Nitrate Effects on Nodule Oxygen Permeability and Leghemoglobin

Nodule Oximetry and Computer Modeling

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Two current hypotheses to explain nitrate inhibition of nodule function both involve decreased O₂ supply for respiration in support of N₂ fixation. This decrease could result from either (a) decreased O₂ permeability (P₀) of the nodule cortex, or (b) conversion of leghemoglobin (Lb) to an inactive, nitrosyl form. These hypotheses were tested using alfalfa (Medicago sativa L. cv. Weevitchek) and birdsfoot trefoil (Lotus corniculatus L. cv. Fergus) plants grown in growth pouches under controlled conditions. Nodulated roots were exposed to 10 mM KNO₃ or KCl. Fractional oxygenation of Lb under air (FOL₀), relative concentration of functional Lb, apparent P₀, and O₂-saturated central zone respiration rate were all monitored by nodule oximetry. Apparent P₀ and FOL₀ in nitrate-treated nodules decreased to <50% of values for KCl controls within 24 h, but there was no decrease in functional Lb concentration during the first 72 h. In nitrate-treated alfalfa, but not in birdsfoot trefoil, FOL₀, apparent P₀, and O₂-saturated central zone respiration rate decreased during each light period and recovered somewhat during the subsequent dark period. This species difference could be explained by greater reliance on photoreduction of nitrate in alfalfa than in birdsfoot trefoil. Computer simulations extended the experimental results, showing that previously reported decreases in apparent P₀ of Glycine max nodules with nitrate exposure cannot be explained by hypothetical decreases in the concentration or O₂ affinity of Lb.

Nitrate exposure can inhibit both nodulation of legumes and N₂ fixation by existing nodules (Streeter, 1988). Modification of the response of crop and forage legumes to nitrate may be desirable in some agricultural contexts, e.g. to increase the nitrogen contribution from a green manure crop or to decrease total nitrogen supply where nitrate inhibition by elevated external O₂ (Vessey et al., 1988). A decrease in FOL is not predicted by the direct carbohydrate limitation or nitrite toxicity hypotheses. In fact, inhibition of respiration by carbohydrate limitation or nitrite toxicity would be expected to decrease the capacity to consume O₂ diffusing into the nodule, thereby increasing central zone O₂ concentration and FOL.

Decreased nodule O₂ permeability has been implicated in legume responses to several forms of stress (Hunt and Layzell, 1993). Past estimates of P₀ in nitrate-treated nodules were based on attempts to calculate the O₂ flux into nodules from net CO₂ exchange rates of nodulated roots. Although several variations on this approach (Schuller et al., 1988; Vessey et al., 1988; Minchin et al., 1992) all appeared to show a decrease in P₀ with nitrate exposure, a reexamination of nitrate effects by an independent method seemed worthwhile. Therefore, in this paper we report nitrate effects on P₀ in alfalfa (Medicago sativa) and birdsfoot trefoil (Lotus corniculatus) as measured by nodule oximetry (Denison and Layzell, 1991). This method estimates P₀ of intact, attached nodules by spectrophotometric monitoring of changes in FOL in response to changes in external O₂ concentration.

Nodule O₂ permeability is defined here explicitly by Fick's law of diffusion

\[ F = P_0 \times A \times (O_{\text{e}} - O_{\text{i}}) \]  

where \( F \) is the total inward O₂ flux (mol/s) across the gas diffusion barrier in the nodule cortex (Tjevskema and Yocum, 1973), \( P_0 \) is the O₂ permeability (m/s) of this barrier, \( A \) is the barrier surface area (m²), and \( O_{\text{e}} \) and \( O_{\text{i}} \) are the gas-phase concentrations of O₂ (mol/m³) in the external environment and central zone airspaces, respectively. Lb facilitates O₂ diffusion within infected cells (Appleby,

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\[ \text{Abbreviations: FOL, fractional oxygenation of Lb; FOL}_{\text{air}} \text{, steady-state FOL of nodules under air; } K_{\text{mv}}, \text{ central zone Michaelis constant for } O_2 \text{ consumption; } Lb, \text{ leghemoglobin; } O_{\text{ov}}, \text{ central zone gas-phase } O_2 \text{ concentration; } O_{\text{cv}}, \text{ central zone dissolved } O_2 \text{ concentration; } P_{\text{cv}}, \text{ nodule } O_2 \text{ permeability; } V_{\text{max}}, \text{ O}_2{-}\text{saturated central zone rate of } O_2 \text{ consumption.} \]
so nitrate-induced changes in Lb concentration or O₂ affinity could also affect O₂ supply. Conversion of Lb to a nitrosyl form that does not bind O₂ was reported for Lb extracted from nitrate-treated soybean (Glycine max) nodules by Kanayama et al. (1990), who suggested that the resulting decrease in O₂ supply was the main cause of decreased nitrogen fixation. In Pisum sativum nodules, acetylene reduction rates decreased to 40% of controls after 2 d of nitrate exposure, whereas total Lb concentration was unchanged over the same period (Chen and Phillips, 1977). However, conversion of Lb to a nonfunctional form might not have been detected. Sprent et al. (1987) did not find significant concentrations of nitrate or nitrite in the central zone of three legume species after 3 d of exposure to 10 mM nitrate. However, Hunt and Layzell (1993) suggested that nitrite might have been sequestered as nitrosylhemoglobin, and that “changes in the affinity of Lb for O₂, or in the concentration of functional Lb, [might] account for the changes in nodule permeability . . . .” To test this hypothesis, we have used nodule oximetry to measure changes, if any, in the concentration of functional Lb (i.e. Lb that is able to bind O₂ reversibly) in nodules exposed to nitrate.

To explore possible interactions between Lb and Pₐ further, a previously published model of O₂ diffusion in nodules (Denison, 1992) was modified to incorporate recent data on the sizes and spacing of intercellular air spaces in soybean nodules (van Cauwenbergh et al., 1993). The new model was then used to determine (a) how hypothetical changes in the concentration or O₂ affinity of Lb would affect nodule activity and (b) whether such changes would alter Pₐ estimates obtained by gas exchange. If so, then reported changes in apparent Pₐ with nitrate exposure might actually indicate changes in Lb-facilitated diffusion of O₂. The modeling results were expected to be most directly applicable to interpretation of previous reports of nitrate effects on soybean nodules, but it was anticipated that some of the qualitative conclusions might be more widely applicable.

