Synthesis and Accumulation of Nitrite in Soybean Nodules Supplied with Nitrates

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ABSTRACT

Nodulated soybean plants (Glycine max [L.] Merr.) were grown in sand culture without combined N or with a continuous supply of nitrate in nutrient solution. Moderate nitrate concentration (30 milligrams N per liter) had little effect on nodule weight/plant while high nitrate concentration (100 milligrams N per liter) depressed nodule weight/plant by 70 to 80% with harvests 30 to 60 days after planting and initiation of nitrate treatments.

The effect of nitrate supply on ammonium, amino, and ureide nitrogen concentrations in nodules was small and inconsistent. In contrast, nitrate and nitrite concentrations in nodules were directly proportional to nitrate supply and inversely proportional to nodule weight/plant. Correlations between nitrate or nitrite concentration in nodules and nodule weight/plant were highly significant.

Cytoplasm from soybean nodules was found to contain NADH-dependent nitrate reductase activity (typical activity was 0.1 micromole per milligram protein × hour). A Rhizobium japonicum mutant (derived from strain 61A76) lacking nitrate reductase was employed to show that the cytosol enzyme activity is of host origin. Growth of nodules formed by the mutant lacking nitrate reductase was inhibited by nitrate. These nodules did contain nitrite although concentrations of nitrite (about 0.3 micromole N per gram fresh weight) were low relative to nitrite concentrations (about 1.5 micromole N per gram fresh weight) in nodules formed by R. japonicum strain 61A76. The overall results support the idea that the depression of legume nodule growth by nitrate is directly related to the metabolism of nitrate in nodules.

(b) it deoxygenates and oxidizes leghemoglobin in vitro resulting in the formation of ferric leghemoglobin (18); and (c) it (i.e. nitrous acid) reacts spontaneously with primary and secondary amines.

There have been very few reports demonstrating the actual presence of nitrite in nodules on plants supplied with nitrate. Rigaud has reported 140 to 780 nmole NO₂⁻/g fresh weight of soybean nodules 6 to 24 h after supplying plants with 7 mM NO₂⁻ (17). More recently, Manhart and Wong have reported high NO₂⁻ concentrations in lupine and cowpea nodules supplied with very high (15 mM) nitrate (13). They also showed that the amount of NO₂⁻ accumulated in nodules was positively correlated with NR² activity in bacteroids (13). In both of these studies, the main interest was in exploring the inhibition of nitrogenase activity by nitrate over periods of 1 to 5 d.

After the discovery of NR in Rhizobium japonicum bacteroids (5) it was generally assumed that the synthesis of nitrite in nodules occurs in the bacteroids. However, Gibson and Pagan showed that growth and N₂-fixing activity of Macroptilium atropurpureum and Trifolium subterraneum nodules formed by Rhizobia lacking NR were still depressed when plants were supplied with nitrate (6). This observation has recently been extended to Vigna unguiculata and Lupinus angustifolius nodules (13). Coupled with the fact that nitrate was not detected in these nodules (6, 13), these observations make it less likely that the generation of nitrite in nodules may be the mechanism by which nitrate inhibits nodule growth and activity.

The work reported here was focused on the effect of nitrate on soybean nodule growth. The objectives were to (a) document the occurrence of nitrite in nodules supplied with moderate [NO₃⁻] for long (>30 d) periods of time; (b) assess, quantitatively, the association between [NO₃⁻] in nodules and nodule growth; and (c) determine if a mechanism other than bacteroid NR exists in soybean nodules for the generation of nitrite.

MATERIALS AND METHODS

Plant Culture. Soybean seeds (Glycine max [L.] Merr.), cv. Beeson, were planted in silica sand. Variations in nitrate concentration in nutrient solutions were provided by balancing nitrate and chloride. Details on nutrient solution composition and plant growth conditions have been published (22).

Plants for experiments 1 and 2 were grown outdoors during the summer months without alteration of the natural photoperiod. Plants for the preliminary experiment and for experiments 3 and 4 were grown in a greenhouse between January and May with supplemental light providing a photoperiod of 15 h. For the preliminary experiment, fluorescent and incandescent lamps provided about 200 μE m⁻² s⁻¹ (400–700 nm). For experiments 3 and 4 metal halide lamps were used to provide about 1000 μE m⁻² s⁻¹ (400–700 nm).

