Effect of pO$_2$ on Growth and Nodule Functioning of Symbiotic Cowpea (Vigna unguiculata L. Walp.)$^1$

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ABSTRACT

Nodulated cowpea (Vigna unguiculata L. Walp. cv Vita 3:Bradyrhizobium CB 756) plants were cultured with their whole root system or crown root nodulation zone maintained for periods from 5 to 69 days after planting in atmospheres containing a range of pO$_2$ (1-80%, v/v) while the rest of the plant grew in normal air. Growth (dry matter yield) and N$_2$ fixation were largely unaffected by pO$_2$ from 10 to 40%. Decrease in fixation at pO$_2$ below 5% was due to lower nodulation and nodule mass and, at pO$_2$ above 80%, to a fall in specific N$_2$-fixing activity of nodules. Root:shoot ratios were significantly lower at pO$_2$ below 2.5%. The effect of pO$_2$ on nitrogenase activity (acetylene reduction), both of whole nodulated root systems and crown root nodulation zones, varied with plant age but was generally lower at supra- and subambient extremes of O$_2$. H$_2$ evolution showed a sharp optimum at 20% O$_2$ but was at most 4% of total nitrogenase activity. The ratio of CO$_2$ evolved to substrate ($C_2H_4$+H$^+$) reduced by crown root nodulation zones was constant (6 moles CO$_2$ per mole substrate reduced) from 2.5 to 60% O$_2$ but at levels below 2.5 and above 80% O$_2$ reached values between 20 and 30 moles CO$_2$ per mole substrate reduced. Effects of long-term growth with nonambient pO$_2$ on adaptation and efficiency of functioning of nodules are discussed.

Lowered nitrogenase activity of legume root nodules under a variety of imposed experimental conditions have been interpreted as being due to a limitation of O$_2$ supply to bacteroids (4, 8, 12, 17, 29). Recent evidence further suggests that nodules of some symbioses are able to protect nitrogenase from O$_2$ damage by a rapid increase in resistance to diffusion of gases (21, 29). This response appears to be mediated, in part, by operation of a biologically controlled “variable diffusion barrier” possibly located at, or close to, the inner cortex of the nodule (13, 26, 30, 31).

Although the mode of operation of this diffusion barrier remains unknown, it appears that many of the changes in gaseous diffusion resistance of nodules relate to changes in gaseous conductance. A temperature effect on gaseous permeability has been reported for the non-legume, Alnus rubra (28), as well as for soybean nodules (17, 24, 25, 27), while in the latter, permeability to gases has been shown to change with water status (9). Thus, mechanisms regulating O$_2$ supply may underlie variation in field performance of N$_2$ fixation in legumes. Few studies have examined directly the response of fixation to changes in the permeability of nodules, although decreasing O$_2$ concentrations from ambient to 10% around nodulated soybean roots resulted in a 63% increase in the permeability of nodules to gas, while increasing the rhizosphere O$_2$ concentration led to a decrease in permeability (26). These data suggest that soybean nodules are able to regulate the permeability of their tissue components to O$_2$ in response to changing rhizosphere pO$_2$ and, as a consequence, maintain N$_2$ fixation at relatively constant rates.

The present study reports the effect of O$_2$ level on plant growth, nodule development, N$_2$ fixation, and nitrogenase activities of intact nodules on cowpea plants cultured either with their whole nodulated root systems or their root crown nodulation zones alone maintained in a range of sub- and supra-ambient O$_2$ throughout extended periods of growth.

MATERIALS AND METHODS

Plant Material

Cowpea (Vigna unguiculata L. Walp. cv Vita 3) seeds were inoculated with a pea suspension of Bradyrhizobium strain CB 756 and allowed to germinate in N-free silica sand in freely draining pots. Seventeen seeds were sown per pot and left to grow in a naturally lighted glasshouse in which the air temperature was controlled to provide a daytime maximum of 30 °C and minimum at night of 20 °C. At 5 or 8 DAP$^3$, infected seedlings were selected for uniformity of size and appearance, and the sand was gently washed away from their roots. At this stage in their development, the seedlings bore a pair of unifoliate leaves and no nodules were evident on the root system.

