**Diffusion Limitation of Oxygen Uptake and Nitrogenase Activity in the Root Nodules of Parasponia rigida Merr. and Perry**

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**ABSTRACT**

*Parasponia* is the first non-legume genus proven to form nitrogen-fixing root nodules induced by rhizobia. Infiltration with India ink demonstrated that intercellular air spaces are lacking in the inner layers of the nodule cortex. Oxygen must diffuse through these layers to reach the cells containing the rhizobia, and it was calculated that most of the gradient in 02 partial pressure between the atmosphere and rhizobia occurs at the inner cortex. This was confirmed by O2 microelectrode measurements which showed that the O2 partial pressure was much lower in the zone of infected cells than in the cortex. Measurements of nitrogenase activity and O2 uptake as a function of temperature and partial pressure of O2 were consistent with diffusion limitation of O2 uptake by the inner cortex. In spite of the presumed absence of leghemoglobin in nodules of *Parasponia rigida* Merr. and Perry, energy usage for nitrogen fixation was similar to that in legume nodules. The results demonstrate that O2 regulation in legume and *Parasponia* nodules is very similar and differs from O2 regulation in actinorhizal nodules.

*Parasponia* in the Ulmaceae is the first non-legume genus proven to form root nodules induced by rhizobia. *Parasponia* nodules differ from those of legumes in having a central vascular bundle and infection threads that persist in mature nodules (2, 15, 17). Leghemoglobin is absent (5), which makes the study of O2 uptake in these nodules of special interest since leghemoglobin is believed to play an important role in oxygen transport in legume nodules (4, 9). In the present work, we have studied internal 02 uptake, nitrogenase activity, and the distribution of intercellular air spaces. Our findings are similar to previous results with soybean nodules (8-10, 13, 14). In both cases, microaerophilic conditions for the rhizobia are achieved by the presence of specialized cortical cells that completely surround the tissue containing the rhizobia. These cortical cells lack intercellular air spaces and thus restrict O2 diffusion to the rhizobia.

**MATERIALS AND METHODS**

Plants of *Parasponia rigida* Merr. and Perry were grown from seed (obtained from Papua New Guinea) in pots containing a mixture of equal parts of peat, vermiculite, and sand. The pots were watered daily with tap water and weekly with nitrogen-free nutrient solution. The seedlings were nodulated by drenching the pots with cultures of a rhizobium strain isolated from *Parasponia parviflora* Mig. in Indonesia. Some of the nodulated seedlings were transferred to aeroponics boxes (19), and these were used for anatomical studies and O2 electrode measurements. Other seedlings were planted outdoors, where they grew during the summer months. These were used for studies of gas exchange. The plants were about 1.5 years old when nodules were harvested.

Nodules were vacuum-infiltrated with India ink by placing them under vacuum for 5 min before immersion in ink followed by release of the vacuum. Prior to infiltration, the larger particles in the ink (Higgins India ink; Faber Castell Corp., Newark, NJ) were removed by centrifugation at approximately 12,000g for 30 min.

Oxygen microelectrode measurements were performed as previously described (14), using model 721 microelectrodes from the Transidyne General Corp. (Ann Arbor, MI). These electrodes were glass-insulated platinum with a tip diameter of 2 μm and a 10 μm length of exposed platinum. The microelectrodes were maintained at −0.50 v with respect to a calomel reference electrode, and current was measured with a Keithley model 610C electrometer. The nodule was held against the reference electrode in a modified plastic syringe body (14), and the microelectrode was advanced into the nodule using a micromanipulator. The electrodes and nodule were enclosed in a grounded aluminum box. The electrode path through the nodule was determined by enlarging the hole left by the electrode and then taking freehand sections.