The same computer model was also used to assess two possible sources of error in Pₐ estimates made by nodule oximetry. The standard nodule oximetry calculations (Denison and Layzell, 1991; Denison et al., 1992a) assume (a) uniform conditions throughout infected cells and (b) instantaneous equilibration between FOL and Oₓ. Decreases in Lb-facilitated O₂ diffusion could perhaps lead to significant violations of these assumptions. The effects of nonuniform or nonequilibrium conditions cannot be tested simply by changing the parameters used by the standard nodule oximeter algorithms because the assumptions of uniform conditions and instantaneous equilibrium are inherent to those algorithms. Instead, the possible effects of departures from these assumptions were evaluated using the computer model, which allowed the possibility of non-equilibrium conditions and significant O₂ gradients within infected cells.

A second type of possible error in Pₐ estimates from nodule oximetry results from discrepancies between the actual Lb concentration and O₂ affinity for a particular nodule and the representative values (based on published data) assumed by the nodule oximetry algorithms. Although nodule oximetry can measure changes in relative Lb concentration within a given nodule (Denison et al., 1992a), absolute values for Lb concentration and O₂ affinity in a given nodule are generally not known. This source of error could be evaluated simply by running the nodule oximetry algorithms with various assumed values, but that would not address possible interactions between errors in assumed parameter values and errors due to nonuniform or nonequilibrium conditions. Therefore, the same computer model was used to simulate both types of potential error.

**MATERIALS AND METHODS**

**Growth of Plants and Nodule Oximetry**

Birdsfoot trefoil (Lotus corniculatus L. cv Fergus) and alfalfa (Medicago sativa L. cv Weevlchek) were grown in growth pouches under controlled conditions as previously described (Denison and Layzell, 1991; Denison et al., 1992a), except that the nitrogen-free nutrient solution of Vessey and Layzell (1987) was modified by substituting N,N’-ethylenebis-2,2’-hydroxyphenylglycine (Chaney and Bell, 1987) for Fe-Sequestrene 330.

Nodule oximetry was used for repeated measurements of FOL_air, relative concentration of functional Lb, V_max, and apparent Pₐ, using attached nodules as previously described (Denison and Layzell, 1991; Denison et al., 1992a). This method determines FOL at any point in time by fiber-optic spectrophotometry of Lb using the ratio of red (660 nm) to IR (820 nm) transmittance through the intact nodule. Each oximeter assay includes a calibration procedure that assumes complete deoxygenation of Lb under pure N₂ and full oxygenation of Lb under elevated external O₂ at steady state. Relative Lb concentration is estimated by comparing the magnitude of the spectrophotometric change, with a transition from deoxygenation to full oxygenation, to a reference value for the same individual nodule (Denison et al., 1992a). Quantitative estimates of apparent Pₐ and V_max determined from the rates of change in FOL after a change in the external O₂ concentration, were based on an assumed Lb concentration of 0.68 mM (Bergersen, 1982).

Although the magnitude of changes in transmittance at 660 nm with changes in FOL is only about half of that at wavelengths such as 560 or 580 nm, the timing of responses to changes in external O₂ is similar (data not shown). High-intensity light-emitting diodes are not yet available at the latter two wavelengths. Approximate agreement between oximetry and other estimates of central zone Kₘ (Denison and Layzell, 1991) and V_max (Denison et al., 1992b) further increases our confidence that nodule oximetry measures FOL rather than some other O₂-sensitive change in nodule spectral properties.

**Nitrate Treatments**

One nodule per pouch was selected before random assignment of pouches to treatments. Each selected nodule
was assayed at about 9:00 AM, 12:00 noon, and 3:00 PM each day (during the normal light period of 6:00 AM to 6:00 PM) for four successive days, then again after 6 d of nitrate exposure. Immediately after the second assay on the 1st d, the nutrient solution in five replicate growth pouches was supplemented with KNO₃ to give a concentration of 10 mM; five control pouches received 10 mM KCl. Each solution was replaced daily. The entire experiment was repeated a second time for both birdsfoot trefoil and alfalfa.

**Modeling Changes in Lb Concentration and O₂ Affinity**

An improved version of an earlier simulation model (Denison, 1992) was used to evaluate the effects of hypothetical changes in Lb concentration or O₂ affinity. The model is dynamic (i.e. steady-state conditions are not assumed) and includes a fairly detailed treatment of respiration and the diffusion of O₂ and oxygenated Lb in infected cells.

The current model differs from the earlier model mainly in the details of nodule anatomy. The earlier model assumed that the entire surface area of each infected cell was fully exposed to the network of air spaces (Bergersen and Goodchild, 1973) that aerates the nodule interior. This assumption would tend to overestimate O₂ supply to the interiors of individual cells, thereby diminishing the apparent importance of Lb. The current model still simplifies nodule anatomy, but should allow qualitatively accurate conclusions concerning the effects of changes in Lb concentration and O₂ affinity.

In the current model, the network of interconnected air spaces in the nodule interior was modeled as a set of regularly spaced parallel air-filled tubes of constant diameter. Spacing and dimensions of these air spaces were based on recent data for soybean from van Cauwenbergh et al. (1993). The model has 1 air space per 1000 mm² of cross-sectional area, and air spaces occupy 0.5% of total cross-sectional area. Each air space occurs at the junction of four cells (diamond-shaped in cross-section) with 34-mm sides (Fig. 1, top). Previous nodule models have treated cells as spheres (Sheehy and Bergersen, 1986; Denison, 1992) or cubes (Thumfort et al., 1994).

