Adaptation of Nitrogen Fixation by Intact Soybean Nodules to Altered Rhizosphere pO$_2$

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ABSTRACT

The N$_2$-fixing legume nodule requires O$_2$ for ATP production; however, the O$_2$ sensitivity of nitrogenase dictates a requirement for a low pO$_2$ inside the nodule. The effects of long term exposures to various pO$_2$s on N$_2$[C$_3$H$_2$] fixation were evaluated with intact soybean (Glycine max [L.] Merr., var. Wye) plants. Continuous exposure of their rhizosphere to a pO$_2$ of 0.06 atmospheres initially reduced nitrogenase activity by 37 to 45% with restoration of original activity in 4 to 24 hours and with no further change in tests up to 95 hours; continuous exposure to 0.02 atmosphere of O$_2$ initially reduced nitrogenase activity 72%, with only partial recovery by 95 hours. Similar exposures to a pO$_2$ of 0.32 atmospheres had little effect on N$_2$[C$_3$H$_2$] fixation; a pO$_2$ of 0.89 atmosphere initially reduced nitrogenase activity by 98% with restoration to only 14 to 24% of that of the ambient O$_2$ controls by 95 hours. Re-exposure to ambient pO$_2$s of plants adapted to subambient pO$_2$s reduced N$_2$[C$_3$H$_2$] fixation to similar magnitudes as the reductions which occurred upon initial exposure to variant pO$_2$s conditions, and a time period was required to readapt to ambient O$_2$. It is concluded that the N$_2$[C$_3$H$_2$]-fixing system of intact soybean plants is able to adapt to a wide range of external pO$_2$s as probably occur in soil. We postulate that this occurs through an undefined mechanism which enables the nodule to maintain an internal pO$_2$ optimal for nitrogenase activity.

Oxygen is essential for ATP production by oxidative phosphorylation to sustain the symbiotic N$_2$ fixation process in legume nodules; however, the nitrogenase enzyme is remarkably sensitive to O$_2$ with rapid inactivation occurring upon exposure to ambient O$_2$ concentrations (4). Recent research findings reveal the systems used by the legume nodule to satisfy these O$_2$ needs. It has been suggested (4) and there is evidence (6, 23) that leghemoglobin in the soybean (Glycine max [L.] Merr.) nodule facilitates a high O$_2$ flux to the bacteroids at a very low O$_2$ concentration estimated (2, 23) to range from 0.005 to 0.010 mm Hg, or lower. Direct measurements of the pO$_2$ in the central tissue of the nodule show extremely low levels and provide evidence for a barrier in the inner part of the nodule cortex which limits O$_2$ diffusion from the external environment (22). Other researchers suggest that there is a continuous network of intercellular spaces which permits gaseous diffusion of O$_2$ from the external atmosphere to the host cells of the cortex, the interstitial cells, and the mitochondria at the periphery of the bacteroid-containing cells, all of which represent a continuum of O$_2$ sinks (5).

However, in spite of these elaborate mechanisms, the N$_2$-fixing activity of the soybean nodule appears to be limited by the pO$_2$ in air since maximal rates of N$_2$ fixation, measured in short term experiments using intact nodules, usually are increased by supra-ambient pO$_2$s (3, 7). A comprehensive study, using field-grown plants throughout the complete growth cycle, gave maximal rates of N$_2$ fixation at 0.26 to 0.41 atm O$_2$ with substantial decreases at subambient pO$_2$s (9).

In the soil environment of the nodule, pO$_2$s greater than 0.21 atm probably do not occur naturally, while subambient pO$_2$s probably exist under various environmental circumstances (4, 17, 21). Thus, an ideal legume N$_2$-fixing system should be able to adapt rapidly to a range of subambient pO$_2$s such as occur in soil so as to maximize N$_2$ fixation. The aerobic N$_2$-fixing bacterium Azotobacter possesses mechanisms that protect its nitrogenase from O$_2$ extremes (18), but based on previous research findings (3, 7), it would appear that agriculturally important legumes cannot successfully cope with O$_2$ fluctuations in the rhizosphere. Through the use of long term, intact plant studies, we have discovered and report here that the N$_2$-fixing system in the soybean nodule can adapt to such a variant range of rhizosphere pO$_2$s. A preliminary report of this work has been presented (9).