Gas exchange of excised nodules was measured in 30-ml disposable plastic syringes, using a gas volume of 13 to 17 ml and a nodule fresh weight of 0.10 to 0.16 g per ml of gas volume. Gas mixtures were made from air plus additions of N2, O2, and acetylene (generated from CaC2). The final acetylene content was 10% (v/v). Gas composition was measured at the beginning and end of a 7-min incubation period using a Carle model 1153 gas chromatograph equipped with a thermal conductivity detector employing thermistors. Measurements were made of CO2, ethylene, acetylene, O2, and N2 using a column (1.7 mm i.d., 0.95 m) packed with a mixture of 75% (v/v) Porapak N (50–80 mesh) and 25% Porapak R (80–100 mesh) and a second column packed with Molecular Sieve 5A (80–100 mesh; 1.7 mm i.d., 0.85 m length). The columns were operated at 35°C with helium carrier gas, series/bypass valve, and injection by sampling loop. Water vapor was removed by passing the samples through metal tubing (1.0 mm i.d., 8 cm length) which was maintained at −20°C and placed upstream of the sampling loop. A computing integrator (Columbia Scientific Industries model 3A) was used to measure peak area and to actuate motorized valves on the chromatograph. Analysis time was 5.8 min. During the 7-min assay period, there was a 0.9% loss of CO2 due to absorption into the plastic and rubber of the syringe used as an assay vessel. Nitrogenase activity and respiration were linear for at least 3 h after nodule detachment, which allowed the effects of P02 or temperature to be measured using a single sample of nodules. In Figure 6, measurements were...
made in the sequence of 21, 24, 28, 21, 18, 15, and 12°C, with 8 min being allowed for equilibration after each temperature change. In Figure 7, the sequence was 20, 15, 10, 5, 20, 25, 30, 40, and 50 kPa O₂.

The volumes of intercellular air spaces in the lenticels and bacterial zone were estimated from their areas in nodule cross-sections.

RESULTS

Distribution of Intercellular Air Spaces. Figures 1 and 2 show freehand sections of nodules after vacuum infiltration of India ink into the intercellular air spaces. As previously observed, cells infected by rhizobia occur in zones between the central vascular bundle and uninfected cortical cells at the exterior of the nodule (2, 15, 17). The vascular bundle is connected to the cortical tissue by one or more ray-like areas of uninfected tissue. The India ink entered the nodule via lenticels on the nodule surface (Fig. 1) and filled spaces between cells in the middle cell layers of the cortex. Ink was also observed between some of the uninfected cells that connect the cortex to the vascular bundle (Fig. 2). Ink did not infiltrate into a layer about four cells thick at the exterior of the nodule and another layer about seven cells thick adjacent to the infected tissue. The layer in the inner cortex may correspond to the layer of densely staining cells noted by Trinick (15). Thus, India ink was observed only in the lenticels, the middle part of the cortex, and in the tissue connecting the cortex and vascular bundle. Ink was never observed in the infected tissue, indicating that air spaces do not pass directly from the atmosphere to the infected cells. Although the infected tissue appears dark in Figures 1 and 2, this darkness is due to the density of the cell contents and is not localized in the intercellular air spaces.

To confirm these results, we vacuum-infiltrated nodules with a variety of dyes dissolved in ethanol or water. The same distribution of intercellular spaces was found, with the dyes never penetrating into the infected tissue. Photographs were not possible, because the dyes rapidly diffused out of the intercellular spaces. We also confirmed that the ink- or dye-filled spaces observed were normally filled with air. In freehand sections, air was retained in many of the intercellular spaces and was readily observed as dark areas resulting from the difference in light refraction between the air-filled spaces and the plant cells (3). Using this technique, the intercellular air spaces observed corresponded to the spaces that were filled with India ink after infiltration. By the use of light refraction, intercellular air spaces were also observed in the bacterial zone but not in the inner cortex (Figs. 3 and 4).