Regular spacing of parallel air spaces allowed a cross-section of the nodule interior (normal to the tubular air spaces) to be tiled by hexagonal regions surrounding each air space. Diffusion of O₂ and Lb was modeled in two dimensions using cylindrical coordinates for eight concentric layers surrounding the air space and inscribed in the hexagonal region (Fig. 1, bottom). Consumption of O₂ by respiration, as a function of O₂ concentration, was modeled for each cylindrical layer.

The metabolic activity of that part of the hexagon beyond the outermost cylindrical layer was assumed to be zero, which simplified the model and greatly reduced computation time. The excluded zone accounted for only 9.6% of the total cross-sectional area (or volume), and presumably a somewhat lower fraction of total metabolic activity, due to the distance from the air space.

Because the diffusion of O₂ in the gas phase is 4 orders of magnitude higher than in the liquid phase, gas-phase O₂ concentration was assumed to be uniform throughout the network of air spaces. As in the earlier model, the network of internal air spaces was assumed to connect to the inner surface of a diffusion barrier surrounding the nodule interior. Airspace O₂ concentration was simulated dynamically, decreasing in response to O₂ consumption within the central zone and increasing with inward diffusion of O₂ through the diffusion barrier from the external environment.

Parameter values for a standard nodule are given, with sources cited, in Table I. Because the anatomical data of van Cauwenbergh et al. (1993) on air-space spacing and area were based on soybean nodules, values appropriate for soybean were also used for other model parameters. Assumed values for \( V_{\text{max}} \), \( K_{\text{m}} \), and the Lb "off" constant differ somewhat from those in the previous model (Denison, 1992), which used values for birdsfoot trefoil. These differences are probably less important than the substantial reduction in exposed surface area of infected cells implicit in the anatomical assumptions of the current model. Predictions of the current model would not be expected to agree quantitatively with our data for alfalfa or birdsfoot trefoil, although some qualitative conclusions could be applicable. Copies of the new model (on IBM/PC-compatible floppy disk) are available from the senior author.

Nodule function in air was evaluated by assuming an external gas-phase O₂ concentration of 8.3 mol/m³ (about 20 kPa) and running the model until essentially steady-
Table 1. Default parameter values used in nodule simulation model, with sources

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility of O₂ in cells</td>
<td>2.69 × 10⁻²</td>
<td>Altman and Dittmen, 1971</td>
</tr>
<tr>
<td>Diffusivity of O₂ in cells</td>
<td>7.5 × 10⁻¹⁰ m² s⁻¹</td>
<td>White, 1974</td>
</tr>
<tr>
<td>Diffusivity of O₂ in airspace</td>
<td>1.78 × 10⁻³ m² s⁻¹</td>
<td>White, 1974</td>
</tr>
<tr>
<td>Diffusivity of Lb</td>
<td>1.1 × 10⁻⁷ m² s⁻¹</td>
<td>White, 1974</td>
</tr>
<tr>
<td>Lb concentration</td>
<td>0.68 mol m⁻¹</td>
<td>Berghersen, 1982</td>
</tr>
<tr>
<td>Lb &quot;on&quot; constant</td>
<td>1.2 × 10⁻³ m³ mol⁻¹ s⁻¹</td>
<td>Gibson et al., 1989</td>
</tr>
<tr>
<td>Lb &quot;off&quot; constant</td>
<td>5.6 s⁻¹</td>
<td>Gibson et al., 1989</td>
</tr>
<tr>
<td>Radius of airspace</td>
<td>1.26 × 10⁻⁶ m</td>
<td>van Cauwenberge et al., 1993</td>
</tr>
<tr>
<td>Radius of modeled cylinder</td>
<td>1.7 × 10⁻⁵ m</td>
<td>van Cauwenberge et al., 1993</td>
</tr>
<tr>
<td>Radius of nodule central zone</td>
<td>1.0 × 10⁻³ m</td>
<td>Arbitrary</td>
</tr>
<tr>
<td>Nodule O₂ permeability</td>
<td>1.05 × 10⁻⁶ m s⁻¹</td>
<td>Denison et al., 1992b</td>
</tr>
<tr>
<td>Vₘₐₓ for O₂ consumption</td>
<td>4.66 × 10⁻² mol m⁻³ s⁻¹</td>
<td>Denison et al., 1992b</td>
</tr>
<tr>
<td>Kₘ for O₂ in central zone</td>
<td>1.22 × 10⁻⁵ mol m⁻³</td>
<td>Denison et al., 1992b</td>
</tr>
</tbody>
</table>

state conditions were achieved. Model predictions of total O₂ consumption and gradients of O₂ and FOL were recorded. Estimates of apparent Pₒ that would be obtained by gas exchange were made using Equation 1, with the common simplifying assumption that Qₐ = 0 (Witty et al., 1986; Weisz and Sinclair, 1987).

Model predictions of apparent Pₒ and Vₘₐₓ that would be obtained by nodule oximeter were also evaluated by running the model under the nonsteady-state conditions characteristic of the nodule oximeter assay. The external atmosphere was assumed to change sequentially from air, to pure N₂, to pure O₂, and back to N₂. Each gas was maintained until steady-state conditions were achieved. Model predictions for average FOL (weighted by layer volume) were then analyzed, using the standard nodule oximeter algorithms described by Denison and Layzell (1991), as if they were FOL data from a nodule oximeter assay of a real nodule.

The effects of hypothetical changes in the concentration or O₂ affinity of Lb were evaluated by changing the values assumed for the Lb concentration or the Lb "on" constant, and then repeating the procedures described in the previous two paragraphs. Two cases were considered in which either Lb concentration or O₂ affinity was assumed to be only half of the value for the standard nodule of Table I. A more extreme case, in which Lb concentration was assumed to be only 5% of that in the standard nodule, was also simulated.

Analysis of the resulting model predictions using the standard nodule oximeter algorithms assumed either (a) the default values for Lb concentration and O₂ affinity given in Table I, or (b) the modified values assumed by the model for each case. The first approach represents standard practice in the use of the nodule oximeter, in which published values of these parameters are assumed. The second approach represents an ideal case in which Lb concentration and O₂ affinity are somehow measured for each nodule. Simulation of this "corrected" version of the nodule oximeter assay allowed the effects of nonuniform and non-equilibrium conditions to be evaluated independently of errors resulting from use of assumed rather than measured parameter values.