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2 Abbreviation: NR, nitrate reductase.
Commercial inoculant (Agricultural Laboratories, Inc., Columbus, OH) was used to form root nodules in the preliminary experiment and in experiments 1 and 2. Pure strains of *Rhizobium japonicum* were used essentially the same with respect to growth rate in culture, number of nodules formed, nodule weight/plant, acetylene reduction activity of nodules, plant growth in zero-N solution, and complete decomposition of ureides. Extracts of nodules were analyzed for nitrite at very low concentrations in nodule exudate flow by vacuum infiltration with 125 µl of KOH-diluted 60% HCl. This concentration of HCl will conserve NH₄⁺ but will not hydrolyze amides. Extracts were centrifuged at 12,000g for 5 min. Volume of each tube was recorded after centrifugation and portions of the extract were analyzed for NH₄⁺ concentration by microdiffusion and Nesslerization.

For analysis of other nitrogenous constituents, ethanol extracts of nodules (22) were employed. Nitrate was analyzed by the method of Woolley et al. (26) using 100 mg portions of the reagent powder. Ureide was analyzed as described previously (21). Amino nitrogen was measured by the method of Moore and Stein (15). Prior to amino-N determinations, ammonia was removed from samples by drying portions of extract with 125 µl of KOH-borate buffer (9.28 g boric acid plus 32.5 ml of 6.0 N KOH diluted to 500 ml with methanol).

**Nitratreductase.** For *in vivo* NR, 250 mg of leaf tissue was vacuum infiltrated with 0.1 ml phosphate buffer (pH 7.5) containing 1% (v/v) propanol and 0.01% (v/v) Triton X-100 as a wetting agent. Nitrate was omitted from the infiltration medium in order to provide a more accurate estimate of *in situ* nitrate reduction rate (3). Mixtures were incubated at 30°C in a shaking water bath in the dark. Samples taken after 0, 15, 30, and 45 min were analyzed for NO₃⁻, and enzyme activity was estimated from a period of linear increase in [NO₃⁻] and from standards added to samples (23).

For determinations of nodule NR about 1 g fresh nodules were chilled and extracted at 2°C with 5 ml 25 mM phosphate buffer (pH 7.4), containing 0.2 M mannitol and 2.5 mM dithioerythritol. Moist, insoluble PVP (2.5 g) was mixed with crude extract. After standing for 10 min, the extract was filtered through four layers of cheesecloth and centrifuged (2°C) at 12,000g for 15 min. The supernatant was used for cytosol assays.

For determination of bacterial NR, the bacteroid pellet was rinsed twice with 0.2 ml phosphate buffer (pH 7.5) and then resuspended in 5.0 ml of the same buffer. The reaction mixture consisted of 400 µmol phosphate buffer (pH 7.5), 40 µmol KNO₃, 100 µmol succinate (pH 7.5), and 25 to 50 µl bacteroid preparation (about 50 µg protein) in a total volume of 3.4 ml. Boiled bacteroids served as the control. Mixtures were incubated aerobically for 30 to 60 min at 30°C and reagents for colorimetric determination of nitrite (23) were added to stop the reaction. Tubes were then centrifuged at 15,000g and A₅₂₀ of the supernatant was determined. Protein content was determined by the method of Bradford (2).

**RESULTS**

A preliminary experiment was conducted to characterize the effect of nitrate on nodule growth using the sand culture system to be employed in subsequent experiments. Little effect of 15 or 30 mM NO₃-N/L on nodule weight/plant was found, while 60, 100, and 150 mg N/L reduced nodule weight, relative to 0-N controls, by about 40, 80, and 85%, respectively. Stem exudate was also collected from these plants to assess the effect of nitrate treatments on nitrogen nutrition. Results showed that a drastic reduction in ureide concentration accompanied the expected increase in nitrate concentration while concentration of amino-N was apparently not altered by nitrate treatment (Table I). Declines in ureide concentration of stem exudate in response to nitrate supply are interpreted as indicating a decline in N₂-fixation activity in nodules (14, 16). Since there was an apparent positive effect of nitrate supply on exudate flow rate (Table I), the decline in quantity of ureide transported may not be as great as the decline
Exudate collection began 2 h after the beginning of the photoperiod and lasted 1.5 to 2 h. Stems were severed with a razor blade about 3 cm above the surface of the sand; stumps were washed with water and fitted with pieces of latex rubber tubing. After measuring volume of liquid collected, exudate was frozen until analyzed. Data are averages of two replicates (10-15 plants/replicate) sampled at 41 d after planting.

