**Communication**

**Regulation of O₂ Concentration in Soybean Nodules Observed by in Situ Spectroscopic Measurement of Leghemoglobin Oxygenation¹**

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**ABSTRACT**

A fiber optic spectrophotometric system was used to monitor the *in vivo* oxygenation of leghemoglobin in intact, attached soybean root nodules (*Glycine max* L. Merr. × USDA 16 Bradyrhizobium japonicum) which were flattened during development by growth in narrow, glass-walled cuvettes. When equilibrated at an external pO₂ of 20 kilopascals, leghemoglobin was 36.6 ± 5.4% oxygenated, a value estimated to represent an infected cell O₂ concentration of 21.5 nanomolar. Increasing the external pO₂ from 20 to 25 kilopascals caused a rapid increase in leghemoglobin oxygenation, followed by a recovery to the initial level, all within 7.5 minutes. At 25 kilopascals O₂, the rates of H₂ and CO₂ evolution were similar to those at 20 kilopascals. Since respiration had not increased, the results support the proposal that nodules adapt to increased external pO₂ by regulating their resistance to O₂ diffusion.

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N₂ fixation by *Rhizobium* bacteria in symbiosis with legumes requires a large supply of reductant and ATP from aerobic respiration, yet nitrogenase, the N₂-fixing enzyme, is irreversibly inhibited by even low levels of O₂ (13). Therefore, it has been proposed that the legume root nodule must be capable of regulating its O₂.² (14).

Tjeckema and Yocum (18) provided evidence that a barrier to O₂ diffusion is present in the nodule cortex surrounding the infected cells. It has been suggested (16–18) that this barrier consists of a continuous layer of cells with water-filled intercellular spaces. Gas exchange experiments have indicated that the barrier is physiologically regulated (7, 14, 21, 23), presumably through changes in thickness of the water-filled layer (8, 15). Wittry et al. (24), using O₂ microelectrodes, provided direct evidence that O₂ is regulated in response to changes in O₂; however, this technique was invasive and was not sufficiently sensitive to provide an estimate of O₂ under ambient conditions. An aim of the present study was to provide evidence for regulation of O₂ using a noninvasive technique of much higher sensitivity.

Upon increasing O₂, an immediate, temporary decline in nitrogenase activity is observed (7, 21), which cannot be accounted for by destruction and *de novo* synthesis of nitrogenase (7). Either of two mechanisms could account for this decline: (a) an immediate, overcompensating increase in the diffusion resistance may occur, causing a rapid decrease in O₂ and limiting respiration to support nitrogenase activity; or (b) a more gradual increase in the diffusion resistance may occur, allowing O₂ to undergo a transient increase, which in turn causes reversible inhibition of nitrogenase. A further aim of this study was to investigate these two hypotheses by direct measurement of O₂.

The infected cells of the nodule contain *Lb*, a myoglobin-like protein that binds reversibly with O₂ with high affinity. *Lb* serves as a carrier of bound O₂ to the bacterial electron transport chain while maintaining a low O₂ (3, 22). Spectrophotometric measurement of oxygenation-dependent changes in the optical absorbance of *Lb* is a highly sensitive, noninvasive means for monitoring O₂. However, it is difficult to obtain meaningful absorbance spectra from intact soybean nodules due to their thickness (about 5 mm), which results in high light absorbance and scattering, and their spherical shape and heterogeneous structure, which result in preferential transmission of light through the non-*Lb*-containing cortex (4, 9). Therefore, in most studies *Lb* oxygenation has been measured in nodule slices (2, 4). Although Klucas et al. (9) measured *Lb* oxygenation in intact, attached nodules of sweet clover, they did not study the regulation of O₂ under changing environmental conditions. In the present study, an improved spectroscopic technique is described, which was used to measure O₂ in intact, attached soybean nodules that were flattened during development by growth in narrow glass-walled cuvettes. This technique was used to obtain evidence for regulation of O₂ with increases in O₂ and to determine the speed with which this regulation occurs.