Measurements of Internal Pₐ. With air in the chamber which enclosed the nodule and electrodes, the O₂ microelectrode current was highest in the nodule cortex, decreased rapidly at the boundary between the cortex and bacterial zone, and was very low within the bacterial zone (Fig. 5). Most of the low electrode current in the bacterial zone was due to reduction of substances other than O₂, since the current was about the same whether N₂ or air was present in the electrode chamber. But the high electrode current in the cortex was clearly due to O₂, since the current was about 50 times lower with N₂ in the electrode chamber than with air. The increase in electrode current near the nodule surface during N₂ flushing may have been due to traces of O₂ in the electrode chamber. From the results in air and N₂, it is clear that the average Pₒ₂ in the nodule cortex is much higher than in the bacterial zone.

Nitrogenase Activity and Respiration as a Function of Temperature and Pₒ₂. Nitrogenase activity was optimal and relatively independent of temperature between 18°C and 28°C, but decreased to low levels as temperature decreased from 18 to 12°C (Fig. 6). Similar results were found by Trinick (16), except that he found optimal activity between 25 and 35°C. Similar results have been observed for legumes such as soybeans, where nitrogenase activity is optimal and relatively independent of temperature over the range of approximately 15 to 30°C (7). In contrast to nitrogenase activity, CO₂ evolution and O₂ uptake were temperature dependent between 18 and 28°C, but the Q₁₀ for O₂ uptake was only 1.4, which is less than the usual value of 2 to 3 for plant tissue.

At a constant temperature of 22°C, we found maximum nitrogenase activity at 26 kPa O₂ (Fig. 7). This is somewhat higher than the optimum of 20 kPa found by Trinick (16). The uptake of O₂ and evolution of CO₂ increased almost linearly with increased Pₒ₂ between 5 and 40 kPa, which is similar to previous results with soybean nodules (7, 13). The evolution of CO₂ is somewhat understated in both Figures 6 and 7, inasmuch as no correction was made for retention of respired CO₂ in the tissue at the end of the assay. Extrapolation from measurements on nodules of Alnus rubra indicate that CO₂ evolution was understated by approximately 9% in Figure 6 and 14% in Figure 7.

Calculation of Pₒ₂ Gradients. As illustrated in Figure 8, O₂ enters the nodule through lenticels and then diffuses in intercellular air spaces through the central part of the nodule cortex. However, air spaces are absent in the inner cortex, and O₂ must diffuse through the cell contents of this tissue to reach the bacterial zone. Within the bacterial zone, air spaces are present through which O₂ diffuses before entering the rhizobia-containing cells. The Pₒ₂ gradient across each of these components of the pathway of O₂ diffusion from the atmosphere to the rhizobia can be estimated by calculation from our values for nodule dimensions and O₂ consumption rates. The Pₒ₂ gradient across the air-space-free layer in the inner cortex was calculated from Fick's law of diffusion:

\[
Q = DA \frac{dC}{dx}
\]

where \( Q \) is the rate of material transfer through area (A), \( D \) is the diffusion coefficient, and \( dC \) is the change in concentration in the distance \( dx \) in the direction of diffusion. Using the above, we calculated \( dC \) at the inner cortex, assuming the diffusion coefficient of O₂ in pure water (2.13 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}), A as that of a sphere of 0.22 cm diameter (the diameter at the inner cortex), a value for \( dx \) of 0.010 cm (the thickness of the inner cortex), and a value for \( Q \) of 4.5 \times 10^{-11} \text{ mol sec}^{-1} \text{ cm}^2 based on a nodule diameter of 0.25 cm and assuming that all nodule respiration occurs interior to the inner cortex. From the above, we calculated a value for \( dC \) equivalent to 11 kPa which is a substantial part of the maximum Pₒ₂ gradient of 20 kPa. But the solubility and diffusion of O₂ are probably substantially less in nodule tissue than in pure water: O₂ diffuses through muscle tissue at one-third to one-half of the rate through pure water (18). Thus, 11 kPa is the minimum value for the Pₒ₂ gradient across the inner cortex, with the actual value being greater.