RESULTS

Qualitative Changes in Nodule Oximeter Data with Nitrate Exposure

Nodule oximeter assays for a typical birdsfoot trefoil nodule at various times after exposure to 10 mM KNO₃ are shown in Figure 2. Drift in the ratio of red/IR transmittance, if any, was corrected as previously described (Deni-
son and Layzell, 1991). Differences with duration of nitrate exposure are apparent in FOL\textsubscript{air} in rates of change in FOL after changes in external O\textsubscript{2}, and in the overall magnitude of changes in the red/IR ratio.

Under steady-state conditions, FOL represents a balance between influx of O\textsubscript{2} from the atmosphere (increasing with P\textsubscript{O}) and O\textsubscript{2} consumption by infected cell respiration. The standard nodule oximeter assay perturbs the normal steady state by varying external O\textsubscript{2} concentration and estimates apparent P\textsubscript{O} and V\textsubscript{max} from the resulting changes in FOL with time. For example, the decrease in FOL (after a decrease in external O\textsubscript{2} concentration) will be more rapid in a nodule with greater V\textsubscript{max}.

Prior to nitrate exposure (Fig. 2A), the red/IR ratio under air was appreciably higher than its steady-state value under N\textsubscript{2} (0 kPa O\textsubscript{2}); calculated FOL\textsubscript{air} was 29.3%. After 48 h of exposure to nitrate (Fig. 2B), the red/IR ratio under air was almost indistinguishable from that under N\textsubscript{2}; calculated FOL\textsubscript{air} decreased to 6.1%. A slower response to an increase in external O\textsubscript{2} was apparent after 48 h, despite use of a higher external O\textsubscript{2} to saturate Lb. This higher O\textsubscript{2} concentration (90 versus 70 kPa, assuming a total atmospheric pressure of 100 kPa) was selected automatically by the nodule oximeter because of the sluggish response to the brief early exposure to 50 kPa O\textsubscript{2}. A slower increase in FOL after increases in external O\textsubscript{2} usually indicates lower P\textsubscript{O}. An increase in V\textsubscript{max} could be an alternative explanation for slower increases in FOL in some situations, but that was clearly not the case for the nitrate-treated nodule shown in Figure 2B. The slow decrease in FOL after switching from 100 to 0 kPa O\textsubscript{2} indicates that V\textsubscript{max} was actually much lower after 48 h of nitrate exposure. Any change in K\textsubscript{m} would have affected only the lower part of the curve. (Calculation of P\textsubscript{O} by the oximeter algorithms corrects for any changes in either V\textsubscript{max} or K\textsubscript{m}.)

The lack of any additional increase in the red/IR ratio during the brief exposure to 100 kPa O\textsubscript{2} (Fig. 2B) confirms that Lb was already O\textsubscript{2} saturated by the exposure to 90 kPa O\textsubscript{2}. This test is performed by the nodule oximeter whenever a slow response to increased external O\textsubscript{2} suggests that Lb within the nodule may not be fully oxygenated.

The vertical axes in Figure 2, A to C, differ in absolute values, but the same vertical scale factor was used in each panel to illustrate the decrease in peak height with duration of nitrate exposure. The maximum and minimum red/IR values in each panel are assumed to correspond to full oxygenation and full deoxygenation of Lb, as indicated in Figure 2A. Therefore, changes in peak height for a given nodule indicate changes in functional Lb concentration (Denison et al., 1992a). For the nodule shown in Figure 2, there was little change in functional Lb concentration during the first 48 h, but calculated concentration of functional Lb after 145 h was only about 25% of that at time zero.

### Time Course of Nitrate Effects on Calculated Parameters

Both apparent P\textsubscript{O} and V\textsubscript{max} gradually decreased in control (KCI-treated) nodules over the course of the experiments (Table II). Similar decreases with repeated oximeter assays have previously been reported (Denison and Layzell, 1991) and attributed to slight mechanical damage or effects of O\textsubscript{2} exposure. Trends during the first 72 h of nitrate exposure (Fig. 3) are therefore presented as the ratio of mean values (n = 5) for nodules exposed to KNO\textsubscript{3} and KCI. A second experiment with each species gave qualitatively similar results (data not shown).

There was little or no decrease in the concentration of functional Lb during the first 72 h of nitrate exposure in either species (Fig. 3). Some of the day-to-day variation in measurements of relative Lb concentration is probably due to slight differences in probe placement over time, especially for the indeterminate alfalfa nodules (Denison et al., 1992a). After 145 h, functional Lb concentration in nitrate-treated birdsfoot trefoil nodules was significantly lower than before nitrate exposure (Table II).

FOL\textsubscript{air} for nodules of each species decreased within 24 h of nitrate exposure, relative to KCl controls. This decrease (Fig. 3, A and B) was consistent with decreased apparent P\textsubscript{O} (Fig. 3, C and D) with nitrate exposure. Apparent V\textsubscript{max} also decreased, particularly in birdsfoot trefoil. This decrease in apparent V\textsubscript{max} would have been expected to cause an increase in FOL\textsubscript{air} if there had been no decrease in P\textsubscript{O}-

### Table II. Effects of exposure to 10 mM KCl or KNO\textsubscript{3} for the times indicated on functional Lb concentration, FOL\textsubscript{air}, P\textsubscript{O}, and V\textsubscript{max} (mean ± SD, n = 5) as measured by nodule oximetry