MATERIALS AND METHODS

Plant Culture. Two long term experiments were conducted to study N$_2$[C$_3$H$_2$] fixation responses by nodules exposed to various rhizosphere pO$_2$s, and culture of the intact Wye soybeans used in both of the experiments was similar. Seeds treated with Agway, peat-based Rhizobium japonicum inoculum were planted in sterile silica sand contained in 18-cm diameter pots modified to serve as incubation vessels (8, 19) for assays of N$_2$[C$_3$H$_2$] fixation. Seedlings emerged after 5 days. The seedlings were inoculated with 50 ml of a 20% (v/v) peat-based inoculant drench to further insure nodulation and they were thinned to one plant/pot 1 week after planting. The plants were supplied with 150 ml/pot of N-free, Hoagland nutrient solution (14) on alternate days and deionized water on other days. In the first experiment, 200 ml/pot of full strength, complete Hoagland solution were applied once 2 weeks after planting, and in the second experiment, a 200 ml/pot application of 10% (v/v), complete Hoagland solution was applied 3 weeks after planting to provide the plants with a N source until the nodules became functional. The soybeans were grown in a controlled environment room having a 24/18 C day/night temperature cycle, a 46,300 lux, 12-hr photoperiod, and a 75% relative humidity. Exposures of the nodulated intact roots to variant pO$_2$s were commenced when the plants were 42 days of age and in the pod-elongation stage of development.

Assay for N$_2$[C$_3$H$_2$] Fixation. In both experiments, the acetylene-ethylene procedure (13) was used to measure N$_2$[C$_3$H$_2$] fixation.
fixing activity. Intact plant incubations for measurement of nitrogenase activity were similar to those previously described (8, 19), and after 15 and 30 min of incubation, 10-cm² gas samples were withdrawn and placed into 10-ml evacuated blood sample tubes (11) for subsequent gas chromatographic assay (8, 13) of C₂H₂ and C₂H₄. Because the C₂H₄ was used in the first and second experiments was insufficient to saturate nitrogenase (0.026 and 0.028 atm, respectively) N₂[C₂H₂] fixation rates were adjusted to maximum velocity using the relationship $V_m = V + (V/Km)$ where $V$ is the velocity of the reaction at substrate concentration $[S]$ and $Km$ is the average reported Michaelis-Menten constant of 0.006 atm of C₂H₂ for nitrogenase (12). Incubation vessels contained from 775 to 710 cm³ of free air space in the first and second experiments, respectively. At field capacity conditions under which all assays were performed, the moisture content of the sand averaged 15.2%, and solubilities of C₂H₂ and C₂H₄ were considered in rate computations of N₂[C₂H₂] fixation.

**Experimental Design.** Four rhizosphere P₂O₅ were used in the first long term study to evaluate P₂O₅ effects on N₂[C₂H₂] fixation by nodulated intact plants. Exposure to a desired P₂O₅ was accomplished by purging N₂O₅CO₂ gas mixtures through the incubation vessels from top to midside port at a rate of 200 cm³/min between assays. Concentrations of O₂ in the N₂O₅CO₂ purge mixtures were measured twice daily with an O₂ electrode, and P₂O₅ averaged 0.06 ± 0.0, 0.21 ± 0.0, 0.32 ± 0.01, 0.89 ± 0.01 atm for the four treatments. No attempt was made to control the P₂O₅ in the purge mixtures of the first experiment; however, calculations based on gas dilutions indicated that the P₂O₅ ranged from approximately 65 to 320 µl/l for the various treatments. The P₂O₅ of 0.06 atm was obtained by addition of N₂ to air, whereas the P₂O₅ of 0.32 and 0.89 atm were obtained by addition of O₂ to air. Incubations for measurement of N₂[C₂H₂] fixation at the four O₂ concentrations were performed at 24°C in different ArO₂C₄H₄ gas mixtures when the experiment was initiated and after purging with the equivalent O₂-containing N₂O₅CO₂ gas mixture for average times of 3.9, 23.7, and 47.4 hr. Initially, there were four replications of each of the four P₂O₅ treatments, but following each incubation for measurement of N₂[C₂H₂] fixation, with the exception of the ambient P₂O₅ treatment, one plant of each nonambient P₂O₅ treatment was immediately reincubated under ambient O₂ conditions. Subsequent purgings and N₂[C₂H₂] fixation assays on these plants were conducted under ambient O₂ conditions for the duration of the study to test the reversibility of the previously imposed variant P₂O₅ treatment effects. After the N₂[C₂H₂] fixation assays were completed, the silica sand was gently washed from the roots and nodules, the nodules were stripped from the roots, and the nodule fresh weight/stem was recorded. The data were expressed on a specific activity basis (µg N₂[C₂H₂] fixed/g nodule fresh weight/hr). To test P₂O₅ treatment effects, the N₂[C₂H₂] fixation specific activity data were subjected to a $\chi^2$ transformation to obtain homogeneous data, and a completely random analysis of variance with unequal replication was performed. Transformed transformation means were compared by Duncan's new multiple range test as modified by Kramer for unequally replicated treatments (20).