<table>
<thead>
<tr>
<th>Nitrate in Nutrient Solution</th>
<th>Exudate Composition</th>
<th>Exudate Flow Rate</th>
<th>Total N Transporta</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg N/L</td>
<td>µg N/ml exudate</td>
<td>µl/plant·h</td>
<td>µg N/ plant·h</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>572</td>
<td>10.8</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>406</td>
<td>25.8</td>
</tr>
<tr>
<td>30</td>
<td>155</td>
<td>204</td>
<td>66.6</td>
</tr>
<tr>
<td>60</td>
<td>237</td>
<td>82</td>
<td>106.2</td>
</tr>
<tr>
<td>100</td>
<td>247</td>
<td>42</td>
<td>75.6</td>
</tr>
<tr>
<td>150</td>
<td>249</td>
<td>34</td>
<td>98.4</td>
</tr>
</tbody>
</table>

* Sum of nitrate-N, amino-N, and ureide-N.

Fig. 2. Effect of nitrate supply on soybean nodule growth rate. Plants were grown outdoors in sand culture and supplied with nutrient solution containing either 0, 30, or 100 mg N/L. Correlation coefficients for the regression lines are 0.97 (0-N), 0.98 (30-N), and 0.98 (100-N). Each point represents the average of two replicates. Experiment 1.

increase in plant vigor in response to nitrate. Use of a moderate nitrate concentration in experiments of this type provides a useful control because it permits one to impose major changes in nitrogen nutrition (Table I) with nil or slightly positive effects on nodule growth rate (Fig. 2).

In a second experiment with more replications, 100 mg NO₃⁻/N/L supplied for 36 d resulted in about a 70% decline in nodule weight/plant relative to 0-N or 30-N controls (Table II). Number of nodules/plant was reduced 30% and specific acetylene reduction activity was reduced 25% by the 100-N treatment (22). There were generally no significant effects of the 100-N treatment on concentration of ureide, ammonium, or amino nitrogen in nodules (data not shown). The overall mean concentration of ureide, ammonium, and amino nitrogen in these nodules was 190, 103, and 340 µg N/g fresh weight, respectively.

There were consistent, major effects of nitrate treatments on nitrate and nitrite concentrations in nodules (Table II). Nitrate concentration in roots was 3 to 4 times the nitrate concentration in nodules. The very low concentration of nitrate in nodules not supplied with nitrate probably reflects analytical error.

The nitrite concentrations are considered more important because of the potential inhibitory effects of nitrite. The amounts found represent concentrations of about 30 to 100 mmol/g fresh weight. It should be noted that nitrite was detected in 30-N nodules, the growth of which was not inhibited relative to the 0-N control.

NR activity was found in cytosol (Table II). (Estimates of activity per unit fresh weight can be made by assuming an average protein concentration of 12 mg/g fresh weight.) Cytosol NR activity did not increase markedly in response to nitrate supply. In contrast, NR activity in leaf blades increased 13-fold in response to 100 mg NO₃⁻/N/L (Table II). The results for nodule cytosol are anomalous because of the well known substrate inducibility of this enzyme.

These results (Table II) were obtained with nodules formed by Rhizobia in commercial inoculant. Bacteroids in these nodes did have NR (data not shown) and, even though care was taken not to disrupt bacteroids during preparation of fractions, there was a chance that at least some of cytosol NR originated in bacteroids. The cytosolic origin of this enzyme activity was con-
Table III. Effects of Nitrate on Nodules Formed by R. japonicum which Express (61A76) or Do Not Express (NR-6) NR

Mean NR activity of 61A76 bacteroids, across all treatments and harvests, was 1.50 ± 0.17 (SE) µmol/mg protein-h. NR could not be detected in any bacteroid preparations from nodules formed by NR-6. For the O-N control, results are from three harvests, 42, 55, and 56 d after planting. Otherwise, the mean and se of three or four replicates are shown, except where indicated by footnote . Experiments 3 and 4.