Culture of Plants with Whole Root Systems Exposed to Different Atmospheres

Selected, 5-d-old seedlings were transferred to $\frac{1}{2}$ strength Hoagland nutrient solution containing no combined N in 3.5 L plastic liquid culture vessels as described previously (2). The level of culture solution was adjusted so that the upper crown root nodulation zone was not wetted but was maintained in a gaseous environment. Five seedlings were sealed into a lid of each pot with Terosan, Heidelberg, FRG, and the shoot system was supported within the normal am-

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$^3$ Abbreviations: DAP, days after planting seed; DM, dry matter.
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Figure 1. Effect of \( pO_2 \) around the nodulated root system of cowpea on dry matter yield (A) and \( N_2 \) fixation (B). Plants were harvested at 22, 38 and 42 DAP. \( N_2 \) fixation was measured as total plant N minus seed N. Values are means ± SE (\( n = 4-6 \)). Where no bar is shown, the SE is smaller than the symbol.

Figure 2. Effect of \( pO_2 \) on nodule number (A), nodule dry matter (B) and nodule specific activity (C) of cowpea plants grown with whole nodulated root systems exposed to different \( pO_2 \) from 5 DAP. Plants were harvested at 22, 38 and 42 DAP. Specific activity was computed using the rates of \( N_2 \) fixation from Figure 1. Values are means ± SE (\( n = 5-6 \)). Where no bar is shown, the SE is smaller than the symbol.
Table I. Effect of pO₂ on Ratio of Root DM to Shoot DM of Cowpea Plants Cultured with Their Whole Nodulated Root Systems Exposed to Different pO₂ from 5 DAP

<table>
<thead>
<tr>
<th>O₂ Level (%)</th>
<th>Root:Shoot Ratio (DM basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 d</td>
</tr>
<tr>
<td>1</td>
<td>0.22 ± 0.07*</td>
</tr>
<tr>
<td>2.5</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>0.51 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>20</td>
<td>0.59 ± 0.16</td>
</tr>
<tr>
<td>40</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td>60</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>80</td>
<td>0.76 ± 0.14</td>
</tr>
</tbody>
</table>

*Mean ± se (n = 4–6). b Not determined.

Each pot via a similar tube fitted with a plastic funnel. Plants were maintained under these culture conditions for varying periods up to 42 DAP.

Culture of Plants with Nodulated Crown Root Region Exposed to Different Atmospheres

Selected 8-d-old seedlings cultured in sand and infected with *Bradyrhizobium* as described above were transferred to liquid culture containers so that the crown root nodulation zone was enclosed in a small growth chamber and separated from the rest of the root system which was bathed in nutrient solution and aerated with ambient air (150–200 mL·min⁻¹). The small growth chambers, which were described previously (14), were fabricated from PVC and had a cylindrical inner space of about 10 mL. A moisturized gas stream, containing a range of O₂ levels, was passed through three or four of the small chambers connected in series at 150 to 200 mL·min⁻¹. There was negligible nodulation of lateral roots so that the full complement of nodules on these plants developed and were enclosed within the small chambers. Plants were maintained under these conditions, without opening the small chambers, for varying periods up to 69 DAP.

Preparation and Use of Gas Mixtures

A range of O₂/N₂ mixtures (1, 2.5, 5, 10, 20, 40, 60 and 80% (v/v) O₂ in N₂) was generated with Wosthoff gas mixing pumps (5) and Hasting's mass flow controllers (Hasting's Flow Controller, Hampton, VA). Industrial grade gases (Commonwealth Industrial Gases, Perth, WA) were used without purification, but the mixtures generated were moistened by bubbling through water at ambient glasshouse temperature. The O₂ and N₂ contents of all mixtures were monitored by gas-liquid chromatographic analysis of samples taken from the supply lines to pots as described previously (2). When gas cylinders were changed, the pots were temporarily sealed to preclude atmospheric contamination. Such periods were infrequent and rarely exceeded 10 min. In these experiments, ambient air was denoted as 20% O₂. The same gas mixing system was also used to generate three-way mixtures comprising 2.5, 5, 10, 20, 40, or 80% (v/v) N₂, 20% (v/v) O₂ and the balance as Ar.