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² Abbreviations: O₂, concentration (nm) of free O₂ in the infected cells of the nodule; O₂, pO₂ partial pressure (kPa) outside the nodule; *Lb*, ferrous leghemoglobin; Y, fractional oxygenation of *Lb* as percent; *Aₗₒ*, nodule absorbance (642 nm) at O₂ = 0 kPa (fully deoxygenated *Lb*); *Aₒₜ*, nodule absorbance (642 nm) at O₂ = 100 kPa (fully oxygenated *Lb*); *Aᵣ*, nodule absorbance at time *t*; *kᵣ*, rate constant for dissociation of O₂ from *Lb*; *kₛ*, rate constant for binding of O₂ with *Lb*. 

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**Keywords:** Oxygenation, Leghemoglobin, soybean root nodules, spectroscopy.
MATERIALS AND METHODS

Plant Material and Growth and Experimental Conditions. Seeds of soybean (Glycine max L. Merr. cv Harosoy 63) were inoculated with Bradyrhizobium japonicum strain USDA 16 and grown as described previously (7) in a growth chamber with 16 h photoperiod, 25°C day, and 20°C night. At age 10 d, seedlings were placed with the root systems enclosed in cuvettes as shown in Figure 1. The cuvettes were then placed in 10 cm diameter plastic pots, covered with moist vermiculite, and returned to the growth cabinet. Plants were used for experiments at age 30 to 32 d, and were kept in a greenhouse at the site of the spectrophotometer (Royal Military College of Canada) on the day of the experiments.

A total period of 5 to 10 min elapsed between removal of a plant from the greenhouse and beginning of the measurements. The assays were carried out at room temperature (approximately 22°C). The shoots were maintained in darkness during the experiments because of the need to prevent stray light from reaching the spectrophotometer. However, this is unlikely to have affected nitrogenase activity since soybeans do not show a pronounced diurnal variation in nitrogenase activity (20), and since the rates of H2 and CO2 evolution were stable.

Gas Exchange and Spectrophotometric Measurements. Each plant chosen had a relatively homogeneous population of nodules that were flattened against the glass walls of the cuvette. For an experiment, a cuvette (Fig. 1) was removed from its growth pot, sealed with silicon rubber (Dow Corning 3110 RTV cured with RTV 4 catalyst), and equipped with 18.5 gauge hypodermic syringe needles for connection to an open circuit gas exchange system to monitor H2 and CO2 evolution, as described previously (7). For spectrophotometric measurements, quartz fiber optic probes were placed on either side of a typical nodule. The illuminating probe (a fiber bundle of 2 mm diameter) was directed to a xenon arc light source equipped with a lens for focusing and a band pass filter to reduce illumination in the red region. The collecting probe (a single fiber of 1.5 mm diameter) was directed to a Tractor Northern TN1710 spectrophotometer. The complete spectrophotometric system is described elsewhere (12). The lag time between the spectrophotometric and gas exchange measurements was approximately 1 min.

Experimental Procedure and Calculations. During each experiment the absorbance spectrum of a selected nodule was monitored over the range of 500 to 650 nm. Each complete scan lasted 40 to 80 ms, and at selected intervals of 30 s or more, 200 sequential scans were averaged to obtain a mean spectrum which covered 8 to 16 s. Meanwhile, the nodule cuvette was flushed with 20 kPa O2 until a stable spectrum and steady state conditions of CO2 and H2 evolution were attained (generally within 10 min). Then O2 was increased to 25 kPa and the absorbance spectrum was monitored until new, steady state rates of gas exchange were attained (after 30–45 min). Finally, the nodules were exposed for 5 to 10 min to and then to 100 kPa O2; to obtain the spectra for the fully deoxygenated and fully oxygenated forms of Lb, respectively. Figure 2 illustrates typical, steady state absorbance spectra obtained sequentially for a single nodule under 0, 20, and 100 kPa O2.