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative [Lb]</th>
<th>FOL\textsubscript{air}</th>
<th>P\textsubscript{O} %</th>
<th>V\textsubscript{max} pmol s\textsuperscript{-1} cm\textsuperscript{-3}</th>
<th>V\textsubscript{max} nmol cm\textsuperscript{-3} s\textsuperscript{-1}</th>
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<tbody>
<tr>
<td>Trefoil</td>
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<tr>
<td>KCl, 0 h</td>
<td>1.00 ± 0.11</td>
<td>26.9 ± 1.7</td>
<td>1.513 ± 0.156</td>
<td>136.4 ± 10.3</td>
<td></td>
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<tr>
<td>48 h</td>
<td>1.01 ± 0.08</td>
<td>25.0 ± 2.2</td>
<td>1.226 ± 0.174</td>
<td>114.7 ± 14.9</td>
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<tr>
<td>145 h</td>
<td>0.94 ± 0.11</td>
<td>24.6 ± 2.7</td>
<td>0.863 ± 0.136</td>
<td>82.3 ± 10.4</td>
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<tr>
<td>KNO\textsubscript{3}, 0 h</td>
<td>1.00 ± 0.05</td>
<td>19.1 ± 3.1</td>
<td>1.152 ± 0.125</td>
<td>116.4 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>0.98 ± 0.06</td>
<td>4.1 ± 1.6</td>
<td>0.340 ± 0.036</td>
<td>39.2 ± 5.7</td>
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<tr>
<td>145 h</td>
<td>0.57 ± 0.18</td>
<td>23.0 ± 13.6</td>
<td>0.311 ± 0.016</td>
<td>30.5 ± 7.5</td>
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<td>Alfalfa</td>
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</tr>
<tr>
<td>KCl, 0 h</td>
<td>1.00 ± 0.18</td>
<td>17.6 ± 4.8</td>
<td>1.749 ± 0.178</td>
<td>175.8 ± 13.8</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>1.27 ± 0.24</td>
<td>22.0 ± 5.6</td>
<td>1.892 ± 0.198</td>
<td>180.5 ± 21.6</td>
<td></td>
</tr>
<tr>
<td>144 h</td>
<td>1.28 ± 0.31</td>
<td>30.0 ± 6.9</td>
<td>1.656 ± 0.256</td>
<td>160.3 ± 28.9</td>
<td></td>
</tr>
<tr>
<td>KNO\textsubscript{3}, 0 h</td>
<td>1.00 ± 0.15</td>
<td>18.6 ± 3.6</td>
<td>1.847 ± 0.207</td>
<td>175.5 ± 15.2</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>1.12 ± 0.20</td>
<td>7.6 ± 2.1</td>
<td>1.011 ± 0.176</td>
<td>147.6 ± 20.0</td>
<td></td>
</tr>
<tr>
<td>144 h</td>
<td>0.93 ± 0.27</td>
<td>5.6 ± 1.3</td>
<td>0.920 ± 0.157</td>
<td>175.2 ± 19.5</td>
<td></td>
</tr>
</tbody>
</table>
tated diffusion accounted for <1% of total O₂ flux through between FOL and O₂, was particularly true for the simulated nodule with only increased gradients of O₂, relative to standard values, for 5% saturated with O₂ (Fig. 3). The assumption of the model predicted only an 8% decrease in apparent P₀ from gas exchange (calculated from O₂ influx) from a 95% decrease in Lb concentration. (Note that the agreement between predictions of O₂ influx and total respiration under steady-state conditions is the result of dynamic simulation, not simply an assumption of the model.)

A decrease in the O₂ affinity of Lb assumed by the model had essentially no effect on apparent P₀ or Vₘₐₓ from simulated nodule oximetry. This parameter is used to calculate O₂ from FOL, but since the oximetry equations that calculate P₀ and Vₘₐₓ are very insensitive to Oₘ, the lack of effect is not really surprising. These equations are: based on total O₂ in the nodule, which is almost entirely present as LbO₂ rather than dissolved O₂.

As one would expect, simulated decreases in Lb concentration did affect model predictions of uncorrected P₀ and Vₘₐₓ from the simulated nodule oximeter assay. A simulated 50% decrease in Lb concentration (without a corresponding correction in the Lb concentration assumed by the oximeter analysis algorithms) actually led to higher estimates of apparent P₀ and Vₘₐₓ (Table III). This is the opposite of our experimental results (also uncorrected) for layer 1. This contrasts with the standard nodule, in which Lb-facilitated diffusion accounted for almost 99% of total O₂ flux. This increase in the importance of diffusion by dissolved O₂, relative to LbO₂, reflects an increased gradient for O₂, as well as the decreased gradient of LbO₂.

Despite these predicted changes in gradients of FOL and O₂, none of the decreases in assumed Lb concentration and O₂ affinity had more than a small effect on predicted total central zone respiration rate or O₂ influx (Table III). The model predicted only an 8% decrease in apparent P₀ from gas exchange (calculated from O₂ influx) from a 95% decrease in Lb concentration. (Note that the agreement between predictions of O₂ influx and total respiration under steady-state conditions is the result of dynamic simulation, not simply an assumption of the model.)

Quantitative comparisons of P₀ and Vₘₐₓ between species are considered unreliable without absolute measurements of Lb concentrations (Denison et al., 1992a). However, some qualitative differences between species were apparent. In birdsfoot trefoil, apparent Vₘₐₓ decreased almost as rapidly as apparent P₀, but in alfalfa the decrease in P₀ was more rapid than the decrease in Vₘₐₓ. This species difference is similar to that previously observed with a detopping treatment (Denison et al., 1992a).

The daily trends in FOLₐᵢʳ, P₀, and Vₘₐₓ also differed between species. Alfalfa nodules showed a diurnal decrease in all three variables during each light period after nitrate exposure, with some recovery each night. In birdsfoot trefoil nodules, all three variables appeared to decrease uniformly over the course of the experiment.

Computer Modeling of Changes in Lb Concentration or O₂ Affinity

Simulated decreases in Lb concentration or O₂ affinity increased gradients of O₂, relative to standard values, for simulated nodules at steady state under air (Fig. 4A). This was particularly true for the simulated nodule with only 5% of the standard Lb concentration. Predicted steady-state FOL gradients were less steep (Fig. 4B) due to the nonlinear relationship between FOL and O₂. A simulated decrease in the assumed O₂ affinity of Lb changed the relationship between FOL and O₂, so that FOL near the air space was lower than for the standard nodule, even though O₂ was higher.