<table>
<thead>
<tr>
<th>NO3⁻ in Nutrient Solution</th>
<th>Duration of Treatment before Harvest</th>
<th>Nodule Fresh Wt</th>
<th>Nodule NO3⁻ Concentration</th>
<th>Nodule NO2⁻ Concentration</th>
<th>NR Activity in Nodule Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg N/L</td>
<td>d</td>
<td>mg/plant</td>
<td>µg N/g fresh wt</td>
<td>µg N/g fresh wt</td>
<td>µg N/g fresh wt</td>
</tr>
<tr>
<td>0</td>
<td>42–56</td>
<td>505 (84)</td>
<td>418 (48)</td>
<td>0.9 (0.1)</td>
<td>0.9 (0.1)</td>
</tr>
<tr>
<td>30</td>
<td>42</td>
<td>199 (57)</td>
<td>172 (27)</td>
<td>24.8 (6.1)</td>
<td>38.2 (5.8)</td>
</tr>
<tr>
<td>30</td>
<td>55</td>
<td>501 (181)</td>
<td>587 (199)</td>
<td>18.2 (3.7)</td>
<td>21.9 (0.3)</td>
</tr>
<tr>
<td>60</td>
<td>56</td>
<td>446 (103)</td>
<td>548 (112)</td>
<td>59.6 (4.1)</td>
<td>65.0 (6.6)</td>
</tr>
<tr>
<td>85</td>
<td>56</td>
<td>110 (28)</td>
<td>137 (26)</td>
<td>178.0 (38.6)</td>
<td>145.5 (10.6)</td>
</tr>
<tr>
<td>100</td>
<td>42</td>
<td>17 (5)</td>
<td>54 (14)</td>
<td>191.4*</td>
<td>166.5 (5.3)</td>
</tr>
<tr>
<td>100</td>
<td>55</td>
<td>49 (13)</td>
<td>110 (19)</td>
<td>217.5 (28.6)</td>
<td>145.0 (12.5)</td>
</tr>
</tbody>
</table>

* Single observation.

Very low enzyme activity was found in 61A76 nodules at 42 d after planting; in samples from 55 and 56 d after planting, mean activity was 60 nmol/mg protein-h.

No sample.

Fig. 3. Relationship between nodule fresh weight and nitrate concentration in nodules. Combined data from experiments 1, 2, 3, and 4 (NR-6 data omitted); nodule fresh weight computed as % of mean O-N nodule weight for each experiment and harvest. The linear regression equation is: fresh weight (%) = 97.1 – 0.48 [NO3⁻]; n = 81, r² = 0.72.

confirmed by employing a R. japonicum mutant lacking NR (Table III). The data in Table III also confirmed that, while cytosol NR increases slightly in response to increasing [NO3⁻] in nutrient solution, nodules grown without nitrate have about half as much activity as nodules grown with high NO3⁻ supply.

Where 61A76 inoculant was used, the 100-N treatment reduced nodule weight/plant by about 95% relative to the O-N control (Table III). Where NR-6 inoculation was used, the 100-N treatment reduced nodule weight by about 80%. This confirms the conclusion previously made by others (6) that the inhibition of nodule growth by nitrate does not require bacteroid NR.

The depression of nodule growth by nitrate when nodules were formed by NR-6 was accompanied by the accumulation of nitrate and nitrite in nodules (Table III). Low concentrations of NO2⁻ are indicated in NR-6 nodules grown without nitrate, but these reaction mixtures did not have the typical color and these very low values probably represent analytical error. Low concentrations of nitrite were again observed in nodules from the 30-N treatment (Table III) and growth of these nodules was not depressed relative to their respective O-N controls.

Nitrate concentrations in 61A76 nodules were similar to nitrate concentrations in NR-6 nodules (Table III). In contrast, nitrite concentrations in 61A76 nodules were generally higher than nitrite concentrations in NR-6 nodules. This suggests that the presence of bacteroid NR may be important in generating nitrite when wild-type nodules are supplied with nitrate.

The relationship between nitrate or nitrite concentration in nodules and nodule weight is illustrated in Figures 3 and 4. These data were assembled from the four experiments reported here and the variation in nodule weight among experiments and harvests made it necessary to express nodule weight as percent of mean nodule weight from the O-N control.

The relationship between nitrate concentration in nodules and nodule weight was statistically significant (Fig. 3). All of the points above the 100% line between 5 and 25 µg N/g fresh weight are from 30-N nodules. These data points tend to confound the attempt to define mathematically the effect of nitrate supply on nodule growth. Thus, it could be argued that these data should be omitted from the regression calculation. Also, it could be argued that if O-N data (cluster of points near 0 µg N/g fresh wt) are omitted the response curve appears to be logarithmic. This was found to be the case with fresh wt (%) = 183 – 74.8 (log