The second long term adaptation study was similar to the first except that longer times of exposure to purge mixtures containing three different P₂O₅ were used between N₂[C₂H₂] fixation incubations, and the balanced experiment was repeated five times. The partial pressure of O₂ in the continuous N₂O₅CO₂ purge mixtures averaged 0.02, 0.06, and 0.21 atm as measured with an O₂ electrode. The P₂O₅ in the purge mixtures was controlled in the range from 333 to 339 µl/l, as determined by IR gas measurements. Initial assays for N₂[C₂H₂] fixation were performed at 24°C in three ArO₂C₄H₄ gas mixtures with P₂O₅ comparable to those of the purge gas mixtures, and subsequent assays were conducted in each of the three P₂O₅ treatments after continuous purging with equivalent O₂-containing N₂O₅CO₂ gas mixtures for 4, 23.9, 47.3, 71, and 95 hr. After continuous exposure to the three P₂O₅ for 99 hr, the intact plants were reincubated in ambient O₂ to test the reversibility of previous P₂O₅ adaptation effects. To confirm that P₂O₅ during N₂[C₂H₂] fixation assays were near those of the treatment purge and incubation gas mixtures, 2-cm³ gas samples were withdrawn with a gas-tight syringe from the rhizosphere of four replicates of the three P₂O₅ treatments at the start and end of the 30-min incubations conducted after 71 hr. The O₂ content of the rhizosphere during incubation was determined with a Perkin Elmer 900 gas chromatograph equipped with a thermal conductivity detector and a specially pretreated (15) stainless-steel columns (2.7 m × 0.3 cm diameter) with a molecular sieve 5A packing connected in series and operated at 11°C. At the end of the experiment, the sand was washed from the roots and nodules, the nodules were stripped from the roots, and the nodule fresh weight was recorded enabling N₂[C₂H₂] fixation rates to be computed on a specific activity basis. Data collected to assess the reversibility of long term P₂O₅ effects were analyzed separately from the variant P₂O₅ exposure data; however, both sets of data were subjected to $\chi^2$ transformations and analyzed as factorial experiments to test P₂O₅ treatment and P₂O₅ incubation time effects and the interaction of these two factors. Duncan's new multiple range test was used to compare treatment means.

**RESULTS AND DISCUSSION.**

The rate of N₂[C₂H₂] fixation (Table 1) was reduced 37% from the ambient O₂ control rates in intact plants following initial exposure of the rhizosphere to a subambient P₂O₅ of 0.06 atm, but after 4 hr of continuous exposure to this low P₂O₅, the N₂[C₂H₂] fixation specific activities were restored and remained at levels comparable to those of the ambient O₂ controls throughout the remainder of the 2-day exposure. The N₂[C₂H₂] fixation specific activities of the ambient O₂ control plants did not differ significantly over the 2-day study. Nitrogenase activity appeared to be slightly, although not significantly, reduced following exposure to 0.32 atm of O₂, and continuous exposure for 2 days gave activity indistinguishable from the controls. Initial incubations conducted in 0.89 atm of O₂ severely reduced the N₂[C₂H₂] fixation specific activity, and subsequent exposure to this supra-ambient P₂O₅ resulted in a 10-fold recovery (Table 1, read horizontally) of the nitrogenase activity. However, the absolute recovery values of N₂[C₂H₂] fixation in 0.89 atm of O₂ were only 14 to 24% of those of the ambient controls, and this lack of total recovery probably was associated with the O₂ sensitivity of the nitrogenase enzyme.