For the case with only 5% of the standard Lb concentration, Lb in the two layers nearest the air space was nearly saturated with O₂ (Fig. 4B), so there was little gradient driving diffusion of oxygenated Lb. As a result, Lb-facilitated diffusion accounted for <1% of total O₂ flux through

Figure 3. Changes, relative to KCl controls, in functional Lb concentration (O), FOLₐᵢʳ (B), Vₘₐₓ (D), and apparent O₂ permeability (E) in attached nodules of alfalfa (A and C) and birdsfoot trefoil (B and D) exposed to 10 mM KNO₃ at time 0. Bars indicate dark period. There were five replicate nodules per treatment.

Figure 4. Gradients in O₂ and FOL predicted by the computer model, as a function of distance from a linear air space (see Fig. 1). The simulations are based on a nodule with normal Lb, plus nodules with decreased Lb concentration or O₂ affinity.
nitrate-treated nodules. When the oximeter analysis algorithms were corrected to use the values assumed by the computer model, halving the Lb concentration decreased apparent \( P_o \) and \( V_{\text{max}} \), but only by <10\% (Table III). Because the nodule oximeter provides only relative estimates of Lb concentration, this correction has generally not been applied to experimental data in the past. Uncorrected values are most useful for interpretation of uncorrected experimental data (Figs. 2 and 3), whereas corrected values would be appropriate in cases where the data were corrected for changes in Lb concentration.

It was not possible to simulate a nodule oximeter assay for the case with Lb concentration of only 5\% of the standard nodule, because simulated FOL changed too rapidly. Lb changed from deoxygenated to fully oxygenated in <1 s in response to a simulated increase in external \( O_2 \) concentration. Changes this rapid have never been seen with real nodules (Denison and Layzell, 1991; Denison et al., 1992b).

**DISCUSSION**

**Mechanism of Nitrate Inhibition**

The experimental results were consistent with the hypothesis that decreased \( N_2 \) fixation by nodules exposed to nitrate is caused, at least in part, by decreased \( P_o \). For each species, average \( P_o \) of nodules exposed to 10 mM KNO\(_3\) for 24 h was less than 50\% of that of nodules exposed to the same concentration of KCl. The decreased \( P_o \) resulted in lower FOL and \( O_p \), which would limit respiration in support of \( N_2 \) fixation. Respiration was also affected more directly. In birdsfoot trefoil, \( V_{\text{max}} \) decreased almost as much as \( P_o \).

The synchrony between changes in \( P_o \) and \( V_{\text{max}} \) may seem surprising at first, but the likely consequences of a lack of synchrony may provide an evolutionary explanation. A decrease in \( V_{\text{max}} \) without a corresponding decrease in \( P_o \) would result in increased \( O_p \), which could lead to \( O_2 \) inactivation of nitrogenase. Legumes have thus been subject to millions of years of natural selection favoring physiological linkages (by mechanisms that are at present unknown) that prevent this situation. A decrease in \( P_o \) without a simultaneous decrease in \( V_{\text{max}} \) is less threatening, and such decreases are sometimes observed (Denison et al., 1992a).

The data appear to disprove the hypothesis that loss of functional Lb is a primary consequence of nitrate exposure in birdsfoot trefoil and alfalfa. Neither species showed any significant decrease in the concentration of functional Lb during the first 72 h. Functional Lb concentration did eventually decrease in birdsfoot trefoil, which confirms the ability of nodule oximetry to detect such losses when they occur. Similar decreases in functional Lb were previously observed in defoliation-induced senescent nodules (Denison et al., 1992a). With additional wavelengths, it might be possible to use a modified nodule oximeter to specifically detect LbNO if it occurs in vivo. The current assay does not distinguish among different forms of Lb, except that it detects only functional Lb that can reversibly bind \( O_2 \).

These results are consistent with the suggestion of Minchin et al. (1989) that early effects of nitrate are restricted to the nodule cortex. However, they do not in themselves prove that nitrate does not reach and exert some influence (other than a change in \( Lb \)) in the nodule central zone. Denitrification by bacteria has been reported in alfalfa nodules after 3 d of exposure to 10 mM nitrate, implying nitrate entry into the central zone by that time (Arrese-Igor et al., 1992). Although our results relieve Lb of direct responsibility for observed changes in \( P_o \), they do not exclude indirect involvement of Lb, e.g. as an \( O_2 \) sensor (Appleby et al., 1988).

**Species Differences**

Species differences in the timing of decreases in \( P_o \) relative to \( V_{\text{max}} \) were somewhat similar to those previously reported after detopping (Denison et al., 1992a). In that paper it was suggested that \( V_{\text{max}} \) may depend primarily on availability of nonstructural carbohydrate in the nodule, and that the slower decrease in \( V_{\text{max}} \) in alfalfa reflected greater root reserves of nonstructural carbohydrates relative to birdsfoot trefoil.

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Table III. Model predictions for standard nodule and nodules with reduced Lb concentration or \( O_2 \) affinity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard Lb</th>
<th>Low Affinity</th>
<th>Low [Lb]</th>
<th>5% Standard [Lb]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb ( ^{on} ) concentration (mol m(^{-3} ))</td>
<td>0.680</td>
<td>0.680</td>
<td>0.340</td>
<td>0.034</td>
</tr>
<tr>
<td>Lb ( ^{on} ) constant (m(^3 ) mol(^{-1} ) s(^{-1} ))</td>
<td>(1.20 \times 10^5)</td>
<td>(6.00 \times 10^4)</td>
<td>(1.20 \times 10^5)</td>
<td>(1.20 \times 10^5)</td>
</tr>
</tbody>
</table>

Steady-state model predictions in air

Gas-phase \( O_2 \) in airspace (mol m\(^{-3} \))

- Average \( O_2 \) (mol m\(^{-3} \))
  - 1.52 \( \times 10^{-3} \)
  - 1.94 \( \times 10^{-3} \)
  - 3.59 \( \times 10^{-3} \)
  - 5.67 \( \times 10^{-1} \)

- Total central zone respiration (mol s\(^{-1} \))
  - 1.10 \( \times 10^{-10} \)
  - 1.10 \( \times 10^{-10} \)
  - 1.10 \( \times 10^{-10} \)
  - 1.02 \( \times 10^{-10} \)

