer at pH 7.0. The macerate was filtered through four layers of cheesecloth. The residue was washed with 10 ml of the K-phosphate buffer and filtered again. Filtrates were pooled and then centrifuged at 500*g* for 3 min to remove plant debris. Bacteroids, collected by centrifuging the supernatant at 10,000*g* for 10 min, were resuspended in 20 ml of the K-phosphate buffer. The suspension was centrifuged at 500*g* for 3 min, then bacteroids again were collected by centrifuging the supernatant at 10,000*g* for 10 min. When microscopically examined, the isolated bacteroid samples contained no plant debris. The bacteroids were then dried to a constant weight in an oven at 80 C.

Nitrogen Fixation Activity of Root Nodules. N_2 fixation activity was assayed by the C_2H_2 reduction technique (6). Upper 5-cm sections of nodulated roots were placed in 25-ml serum bottles, and the bottles were sealed with rubber serum stoppers. C_2H_2 was injected into the bottles to 0.1 atm. The bottles were incubated for 1 h at 30 C. C_2H_4 produced was measured by GC. After being assayed, nodules were detached from the roots, weighed, and counted. C_2H_2 reduction activity was calculated as nmol of C_2H_4 produced/mg nodules·h or nmol C_2H_4 produced/nodule·h.

Nodule Weight and Number of Nodules Per Plant. Lentils grown under the described conditions were mainly nodulated on the upper portion of the tap root. Only the upper 5-cm sections of the roots were used to determine the number of nodules on each plant and weight of nodules. Lower portions of the roots had some smaller immature nodules that were not used.

Leaf and Root NO_3^- Reductase Activity. Leaflets detached from lentils grown under various conditions were used. Nitrate reductase activity was assayed by the *in vivo* method of Nicholas *et al.* (12). Activity was expressed as nmol of NO_2^- formed/g fresh weight·h.

The first 3-cm root tip sections of lentils grown under various conditions were ground with an Omni-Mixer at top speed for 2 min using 6 ml of casein-cysteine grinding medium (14) for each g of fresh materials. After being filtered through four layers of cheesecloth, the homogenates were centrifuged at 27,000*g* for 20 min. The grinding, filtering, and centrifuging were conducted at 4 C. After being centrifuged, the extracts were used for NO_3^- reductase assay by the method of Hageman and Hucklesby (5). Activity was expressed as nmol of NO_2^- formed/g fresh weight·h.

Nitrate Analyses. Nitrate in the growth medium was assayed by the method of Lowe and Hamilton (8). To determine NO_3^- content, 1 g of dry leaf or root was extracted with 10 ml of deionized H_2O by grinding with an Omni-Mixer at top speed for 2 min. A second 10 ml of water was added, and the tissue was reground at top speed for another 2 min. The resulting suspension was filtered through glass wool. The filtrate was analyzed for NO_3^- content (8).

RESULTS AND DISCUSSION

Nitrate has many effects on the development and C_2H_2 reduction activity of lentil root nodules. Lentils grown in a N-free nutrient solution had six times as many nodules per plant as lentils grown in NO_3^- solution (Table I). Nodules from N-free grown plants weighed more than three times as much as nodules from NO_3^--grown plants (Table I).

Nitrate also inhibited C_2H_2 reduction activity of lentil nodules. The activity of each nodule from N-free grown plants was about six times that of a nodule from NO_3^--grown plant (Table I). Compared on nodule weight basis, each mg of N-free grown nodules had twice the C_2H_2 reduction activity as a mg of NO_3^--grown nodules (Table I). Since the amount of bacteroids in each g of nodules was about the same whether the nodules were from NO_3^--grown or N-free grown plants (Table I), the C_2H_2 reduction activity of bacteroids from N-free grown nodules should be also twice as much as that of bacteroids from NO_3^--grown nodules.

The C/N ratio hypothesis (20) and the photosynthate deprivation hypothesis (13, 15) have predicted the inhibitory effects of NO_3^- on lentil nodules. If those hypotheses were correct, adding carbohydrate to the growth medium should alleviate the inhibitory effects of NO_3^-. Thornton (17) and Van Schreven (18) have demonstrated the validity of this assumption.

We have also tested the effects of adding fructose, glucose, and sucrose on the inhibitory effects of NO_3^- on lentil root nodules. The difficulty encountered in conducting this type of experiment is to maintain sugar and NO_3^- concentrations of the nutrient solution. Growth of contaminant microorganisms could decrease the concentrations. By growing lentils under the microbiologically controlled conditions, the NO_3^- concentrations of various samples were maintained at about 15–20 mM throughout the experiment.

Adding fructose to the growth medium affects N_2 fixation activity and nodule number of both NO_3^--grown and N-free grown plants. The number of nodules on each N-free grown plant decreased about 41% when 25 mM fructose was added (Fig. 1). Fructose concentrations higher than 25 mM, did not further reduce nodule number. On the other hand, adding fructose to NO_3^- solution increased the number of nodules from four to eight per plant. At 150 mM fructose, a decrease in number of nodules per plant was also observed (Fig. 1).