Fractional oxygenation of Lb (Y), or percent of Lb bound to O2, was calculated as by Appleby (1):

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Y = \frac{A_{\text{red}} - A_i}{A_{\text{red}} - A_{\text{oxy}}} \times 100
\]  

(1)

Fig. 1. Cuvette for gas exchange and optical absorbance measurements in intact attached soybean nodules. Root systems were enclosed between 3 x 4 cm plates of glass (g) separated with plastic tubing (t) to a width of 1.75 to 2.0 mm and held together by paper clamps (c). Nodules; f, fiber optic probes; ss, silicon sealant; s, syringe tips for open system gas exchange. Arrows indicate the path of gas flow.

Fig. 2. Typical in vivo absorbance spectra of a single soybean nodule which was exposed sequentially to 20, 0, and 100 kPa O2. Each curve was obtained when steady state absorbance was attained (approximately 5 min after a change in pO2). (---), O2 = 0 kPa; (——), O2 = 20 kPa; (-----), O2 = 100 kPa. Fractional oxygenation of this nodule (from Eq. 1) was 25% and the estimated O2 (from Eq. 2) was 12.4 nm.
where $A_{red}$ and $A_{oxy}$ are the absorbances at 642 nm of a nodule with $Lb$ in the fully deoxygenated and fully oxygenated forms, respectively; and $A_i$ is the absorbance at 642 nm of the same nodule at a specific time ($t$) during the experimental treatments. The absorbance at 642 nm was chosen since this region of the spectrum showed the largest absorbance changes while minimizing possible interference from ferric leghemoglobin (6). However, other wavelengths gave similar results and could also be used.

$O_i$ was then estimated from the following equation (6):

$$O_i = \frac{Y(k_i/k_o)_{100 - Y}}{k_i/k_o} \quad \cdots \quad (2)$$

where $k_i/k_o = 37.3 \text{ nm}$. It was assumed that the in vivo properties of $Lb$ are similar to the in vitro properties reported by Appleby (1) and by Bergersen and Turner (6). The estimate of $O_i$ assumed that the steady state spectrum under 100 kPa $O_2$ represented full oxygenation of $Lb$. These assumptions are discussed below.

RESULTS AND DISCUSSION

The methods of the present study have several advantages over those used previously. Flattening of nodules during development reduced the thickness of the cortex and central zone and maximized transmission of light through the central zone. The use of fiber optic probes enabled precise orientation of the light beam through the nodule and collection of the transmitted light. The spectrophotometer allowed extremely rapid, stable and sensitive spectral scans and incorporated signal averaging to reduce noise levels (12). An open circuit gas exchange system made possible rapid changes in $O_i$ with continuous monitoring of $CO_2$ and $H_2$ evolution. (The $H_2$ evolution rate in a symbiosis that lacks uptake hydrogenase provides a measure of nitrogenase activity [7, 11]). Thus, it was possible to obtain meaningful, in vivo spectra of $Lb$ in nodules which displayed normal rates of $H_2$ and $CO_2$ evolution.

The absorbance spectra (Fig. 2) were very similar to those which have been obtained for purified $Lb$ in the oxygenated and deoxygenated states (6). Since $Lb$ is present in nodules at very high concentrations relative to other cellular redox components such as cytochromes (5), it was assumed, as in earlier studies (2, 4, 9), that the changes in the absorption spectra were due entirely to $Lb$ oxygenation.

To obtain a quantitative estimate of $O_i$, the spectra obtained following exposure to 0 and 100 kPa were assumed to represent the fully deoxygenated and fully oxygenated forms of $Lb$, respectively. This assumption has been made in previous studies (2, 4, 9) and is supported by the observation that nodules cannot protect nitrogenase by adapting to large instantaneous changes in external $pO_2$ (7). Therefore, the results for intact nodules in this study most likely provide reasonable estimates of $O_i$, and as such they represent the first determination of $O_i$ in intact, attached soybean nodules. In any case, the qualitative conclusions of this study would not be different if the $Lb$ at an $O_i$ of 0 or 100 kPa were something less than fully deoxygenated or fully oxygenated, respectively.

The estimated mean fractional oxygenation for seven plants at atmospheric $O_i$ (20 kPa) was 31\% (se = 5.4\%), corresponding to a mean $O_i$ of 21.5 \text{ nm}. In comparison, the $O_2$ concentration in water equilibrated with 20 kPa $O_2$ is approximately 250 \text{ \muM}.