- Total \( O_2 \) flux into central zone (mol s\(^{-1} \))
  - 1.10 \( \times 10^{-10} \)
  - 1.10 \( \times 10^{-10} \)
  - 1.10 \( \times 10^{-10} \)
  - 1.02 \( \times 10^{-10} \)

- Apparent \( P_o \) from gas exchange (m s\(^{-1} \))
  - 1.05 \( \times 10^{-6} \)
  - 1.05 \( \times 10^{-6} \)
  - 1.05 \( \times 10^{-6} \)
  - 9.74 \( \times 10^{-7} \)

Uncorrected oximeter assay

- Apparent \( P_o \) by oximetry (m s\(^{-1} \))
  - 1.03 \( \times 10^{-6} \)
  - 1.03 \( \times 10^{-6} \)
  - 1.91 \( \times 10^{-6} \)

- Corrected oximeter assay
  - Apparent \( P_o \) by oximetry (m s\(^{-1} \))
    - 1.03 \( \times 10^{-6} \)
    - 1.03 \( \times 10^{-6} \)
    - 9.56 \( \times 10^{-7} \)

- Apparent \( V_{\text{max}} \) by oximetry (mol m\(^{-3} \) s\(^{-1} \))
  - 4.66 \( \times 10^{-2} \)
  - 4.63 \( \times 10^{-2} \)
  - 9.04 \( \times 10^{-2} \)

- Corrected oximeter assay
  - Apparent \( V_{\text{max}} \) by oximetry (mol m\(^{-3} \) s\(^{-1} \))
    - 4.66 \( \times 10^{-2} \)
    - 4.63 \( \times 10^{-2} \)
    - 4.52 \( \times 10^{-2} \)
With detopping, decreased $P_O$ preceded any significant decrease in $V_{\text{max}}$ (Denison et al., 1992a), so the decrease in $P_O$ was presumably not caused by any direct carbohydrate limitation. That also appears to be true for nitrate exposure, at least in alfalfa. This conclusion is supported by the lack of a consistent decrease in nodule carbohydrates with nitrate exposure (Streeter, 1988; Vessey and Waterer, 1992). The dark-period recovery of $V_{\text{max}}$ in alfalfa also seems inconsistent with explanations based on photosynthetic supply.

The qualitative difference between species in the diurnal pattern of response to nitrate was seen in two separate experiments. It is possible that the indeterminate nodules of alfalfa respond to nitrate differently than the determinate nodules of birdsfoot trefoil, but similar experiments with additional species would be needed to strengthen or refute this hypothesis.

An alternative hypothesis to explain species differences in diurnal pattern is based on differences between the two species in the principal site of nitrate assimilation. Root nitrate reductase activity in alfalfa plants exposed to nitrate is negligible relative to levels in stem and especially in leaf tissues (Vance and Heichel, 1981). In birdsfoot trefoil, root and stem nitrate reductase activity account for about 31 and 52% of total activity, with only 15% in leaves (Monza et al., 1989). Photoreduction of nitrate may therefore be much more important in alfalfa than in birdsfoot trefoil. Diurnal changes in shoot nitrate assimilation could perhaps explain the daytime decline and night recovery observed in alfalfa but not in birdsfoot trefoil. Under this hypothesis, decreases in $P_O$ and $V_{\text{max}}$ would be triggered by some product of nitrate reduction (perhaps some specific nitrogenous compound) rather than by nitrate itself. This explanation is consistent with suggestions that $P_O$ is regulated by a feedback mechanism involving assimilated nitrogen (Heim et al., 1993; Parsons et al., 1993).

Role of $Lb$

Hunt and Layzell (1993) suggested that changes in $Lb$ concentration or $O_2$ affinity might explain observed decreases in apparent $P_O$. For alfalfa and birdsfoot trefoil, our experimental results showed that the decrease in $P_O$ preceded any change in $Lb$ concentration. For the soybean nodules used in some previous studies, computer simulation showed that previously reported decreases in apparent $P_O$ from gas exchange (Schuller et al., 1988; Vessey et al., 1988) could not be explained by hypothetical decreases in $Lb$ concentration or $O_2$ affinity. This does not prove that decreases in functional $Lb$ concentration or $O_2$ affinity do not occur in soybean, but it appears that a decrease in $P_O$ is both necessary and sufficient to explain reported decreases in nodule gas exchange.

Uncorrected apparent $P_O$ from nodule oximetry of soybean nodules was predicted to increase, not decrease, with a hypothetical decrease in $Lb$ concentration, unless the permeability of the nodule cortex decreased enough to counteract the $Lb$ effect. Nodule oximeter $P_O$ measurements for nitrate-treated soybean have not yet been published.

It seems unlikely that the direction of the predicted change in apparent $P_O$ with $Lb$ concentration (the opposite of our results for alfalfa and birdsfoot trefoil) would differ among legume species. The model could be reparameterized to test this hypothesis as appropriate values become available for other legume species.

The additional suggestion of Hunt and Layzell (1993) that changes in $Lb$ might seriously limit nodule activity was supported by the current simulations, in contrast to a simpler, earlier model (Denison, 1992). The importance of $Lb$ in facilitating diffusion within infected cells is apparent from Figure 4. Normal or near-normal $Lb$ concentrations were predicted to maintain relatively uniform concentrations of $O_2$ throughout infected cells. A simulated decrease to 5% of the normal $Lb$ concentration resulted in sharply increased $O_2$ gradients. Predicted $O_2$ concentrations in the layers nearest the air space were almost certainly high enough to inhibit nitrogenase activity. By changing gradients of $O_2$, changes in $Lb$ could significantly affect total nitrogenase activity, even though total central zone respiration and therefore apparent $P_O$ from gas exchange decreased by <10%. However, the simulated nodule with only 5% of the normal $Lb$ concentration also exhibited extremely rapid changes in $F_{\text{OIL}}$ in response to external $O_2$ concentration, unlike any of the hundreds of real nodules we have analyzed by nodule oximetry (including many in situ field assays of two species). This suggests that $Lb$ concentrations low enough to limit nodule function significantly may be uncommon in nature.