At 75 mM, fructose stimulated nodule weight of N-free grown plants more than 2-fold (Fig. 2). But at concentrations higher than 75 mM, stimulation decreased (Fig. 2). Adding 75 mM fructose to NO_3^- solution increased the nodule weight almost 4-fold (Fig. 2).

The C_2H_2 reduction activity of a mg of nodule from N-free grown plants reached maximum at 25 mM fructose (Fig. 3). The activity then decreased to a level lower than that of nodules from plants grown without fructose. This decrease occurred because beyond 25 mM fructose, the C_2H_2 reduction activity of each nodule did not increase (Fig. 4), whereas the weight of each nodule increased steadily as fructose was increased up to 75 mM (Fig. 2). Similarly, C_2H_2 reduction activity of each nodule from NO_3^--grown plants increased only slightly with more than 50 mM fructose (Fig. 4), but the nodule weight increased steadily as fructose was increased to 75 mM (Fig. 2). As a result, C_2H_2 reduction activity of each mg of nodules from NO_3^--grown plants increased up to 50 mM fructose and then decreased (Fig. 3).

Fructose actually did not increase total C_2H_2 reduction activity of the N-free grown lentil plants. The C_2H_2 reduction activity of a plant grown in N-free nutrient solution with 25 mM fructose was 1,316 nmol C_2H_4 formed/h (94 nmol C_2H_4 formed/nodule·h × 14 nodules/plant) (Figs. 1 and 4). The activity of a plant grown without fructose was 1,584 nmol of C_2H_4 formed/h (66 nmol C_2H_4 formed/nodule·h × 24 nodules/plant) (Figs. 1 and 4). Fructose increased total C_2H_2 reduction activity of NO_3^--grown plants by 18-fold. At 75 mM fructose, C_2H_2 reduction activity of NO_3^--grown plant was 720 nmol of C_2H_4 formed/h (90 nmol C_2H_4 formed/nodule·h × 8 nodules/plant) (Figs. 1 and 4). The activity of a plant grown in NO_3^- but without fructose was 40 nmol of C_2H_4 formed/h (10 nmol C_2H_4 formed/nodule·h × 4 nodules/plant) (Figs. 1 and 4).

Glucose and sucrose had effects similar to those of fructose on nodule number, nodule size, and C_2H_2 reduction activity of lentils grown in N-free or NO_3^- solution (data not shown). The results showing that sugars alleviated some inhibitory effects of NO_3^-

Table I. *Effects of Nitrate on the Development and Acetylene Reduction Activity of Lentil Root Nodules*

Plants were grown either with a N-free nutrient solution or with a nutrient solution containing 15 mM NO_3^-. Each value presented represents the mean and SD of 12 samples, except that dry bacteroid content is from three samples.

Nutrient Solution	No. Nodules/ Plant	Fresh Nodule Weight	Bacteroid Content	C_2H_2 Reduction Activity	C_2H_2 Reduction Activity
		mg/nodule	*mg dry wt/g fresh nodules*	*nmol C_2H_4/ nodule·h*	*nmol C_2H_4/mg nodule·h*
N-free	24.1 ± 3.3	6.7 ± 1.8	18.2 ± 1.8	67.9 ± 5.9	10.1 ± 0.9
With NO_3^-	4.0 ± 1.5	2.1 ± 0.8	17.9 ± 2.4	10.8 ± 2.1	4.9 ± 1.0
Inhibition (%)	83.5	71.0	2.0	84.0	51.2

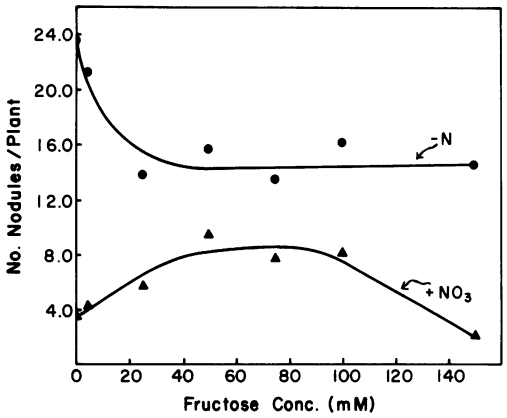

FIG. 1. Effect of fructose on number of nodules on a lentil plant. Experiments were conducted with plants grown in a N-free nutrient solution or a nutrient solution containing 15 mM NO_3^- with indicated concentrations of fructose added to the nutrient solutions.