Re-exposure of the rhizosphere of intact nodulated plants to the ambient P₂O₅ initially reduced N₂[C₂H₂] fixation in those treatments where the intact plants had been previously exposed to P₂O₅ other than ambient. The reductions in nitrogenase activity upon re-exposure to ambient O₂ were particularly evident in plants previously exposed to a P₂O₅ of 0.06 atm for 24 and 48 hr, and reductions in nitrogenase activity were not as evident in the supra-ambient P₂O₅ treatments (Table 1, read vertically). Results of these studies also indicated that a time period was required for re-establishment of the control rate of N₂[C₂H₂] fixation upon re-exposure of the intact plant to ambient O₂.

The second study was conducted to examine: (a) the effect of subambient O₂ concentrations over a longer exposure period; and (b) the range of subambient O₂ concentrations to which the nodule can adapt. The lowest rhizosphere P₂O₅ was 0.02 atm, and it was provided to determine if N₂[C₂H₂] fixation adaptation would still occur under conditions where oxidative phosphorylation and ATP production rates would be expected to be more severely reduced initially. Specific activities of N₂[C₂H₂]
fixation were reduced 72% below the ambient O₂ controls when the nodulated roots of intact plants were first incubated with the rhizosphere containing a pO₂ of 0.02 atm (Table II). Plants continuously exposed to a pO₂ of 0.02 atm only partially recovered their nitrogenase activities, and with the exception of the incubation performed after 3 days, all subsequent N₂[C₂H₄] fixation rates were below those of the ambient O₂ controls. Significant reductions of 45% in nitrogenase activity occurred when the intact plants were initially flushed and incubated with the 0.06-atm O₂ gas mixture, but after 1 day of continuous exposure to this subambient pO₂, the inhibitory effects of low O₂ were overcome, and subsequent N₂[C₂H₄] fixation specific activities were comparable to those of ambient O₂ controls. As in the first experiment, N₂[C₂H₄] fixation specific activities of the ambient O₂ control plants did not differ during the study. The pO₂ treatment × pO₂ incubation time interaction was highly significant because of the differential pO₂ treatment adaptation responses.

The adaptation of the N₂[C₂H₄] fixation system to variant pO₂s was further verified by reincubating in ambient O₂ the intact plants that had been exposed to the two variant and ambient O₂ concentrations for 99 hr. When incubated in ambient pO₂, N₂[C₂H₄] fixation specific activities of plants previously exposed to pO₂s of 0.02 and 0.06 atm were only 29 and 55% of respective 95-hr specific activities, and respective 99-hr nitrogenase activities were only 15 and 58% of those of the ambient O₂ controls (Table III). The 95- and 99-hr ambient O₂ control nitrogenase activities did not differ at the 5% level. Such results again confirmed that sensitivity of N₂[C₂H₄] fixation activity developed upon re-exposure to ambient O₂ in intact plants conditioned, or partially conditioned, to subambient pO₂s. Sensitivity upon re-exposure to ambient O₂ was greatest in those treatments where the rhizosphere pO₂ deviated most from ambient, which resulted in a significant pO₂ treatment × pO₂ incubation time interaction.

Gas chromatographic measurements demonstrated that pO₂s averaged 0.02, 0.07, and 0.20 atm at the start and 0.03, 0.07 and 0.21 atm at the end of the 30-min N₂[C₂H₄] fixation incubations for the low, intermediate, and ambient pO₂ treatments, respectively. These O₂ concentrations averaged slightly higher than those measured for the pO₂ component of the Ar/O₂/C₂H₄ incubation gas mixture; however, the measurements confirmed that the pO₂s in the rhizosphere were within 0.01 atm of those of the variant pO₂ purge streams. The fact that the O₂ concentrations remained stable throughout the incubation period indicated that root and nodule respiration did not consume significant quantities of O₂, and that the incubation vessels were effective in preventing exchange of O₂ from the external environment with that of the rhizosphere. In addition, it has been demonstrated that in soybeans, ¹⁵O-labeled O₂ movement from the shoot to the roots does not occur through the stem (16).