The extremely low value for $O_i$ obtained in this study agrees with an estimate of 10 \text{ nm} based on $Lb$ absorbance of sliced nodules (2) and with the $O_2$ microelecrole measurements of Witty et al. (24), in which nodules under normal conditions displayed $O_2$ which were below the detection limit of the electrode (approximately 1 \text{ \muM}).

In the present study, to observe possible regulation of $O_i$, the absorbance spectrum of a nodule was monitored while $O_i$ was increased from 20 to 25 kPa. Figure 3 shows a typical time course of $Lb$ fractional oxygenation, estimated $O_i$, and associated rates of $CO_2$ and $H_2$ exchange following the increase in $O_i$. In the nodule assayed in Figure 3A, the estimated $O_i$ was 26 \text{ nm} at 20 kPa $O_2$. When $O_i$ was increased to 25 kPa, $O_i$ increased within 2.5 min to approximately 260 \text{ nm}. During the next 2.5 min it declined rapidly to near its original level. Meanwhile, $CO_2$ evolution of the roots and nodules in the cuvette, after an initial peak, declined to 80\% of its initial level, then gradually increased to a steady state at 94\% of initial level (Fig. 3B). Since the roots are insensitive to increases in $O_i$ above atmospheric levels (7) the changes in respiration were attributed to nodule activity only. $H_2$ evolution of the nodules (Fig. 3B), after two initial peaks, declined to 76\% of initial, then returned to a steady state at 93\% of initial, indicating little or no damage to nitrogenase by the transient increase in $O_i$. By comparison, exposure to 100 kPa $O_2$ at the end of the experiment completely eliminated $H_2$ evolution, with a corresponding reduction of $CO_2$ evolution to 57\% of its original level.

The peak in $CO_2$ evolution and the second peak in $H_2$ evolution
followed the peak in $O_2$ by 7.5 min, and this delay could not be accounted for by the time lag of the gas exchange system. Similar peaks have been observed previously (7) and, while they must have a physiological basis, their cause is unknown. Likewise, the transient declines in CO$_2$ and H$_2$O evolution have been observed previously (7, 21) and are considerably more pronounced with larger increases in $O_2$.

For an increase from 20 to 25 kPa O$_2$, a 20% increase in respiration would be required to maintain $O_2$ at its original level (7). Since the steady state rate of CO$_2$ evolution under 25 kPa O$_2$ (Fig. 3B) was slightly lower than the original rate under 20 kPa O$_2$, an increase in respiration could not account for the observed regulation of $O_2$. Although O$_2$ uptake was not measured in the study, it is highly unlikely that reduction of the respiratory quotient (i.e., an increase in O$_2$ uptake without an accompanying increase in CO$_2$ evolution) could adequately compensate for increases in $O_2$. Another possible explanation is that O$_2$ is actively excluded from the infected cells. However, no O$_2$ carrier has been reported in any living system and furthermore such a carrier would require energy, thereby increasing respiratory demand, which was not observed. The most likely explanation is that regulation of $O_2$ is brought about, at least in part, by changes in diffusion resistance of the nodule cortex. This conclusion is in agreement with empirical results and theoretical considerations of previous reports (7, 8, 10, 14, 15, 19, 21, 23).

The transient increase in $O_2$ which follows an increase in $O_2$ demonstrates that the regulatory response is not rapid enough to maintain constant $O_2$ immediately after the change. This supports the suggestion of Hunt et al. (7) that an increasing $O_2$ regulation of the diffusion barrier is slow enough to allow a transient increase in $O_2$, which causes reversible inhibition of nitrogenase activity and the resulting declines in H$_2$ and CO$_2$ evolution.

In summary, this study provides an estimate of $O_2$ in intact, attached nodules of soybean under ambient $O_2$. It demonstrates that nodules are able to regulate $O_2$ in response to an increase in $O_2$. This regulation most likely results from control of the nodule diffusion resistance. Further work is required to determine the nature of the diffusion barrier, the mechanism of its regulation, and its effect, through O$_2$ supply, on the rate of N$_2$ fixation.

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LITERATURE CITED