Measurement of Respiration and H₂ Evolution

Production of CO₂ by whole nodulated root systems of intact water culture-grown plants or by intact segments of the crown root nodulation zone was measured by sampling the gas stream before its entry to and after its exit from the enclosing container in each case. Prior to sampling, the flow rates of gas passing through each pot or small growth chamber were measured with calibrated flow tubes. Then 8 to 10 replicate 1 mL samples were collected from the lines for each pot with gas-tight syringes, and CO₂ content was measured as described previously (1).

H₂ production by enclosed crown root nodulation zones was also measured in 1-mL samples of the flowing gas streams taken as described above. H₂ content was assayed by gas liquid chromatography (3).

Measurement of Acetylene Reduction

An open flow-through system for acetylene reduction assay was used which was similar to that described by Minchin et al. (15). It consisted of a gas-mixing system linked to two gas sampling ports by plastic tubing. Sealed culture vessels to be assayed were introduced into the acetylene-containing gas stream by connecting the inlet of the vessel to the gas line and the outlet to a suba-sealed sampling port. Flow rates of cylinder gas were controlled by valves and calibrated flow tubes to produce streams of either 10% C₂H₂, 20% O₂ and 70% N₂ (v/v), or 10% C₂H₂ and any of the O₂ treatment concentrations used (i.e. 1, 2.5, 5, 10, 40, 60, or 80%) with the balance as N₂. The O₂ content of mixtures was checked by gas chromatographic analysis as described previously (2). The gas line containing uniformly mixed gas from a mixing vessel was divided using a manifold and passed through flow tubes to make two separate, identical streams, each flowing at 150 mL·min⁻¹. In this way, duplicate pots of a treatment could be assayed at the same time. Samples (1 mL) of the gas stream were taken for measurement of ethylene content by gas liquid chromatography (2, 3). Collection of gas from

Table II. Nodule Development, Plant Growth, and N Accumulation in Cowpea with Root System Maintained in Atmospheres Containing 20% O₂ in Different pN₂ from 14 to 29 DAP

<table>
<thead>
<tr>
<th>N₂ Level (%)</th>
<th>Nodule DM mg/plant</th>
<th>Total DM Yield mg</th>
<th>Total N mg/plant</th>
<th>Increment during Exposure to Different pN₂ mg N/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>24.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>454 ± 26</td>
<td>6.0 ± 0.3</td>
<td>221 ± 26</td>
</tr>
<tr>
<td>5</td>
<td>34.4 ± 2.0</td>
<td>623 ± 22</td>
<td>9.7 ± 0.4</td>
<td>390 ± 22</td>
</tr>
<tr>
<td>10</td>
<td>41.3 ± 2.7</td>
<td>637 ± 36</td>
<td>15.6 ± 0.9</td>
<td>404 ± 36</td>
</tr>
<tr>
<td>20</td>
<td>38.7 ± 1.0</td>
<td>637 ± 34</td>
<td>19.8 ± 1.0</td>
<td>404 ± 34</td>
</tr>
<tr>
<td>40</td>
<td>40.4 ± 2.5</td>
<td>633 ± 28</td>
<td>22.4 ± 1.0</td>
<td>400 ± 28</td>
</tr>
<tr>
<td>60</td>
<td>40.0 ± 2.7</td>
<td>637 ± 60</td>
<td>22.2 ± 2.1</td>
<td>404 ± 60</td>
</tr>
</tbody>
</table>

<sup>a</sup>The total DM yield was 232 ± 7 mg/plant and N content 5.6 ± 0.29 mg/plant at the time of application of different pN₂ to nodulated root systems of 14-d-old seedlings. Mean ± se (n = 5–6).

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sampling ports using 1 mL gas tight syringes commenced once the culture vessels with nodulated root systems (or crown root segments) were connected into the acetylene-containing gas stream and continued at recorded time intervals for 30 min or more.