We believe that the adaptation response of the N₂ fixation

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Table I. Specific Activities of N₂[C₂H₄] Fixation by Intact Soybean Roots following Various Times of Exposure to Sub- and Supraambient Rhizosphere pO₂s

<table>
<thead>
<tr>
<th>Mean Time from 0 hr until Reincubation in 0.21 atm O₂</th>
<th>Mean Incubation Times for Plants Continuously Exposed to Indicated pO₂’s (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO₂ atm</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>0.0</td>
</tr>
<tr>
<td>0.11</td>
<td>23.8 (100.4)</td>
</tr>
<tr>
<td>0.32</td>
<td>47.8 (151.5)</td>
</tr>
</tbody>
</table>

*Numbers followed by the same letter do not differ at the 5% level by Duncan’s new multiple range test as modified by Kramer for unequal replication.

Table II. Specific Activities of N₂[C₂H₄] Fixation by Intact Soybean Roots following Various Times of Exposure to Subambient Rhizosphere pO₂s

| Mean Incubation Times for Plants Continuously Exposed to Indicated pO₂’s (hr) |
|----|----|
| pO₂ atm | 0.00 | 4.0 | 23.9 | 47.3 | 71.0 | 95.0 |
| | | | | | | |
| 0.02 | 26.2 (93.8) | 19.3 (84.9) | 36.6 (88.4) | 47.0 (88.4) | 73.0 (88.4) | 65.2 (88.4) |
| 0.06 | 51.8 (159.4) | 136.2 (156.8) | 138.3 (156.8) | | |
| 0.21 | 94.8 (185.5) | 112.5 (128.5) | | | |

*Numbers shown in parentheses are nonreplated N₂[C₂H₄]-fixation specific activities by intact soybean plants previously exposed to nonambient O₂ conditions and reincubated in ambient O₂.

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system of intact soybean nodules to variant PO₂S is different from that previously reported in Azotobacter where both respiratory and conformational protection mechanisms are postulated (18). An impressive feature of one of the O₂ response in Azotobacter is the rapidity with which the N₂ fixation system can either reversibly "switch on" or "switch off" within minutes to protect nitrogenase. Nitrogen fixation activities of intact soybeans were decreased rapidly following exposure to variant PO₂S within a wide range (0.02-0.32 atm O₂), but unlike Azotobacter, control nitrogenase activities were re-established after a considerable period of time of exposure to a variant PO₂. Upon re-exposure of intact plant nodules to ambient O₂ conditions, N₂[C₂H₄] fixation rates were again rapidly repressed, and it appeared that an equivalent lengthy time period was required to re-establish control rates. There are many possible factors that could be involved in the adaptive response of intact plants to variant PO₂S. The response may involve changes in cortical resistance to O₂ diffusion, alterations in vesicle membrane resistance to O₂ transport, changes in leghemoglobin forms (10) or content, conformational changes in nitrogenase, respiratory changes, translocation alterations, or changes in Cyt P-450 which has been implicated in the O₂ transport scheme (1). Presently, the physiological nature of the adaptive response is an enigma; however, one interpretation of the data is that nodules of intact soybean plants exposed to a variant PO₂ have mechanisms that permit the maintenance of an optimal internal PO₂ for ATP production. This capability probably has agronomic significance in soybean production situations where the soil PO₂ is reduced by conditions such as flooding or soil compaction.

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LITERATURE CITED

Table III. Specific Activities of N₂[C₂H₄] Fixation in Ambient pO₂ Following Long Term Exposure to Subambient pO₂s

<table>
<thead>
<tr>
<th>pO₂ atm</th>
<th>Exposed to Indicated</th>
<th>Reincubated in 0.21-atm</th>
<th>O₂ after 99 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>138 N₂[C₂H₄] fixed/g</td>
<td>2.2 13.1 cd</td>
<td>2.2 129.1 a</td>
</tr>
<tr>
<td>0.02</td>
<td>65.2 c</td>
<td>19.1 d</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>137.2 a</td>
<td>75.0 bc</td>
<td></td>
</tr>
<tr>
<td>0.21</td>
<td>113.1 ab</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers followed by the same letter do not differ at the 5% level by Duncan's multiple range test.