Although normal $Lb$ concentration would tend to buffer $O_2$, somewhat against rapid changes, Figure 2 shows that the buffering capacity of $Lb$ is limited. Prior to nitrate exposure (Fig. 2A), this buffering capacity was equivalent to only about 10 s of respiratory $O_2$ consumption at the rate occurring under air. After external $O_2$ increased to 70 kPa, the buffering capacity of $Lb$ to absorb $O_2$ was consumed in less than 7 s. $Lb$ buffering is apparently less important than nodule permeability control or other poorly understood mechanisms that can protect nitrogenase from irreversible inactivation with a brief exposure to 100 kPa $O_2$ (Denison et al., 1992b).

Comparison with the Thumfort Model

Recently, Thumfort et al. (1994) published a model that suggests a role for $Lb$ in "controlling the rate of $O_2$ influx into infected cells." Thumfort et al. assumed that infected cells can be modeled as packed cubes (Fig. 5), which is arguably more accurate than the approach presented here. However, their translation of this cubical assumption into a usable model is only approximate. Rather than modeling the cubes in three dimensions, they represent the interior of infected cells as a series of conceptual "layers" defined by their distance from "the nearest [air] space-cell interface."

Unfortunately, volume elements that are treated identically using this approach may differ greatly in $O_2$ status. This problem is illustrated in Figure 5. The volume elements labeled 1 and 2 would be lumped together in a single layer in the Thumfort model because they are equidistant from the nearest air space. Under the nearest air space...
approximation, they should have the same \( O_i \) and therefore (a) the same rate of \( O_2 \) influx from the adjacent “layer” (not shown) nearer the air space and (b) the same rate of \( O_2 \) consumption from respiration. By conservation of mass, they should therefore have the same steady-state \( O_2 \) efflux to the next “layer.” However, this cannot be true. The efflux from element 1 must be zero (by symmetry), whereas the flux from element 2 must be positive (or all more distant layers would be anaerobic).

A similar problem on a smaller scale was solved for the new model presented here by assuming that a specific 9.6% of cell volume was metabolically inactive, but Thumfort et al. have apparently not made a similar assumption. On the surface of the cube (most of which is not exposed to air spaces), elements 3 and 4 are equidistant from the nearest space, but there are two such spaces for element 4. Therefore, element 4 would have higher \( O_i \) than element 3, which violates the assumption that volume elements in the same “layer” are identical. Most “layers” in a cell would be intermediate in shape between the two “layers” illustrated in Figure 5. Without a three-dimensional model of a cubical cell, we are unable to estimate the magnitude of errors introduced by the nearest air space approximation. Thumfort et al. have apparently developed such a three-dimensional model, but they presented no details supporting their statement that results were “similar.”

One qualitative difference between the predictions of the Thumfort model and the model presented here is particularly interesting. With the Thumfort model, total \( O_2 \) flux into an infected cell (based on respiration rates given in their Table III, assuming conservation of mass and steady-state conditions) was actually predicted to increase somewhat with a decrease in \( Lb \) concentration. This contrasts with the prediction made in our Table III, that decreased \( Lb \) concentration would, if anything, decrease \( O_2 \) flux into infected cells. Changing the assumed diffusivity of \( Lb \) to the value used by Thumfort et al. changed predicted total respiration rate by <1%; \( O_2 \) gradients were similar to those shown in Figure 4 for an \( Lb \) concentration of 0.34 mol m\(^{-3}\).

The two models do agree on one important point. Neither model predicts that changes in \( Lb \) concentration could explain the major decreases in nitrogenase-linked respiration rates (typically to <50% of controls) that actually occur with nitrate exposure (Vessey et al., 1988; Minchin et al., 1992).

**Interpretation of Nodule Oximetry Data**

The usual practice with the nodule oximeter (Denison and Layzell, 1991; Denison et al., 1992a) assumes standard published values for the concentration and \( O_2 \) affinity of \( Lb \) in all calculations. Because actual values for these parameters may vary among species, we have used the nodule oximeter primarily for monitoring treatment effects within a species, and we have made only qualitative comparisons between species.

When the computer model and nodule oximetry algorithms both assumed the same values for \( Lb \) concentration and \( O_2 \) affinity (the “corrected assay”), apparent \( P_O \) and \( V_{max} \) calculated using the oximetry algorithms were similar to values assumed by the model (compare Tables I and III). This shows that errors due to nonequilibrium conditions and FOL gradients within infected cells are relatively small. Because the model assumed uniform conditions within central zone air spaces, the possible effects of gradients over distances greater than cellular dimensions were not addressed by this model.

Discrepancies between the oximetry algorithms and nodule model in assumed values for \( Lb \) concentration appear to be a significant source of potential errors in absolute values of parameters estimated by nodule oximetry. Discrepancies in assumed values for \( Lb \) concentration led to errors in uncorrected simulated oximeter estimates of both apparent \( P_O \) and \( V_{max} \). In the future, we recommend correcting for any significant changes in functional \( Lb \) concentration (also measured by oximetry) when calculating \( P_O \) and \( V_{max} \) from nodule oximetry data. Small random changes in apparent \( Lb \) concentration, as in Figure 3, probably reflect differences in probe placement (Denison et al., 1992a) rather than fluctuations in actual \( Lb \) concentration, so do not require correction. The \( O_2 \) affinity of \( Lb \) is not measured by the nodule oximeter and may vary among species. Fortunately, differences in the \( O_2 \) affinity of \( Lb \) over a physiologically realistic range were found to have almost no effect on oximeter estimates of either \( P_O \) or \( V_{max} \).

Modeling confirmed the validity of nodule oximetry for monitoring treatment effects on individual nodules, provided that measured changes, if any, in the concentration of functional \( Lb \) are included in calculations of \( P_O \) and \( V_{max} \). The ability to simultaneously monitor \( P_O \) or \( V_{max} \) and \( Lb \) concentration should be useful in further clarifying the roles of these variables in nodule function.

Received August 25, 1994; accepted December 30, 1994.

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