Harvest and Analysis of Plant Material

Plants were harvested from individual culture vessels at varying times from 22 to 69 DAP and divided into component parts. DM was measured after air-drying fresh material at 80 °C to a constant weight, and N content in samples of dried milled material was determined by a standard Kjeldahl digestion procedure. N₂ fixation was calculated from total plant N minus seed N. Nodule growth was measured as changes in DM and/or number at each harvest.

RESULTS

Growth and N₂ Fixation

Growth of plants, cultured with their whole nodulated root systems in different pO₂, was not affected by O₂ level around the root system up to 22 d but subsequent growth at sub-ambient O₂, as well as at levels above 20% O₂, was inhibited (Fig. 1A). However, even at 22 d, N₂ fixation declined sharply at low and supra-ambient O₂ (Fig. 1B). Subsequently N₂ fixation showed a broadening plateau with O₂ level, so that by 42 DAP those in 5 to 60% O₂ yielded similar, high rates of fixation. The marked inhibition of fixation at low O₂ was largely a consequence of a lower level of nodulation (Fig. 2A) and nodule mass (Fig. 2B), while at high levels of O₂ (80%) inhibition was due to a sharp fall in the specific N₂-fixing activity of nodules (Fig. 2C), although there was some decline also in both nodulation and nodule mass at 80% O₂. Root growth into the nutrient solution was significantly decreased relative to shoot growth in sub-ambient O₂ compared to that in air (20% O₂) or levels above air and, as a consequence, root:shoot ratios were lower at low pO₂ (Table I).

The gas mixtures containing supra-ambient levels of O₂ also contained progressively sub-ambient N₂ concentrations. However, Table II shows that increases in both total plant and nodule biomass (measured in each case as DM) were unimpaired by 15 d growth at N₂ levels as low as 5% in the gaseous atmosphere surrounding the roots but that each was significantly lower at 2.5% N₂. The amount of N fixed was also significantly lower at N₂ levels below 20%. Thus, where nodulated root systems were maintained in atmospheres containing supra-ambient O₂ levels (40–80%), the lowest N₂ level used to make up the gas mixtures (20% N₂ with 80% O₂) would not have been limiting to N₂ fixation.

Plants which were grown so that only the nodulated root crown was exposed to different pO₂, while the rest of the root system was maintained in liquid culture with an ambient atmosphere, showed relatively normal root development at all O₂ levels used and, as a consequence, root:shoot ratios did not vary between treatments (data not shown). Growth was inhibited at higher and lower O₂ levels, especially at the two extreme concentrations, and this was reflected in plant DM, nodule DM and N₂ fixation (Table III). The specific activity of nodules was relatively constant from 5 to 40% O₂ but fell to less than half that in air at the high and low extremes of O₂. The number of nodules per plant also declined progressively at lower O₂ levels and slightly at 80% O₂ (Table III).

Nitrogenase Activity

Typical time courses of ethylene production by whole nodulated root systems or by nodulated crown root zones following their exposure to a flowing 10% acetylene atmosphere are shown in Figure 3, A and B, respectively. Although pO₂ had a marked effect on the time taken to reach maximum acetylene reduction by whole nodulated roots (Fig. 3A), there was no subsequent decline in nitrogenase activity up to 35 min. A similar response was found for nodulated crown root zones, although at 10 and 20% O₂ a small peak of activity preceded a steady rate of reduction (Fig. 3B). Similar time courses were recorded for all acetylene reduction assays and rates determined from the maximum values obtained, or, where a steady rate occurred, from the average of values after the maximum was reached.

The measurements of nitrogenase activity by acetylene reduction revealed a complex interaction with both pO₂ and...
sequential measurements indicated significantly lower activities at 41 and 42 d for plants developing in 1, 2.5, 5, and 80% O₂ compared to those in air. By 47 and 55 d, however, it was only those grown in 1, 2.5, and 80% O₂ which continued to show lower levels of activity. H₂ evolution by the nodulated root crown at 49 DAP (Fig. 5) also showed a marked response to pO₂. No H₂ could be detected at 1, 2.5, 5, 60, or 80% O₂ but was measurable at 10, 20, and 40% O₂ with a sharp peak at 20%. The highest rates of H₂ evolution were at most 4% those of acetylene reduction (on a molar basis).