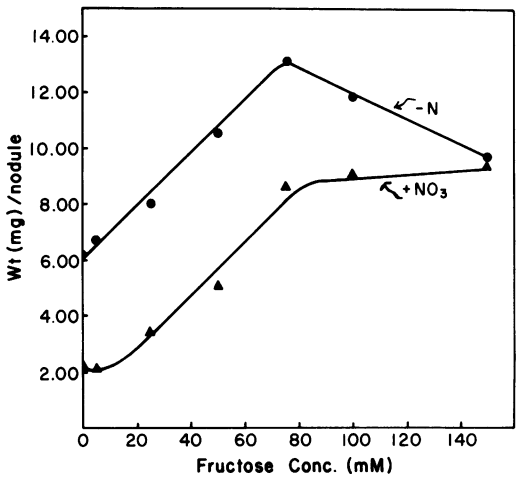

FIG. 2. Effect of fructose on weight of a lentil nodule. Experimental conditions are described in the legend for Figure 1.

seem to support the C/N ratio and photosynthate deprivation hypotheses (13, 15, 20). However, sugars have effects other than those from being a carbohydrate source, as shown by their effects on NO_3^- metabolism of lentil plants. Lentil leaf NO_3^- reductase activity and accumulation of NO_3^- in the leaves both were inhibited about 90% by 75 mM fructose, glucose, and sucrose (Table II). Sugars also inhibited NO_3^- accumulation by the roots, but they did not reduce the root NO_3^- reductase activity (Table III).

Chen and Phillips (2) reported that increasing photosynthate supply in pea plants by CO_2 enrichment cannot alleviate the inhibitory effects of NO_3^-. They concluded that NO_3^- does not inhibit symbiotic N_2 fixation through competition between NO_3^-

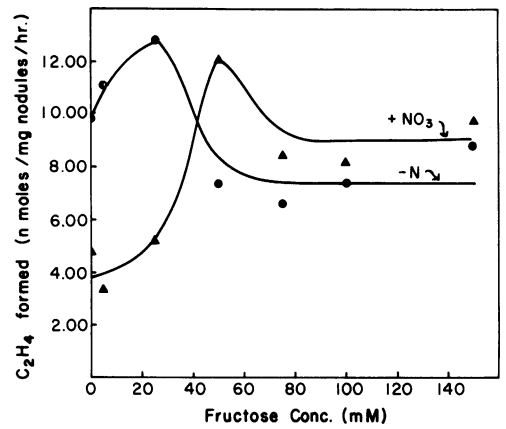

FIG. 3. Effect of fructose on C_2H_2 reduction activity of a mg of lentil nodules. Experimental conditions are described in the legend for Figure 1.

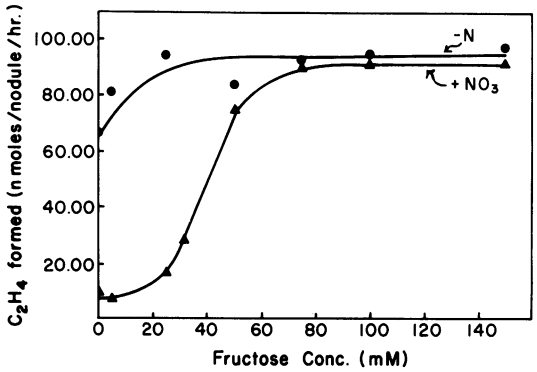

FIG. 4. Effect of fructose on C_2H_2 reduction activity of a lentil nodule. Experimental conditions are described in the legend for Figure 1.

Table II. *Effects of Sugars on Nitrate Reductase Activity and Nitrate Content of Lentil Leaves*

Plants were grown either in a N-free nutrient solution or in nutrient solutions containing 15 mM NO_3^- with or without 75 mM of various sugars as indicated. Each value presented represents the mean and SD of six samples.

Nutrient Solution	Leaf NO_3^- Reductase Activity	Leaf NO_3^- Content
	nmol nitrite/g fresh wt·h	*μmol/g dry wt*
N-Free	48 ± 8	0
With NO_3^-	1,940 ± 187	4.84 ± 0.85
With NO_3^- and fructose	127 ± 27	0.52 ± 0.11
With NO_3^- and glucose	169 ± 19	0.55 ± 0.16
With NO_3^- and sucrose	142 ± 10	0.57 ± 0.08

Table III. *Effects of Sugars on Nitrate Reductase Activity and Nitrate Content of Lentil Roots*

Plants were grown as described in Table II. Each value presented represents the mean and SD of five samples.

Nutrient Solution	Root NO_3^- Reductase Activity	Root NO_3^- Content
	nmol nitrite/g fresh wt·h	*μmol/g dry wt*
N-free	11 ± 5	0
With NO_3^-	250 ± 16	663 ± 43
With NO_3^- and fructose	276 ± 10	115 ± 24
With NO_3^- and glucose	262 ± 21	132 ± 31
With NO_3^- and sucrose	206 ± 15	169 ± 25

reductase and nitrogenase for photosynthate. My results indicate that when sugars were directly supplied to the growth medium of NO_3^--grown lentils, the added sugars can alleviate the inhibitory effects of NO_3^- not only by increasing the carbohydrate supply so that lentils could support both N_2 fixation and NO_3^- reduction, but also by inhibiting the accumulation of NO_3^- and, hence, lowering NO_3^- reductase activity in leaves. Although sugars did not reduce the root NO_3^- reductase activity as measured by the *in vitro* assay (Table III), the lowered NO_3^- accumulation by the roots could reduce the actual *in vivo* NO_3^- reductase activity. Lowered NO_3^- reductase activities in leaves and roots lessen the demand for photosynthate (15), and the photosynthate, in turn, could be used to support N_2 fixation.

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