Fig. 4. Relationship between nodule fresh weight and nitrite concentration in nodules. Combined data for experiments 2, 3, and 4 (NR-6 data omitted); nodule fresh weight computed as % of O-N mean nodule weight for each experiment and harvest. The linear regression equation is: fresh weight (%) = 98.3 – 42.2 [NO2⁻]; n = 48, r² = 0.55.
NITRITE IN SOYBEAN NODULES

DISCUSSION

While ammonium does not inhibit nitrogenase in vitro, ammonium supplied to intact nodules does lower leghemoglobin concentration (1) and acetylene reduction activity (7, 9). Inhibition of nitrogenase in bacteroids by ammonium has recently been reported (19). Ammonium might be generated from nitrate in some bacteroids by nitrate reductase (10, 12) and nitrate reductase (8, 17). For these reasons, [NH₄⁺] in nodules was determined in most experiments reported here. There was no consistent effect of nitrate treatments on [NH₄⁺] of nodules and where treatment effects appeared to be present, they were small (<25%). Ureide and amino nitrogen concentrations in nodules were monitored in most experiments in case the inhibition of nodule growth by nitrate might be related to accumulation of these export products. Again, there was no consistent effect of nitrate on these variables. These negative results do not rule out an effect of nitrate mediated via ammonium, amino acids, or ureides, but they do not support this explanation.

The involvement of nitrite in the effect of nitrate on nodules is more logical. Results showed that a high nitrate supply in nutrient solution (100 mg N L⁻¹, 7.1 mm NO₃⁻) drastically altered the nitrogen nutrition of the plant (Table I) and the growth rate of nodules in sand culture (Fig. 2). Accumulation of high concentrations of nitrate and low concentrations of nitrite in nodules accompanied the depression of nodule growth by nitrate (Tables II and III). Furthermore, the statistical relationships between nitrate or nitrite concentration and nodule growth were highly significant (Figures 3 and 4). These results support the idea that the inhibition of soybean nodule growth by nitrate may be directly associated with nitrate metabolism, possibly with the accumulation of nitrite itself.

The finding that, when nodules are formed by Rhizobia lacking NR, nodule growth and N₂ fixing activity are still inhibited by nitrate has led other workers to conclude that nitrate metabolism is not involved in these inhibitions (6, 13). The finding that bacteroids of many Rhizobium species apparently lack NR (12) supports the idea that bacteroid NR need not be involved in the depression of nodule growth by nitrate. Thus, it is important to recognize that soybean nodule cytosol contains NR which is of host origin (Table III). In over a hundred bacteroid preparations from NR-6 nodules, NR has never been detected; thus, cross-contamination of Rhizobia in the sand culture system cannot account for the results. Evans did not find cytosol NR in soybean nodules (5). The positive results reported here may be related to the use of PVP and diithioerythritol in the extraction of enzyme.

The results reported here raise some new questions. If nitrite is the inhibitory compound, why are very low concentrations of nitrite present in nodules, the growth of which is not inhibited (moderate NO₃⁻ supply)? I can only suggest that nodules are able to tolerate very low nitrite concentrations. The inhibitory effect of nitrite might be offset by increased plant growth and, thereby, increased demand for fixed N when plants are grown with a moderate (30 mg N L⁻¹) nitrate supply. Also, the extent to which nodules can metabolize nitrite may be an important consideration. Bacteroids from Mimosa inquisitiva (8), Phaseolus vulgaris (17), and V. unguiculata nodules (27) have been shown to metabolize nitrite. However, Daniel and Appleby (4) were unable to detect nitrite reductase in bacteroids from soybean nodules, even though enzyme activity was found in R. japonicum cultured anaerobically.

A second question raised by these results is why nodules not supplied with nitrate contain significant amounts of cytosol NR. Induction of NR by metabolites other than NO₃⁻ has been reported (11). However, very low pH was required for expression of the effects (11). The temporal distribution and biochemical properties of this enzyme activity in soybean nodules merit further research.

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LITERATURE CITED

2. BRADFORD MM 1976 A rapid and sensitive method for the quantitative deter-
3. BRETTLER H, CH HANSCH T, CATE 1980 Fate of nitrate during initial nitrate utilization by nitrogen-depleted dwarf bean. Physiol Plant 48: 292-296
4. DANIEL RM, CA APPLEY 1972 Anaerobic-nitrate, symbiotic and aerobic growth of Rhizobium japonicum: effects on cytochrome Pox, other haemoproteins, nitrate and nitrite reductase. Biochim Biophys Acta 275: 347-354
5. EVANS HJ 1954 Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiol 29: 298-301