**Respiration**

CO₂ production by the nodulated root crown of plants at 36 DAP showed a broad optimum between 10 and 40% O₂ with markedly lower values at low O₂ and slightly lower ones at high O₂ (Fig. 6). Taking the sum of the average acetylene reduction rates and H₂ evolution at each O₂ level (from Fig. 5) as a measure of total nitrogenase activity, the molar ratio of CO₂ evolved to substrate reduced was relatively constant at around 6 mol CO₂ released per mol substrate reduced from 2.5 to 60% O₂ but rose very sharply to values of 20 or more at 1 and 80% O₂ (Fig. 6).

**DISCUSSION**

There have been a number of suggestions that supply of O₂ is limiting to N₂ fixation by nodules under normal ambient

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**Figure 3.** Time course measurement of ethylene production by cowpea plants grown with their whole nodulated root system (A) or their nodulated crown zone only (B) exposed to different pO₂ from 5 (A) or 8 (B) to 41 DAP. The pO₂ used for growth was maintained in each case during the assays.

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**Figure 4.** Effect of pO₂ on acetylene reduction by intact nodulated root system of cowpea. Plants (5 d old) were cultured with their whole nodulated root system maintained in different pO₂ and assayed at 18–30 DAP. Values are means ± SE (n = 4).
atmospheres. Minchin et al. (16) cultured pea and soybean plants for 28 d (following seed inoculation) with 10, 21, or 30% O2 and failed to show an effect of O2 on plant growth or N2 fixation. They (16) suggested that the lack of response indicated endogenous regulation of gaseous diffusion resistance in nodules so that, despite variation in external pO2, the same degree of O2 limitation occurred in all treatments. Similar results have been described by Silvester et al. (22, 23) for the Frankia symbioses with Myrica gale and Alnus incana grown in 5 to 40% O2, although earlier studies by Bond (7) and Ferguson and Bond (11), using symbiotic soybean and red clover, have shown reduced growth and N2 fixation at O2 levels below 10%. Generally, a result similar to these was obtained for cowpea in the present study with a broad plateau of plant growth and N2 fixation spanning a 12-fold range of pO2 from 5 to 60%. At very low O2 there was a deficiency of supply, and at high O2 the excess was toxic, possibly as a consequence of inactivation of nitrogenase. Inhibition of N2 fixation and plant growth at supra-ambient O2 was in fact due to high O2 and was not a consequence of N2 limitation in gas mixtures containing as little as 20% N2 (in the case of 80% O2). The response to pO2 found with cowpea (Table II) was similar to that shown by nodulated Lupinus albus (18), indicating an apparent Kn (N2) for N2 fixation in planta of around 10% (v/v). Interestingly, at 22 DAP pO2 did not affect overall plant growth, even though fixation of N2 at low and high O2 was inhibited by as much as 90%, suggesting that, up to this time, input of N was not limiting. Subsequently, the growth of cowpea plants at different pO2 reflected the supply of fixed N which was itself determined by a combination of total mass and specific activity of nodules.

Infection with Bradyrhizobium and, presumably, nodule initiation occurred largely in the period of sand culture in ambient air (5 or 8 d) before plants were transferred to rooting atmospheres of different pO2. This, however, did not eliminate a subsequent inhibitory effect of low O2 on nodulation. Root growth, particularly where plants were grown with their whole root systems in different pO2, was restricted at the lowest O2 levels so that possibly any continuing infection and nodule initiation, which occurred at higher pO2 following transfer of seedlings to liquid culture, might have been limited under these conditions by a lack of infection sites. Alternatively, root development could have conditioned nodulation as well as nodule growth simply as a consequence of the overall effect of lowered root functions on plant nutrition and, as a consequence, the supply of assimilates from the shoot. However, plants cultured so that only their nodules and supporting crown root zone were exposed to nonambient pO2 also showed effects of O2 on nodulation and nodule development as well as on specific N2-fixing activity. Dry matter accumulation by these plants closely reflected levels of N2 fixation, suggesting that the principal effect of O2, whether whole root systems or nodulated root crowns were exposed, was on nodules and their functioning.

The marked effects of pO2 on N2 fixation over long periods of growth were reflected in long-term changes in the acetylene-reducing activity of mature nodules expressed on a per plant basis. The broad optimum between 10 and 40 to 60% O2, shown for plant growth and N accumulation, was also indicated for acetylene reduction with severe restriction of activity at 80% O2 and at levels less than 5% O2. In these respects, acetylene reduction by the nodulated crown root zones of cowpea cultured in different pO2 was similar to that of plants where the whole root system was exposed. Generally, younger nodules showed a quite sharp optimum pO2 for nitrogenase activity.
specific activity close to that of air whether assayed as N₂ fixation (Fig. 2) or as acetylene reduction (Figs. 4 and 5). However, as nodules matured, and presumably became larger, nitrogenase showed a widening optimum PO₂, suggesting that their adaptation to sub- and supra-ambient O₂ was relatively slow and was associated with nodule growth and development. While this does not preclude the operation of an adjustable diffusion barrier in nodules, which responds to changes in O₂ in the short term, it suggests that optimizing O₂ supply over the long term to ensure maximum N₂ fixation may require changes which are more extensive than those associated with short-term adjustment.

The time courses for ethylene production, following exposure of nodulated roots or root segments to acetylene (Fig. 3), provide clear evidence for an adaptation to unfavorable external PO₂ which involved altered gaseous permeability. Rates of acetylene reduction of nodules grown at 1 or 2.5% O₂ and those cultured in 80% O₂ were about equally inhibited compared with those from air culture. However, at low PO₂ maximum rates of ethylene evolution were recorded after 1 or 2 min, while at 80% O₂ 15 to 20 min were required before a constant rate of exchange was reached. At PO₂ intermediate to these extremes, there was an intermediate response, suggesting a continuous range of adjustment by nodules. These data thus appear to support the proposal (16) that nodules adjust to over- or under-supply of O₂ by alteration in diffusional properties.

At both sub- and supra-ambient O₂, evolution of H₂ was inhibited, being essentially zero at levels above 40% and below 10% O₂. Although altered H₂ evolution could reflect changes in overall nitrogenase activity or the relative activities of uptake hydrogenase in materials from different O₂ atmospheres, it seems unlikely that differences in the "relative efficiency" of nodules (10) could account for the observed effects of O₂ on nodule functioning.

As might be expected, where nitorgenase activity and N₂ fixation were inhibited by culture in different PO₂, especially at very low O₂, the respiratory activity of the nodulated crown root zone also declined. No attempt was made to separate the contribution of the root segment to overall CO₂ release within the small enclosing chamber from that of nodules. However, a previous study (14) provides measurements for this symbiosis which indicate that 65 to 85% of the CO₂ lost was from nodules. Thus, changes in CO₂ release due to PO₂ largely reflected changes in nodule functioning.

The relatively constant relationship between CO₂ evolution and nitrogenase activity from 2.5 to 60% O₂ clearly indicates little variation in overall "efficiency" and a close matching between supply of respiratory products and their demand by nitrogenase. Values of around 6 mol CO₂ released per mol substrate reduced (Fig. 6) compared favorably with those determined previously for this and similar symbioses (14, 19, 20). The sharp increase in CO₂ evolved per unit substrate reduced at 1 and 80% O₂ suggests that at these extremes, nodule functioning was especially inefficient, with respiration supporting very much lower levels of nitrogenase activity. At high O₂, this apparent inefficiency may have been due to some sort of "protective" respiration which was utilized to reduce O₂ levels at or close to nitrogenase (6), while at 1% O₂ processes other than N₂ fixation utilized the majority of respiratory products.

In summary, nodules of cowpea were adapted to growth in non-ambient PO₂ such that the input of fixed N to the host plant was maximized. Adaptations from 5 to 10% to 40 to 60% O₂ involved short-term adjustments to gaseous conductance, while at sub- and supra-ambient PO₂ outside this range, more permanent, probably structural, modifications were also involved. This study has not identified the site or mechanism of diffusional control in nodules but has demonstrated that nodulated root systems cultured in either sub- or supra-ambient O₂ may offer useful experimental materials with which to explore these questions more closely.

ACKNOWLEDGMENTS

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