Calculations of the Pₒ₂ gradients across the other components of the O₂ diffusion pathway to the bacteroids show that these gradients are small compared to that calculated for the inner cortex. The Pₒ₂ gradient exterior to the inner cortex is primarily the gradient across the lenticels. This gradient was calculated from equation 1 with the following differences: the lenticels were estimated to occupy 3% of the nodule surface area, and O₂ was assumed to diffuse only through the intercellular air spaces which were estimated to occupy 3% of the lenticel volume. Thus, 0.09% of the total nodule surface area was available for O₂ diffusion. The lenticel thickness was 0.025 cm and 0.18 cm² s⁻¹ was used for \( D \), which is the diffusion coefficient of O₂ in air. From the above, we calculated an O₂ gradient equivalent to 0.09 kPa, which is
small compared to the total gradient of 20 kPa.

There is also a \( P_{O_2} \) gradient in the intercellular air spaces between the inner cortex and the infected cells in the bacterial zone. This gradient can be calculated from the equation:

\[
C_0 - C_r = \frac{V_m}{6D} (R^2 - r^2) \quad (2)
\]

which describes the \( O_2 \) gradient \((C_0 - C_r)\) from the surface of a sphere of radius \( R \) to any radius \( r \); \( V_m \) is the maximum respiration rate per unit volume. The derivation is from Fick’s law of diffusion with the assumption that respiration is \( O_2 \) saturated and uniformly distributed (6). We used a value for \( V_m \) of \( 20 \times 10^{-9} \) mol s\(^{-1}\) cm\(^{-3}\) based on the assumption that respiration in the bacterial zone is twice the rate for the nodule as a whole. The value used for \( R \) was 0.11 cm, and that for \( r \) was 0.04 cm. These are the radii for the inner and outer limits of the bacterial zone. The value used for the diffusion coefficient \( (D) \) was the value for \( O_2 \) in air times 0.03 inasmuch as we estimated that the intercellular air spaces occupy 3% of the total volume in the bacterial zone. Using the above values, the calculated value for \( C_0 - C_r \) was equivalent to 0.015 kPa, which is small compared to the total \( P_{O_2} \) gradient of 40 kPa, which is the external \( P_{O_2} \) at which nodule respiration is \( O_2 \) saturated.

Finally, there is an \( O_2 \) gradient between the air spaces of the bacterial zone and the individual rhizobia within the infected cells. This gradient was again calculated from Fick’s law, using the following derivation and assumptions. The bacterial zone was assumed to be composed of cylinders of radius \( R_c \) containing the rhizobia and supplied with \( O_2 \) diffusing from smaller concentric cylinders of radius \( R_b \) which represent the intercellular air spaces (see Fig. 9). It is assumed that respiration is saturated throughout the cylinder. At any radius, \( r \), between \( R_b \) and \( R_c \), diffusion follows Fick’s law:

\[
Q = 2 \pi D r \frac{dC}{dr} \quad (3)
\]
Respiration exterior to radius \( r \) follows the equation:

\[
Q = \pi 1 V_m (R_e^2 - r^2)
\]

(4)

Setting these two equations equal and integrating from \( R_e \) to \( R_l \), using the boundary condition that \( C = 0 \) at \( r = R_l \):

\[
C_o = 0.5 V_m D^{-1} [R_e^2 \ln (R_l - R_e) - R_e^2 + 0.5 R_e] \quad (5)
\]

Thus, \( C_o \) is the concentration of \( O_2 \) at the intercellular air spaces when \( C = 0 \) at \( R_l \). To calculate \( C_o \), we used the following values: \( R_e = 1 \times 10^{-2} \) cm, \( R_l = 1.2 \times 10^{-2} \) cm, \( D = 1 \times 10^{-5} \) cm s\(^{-1}\), and \( V_m = 20 \times 10^{-6} \) mol s\(^{-1}\) cm\(^{-3}\). The value of \( R_e \) is the estimated radius of the intercellular air spaces and \( R_l \) is one-half of the average distance between intercellular air spaces. From the above, we calculated that the \( P_o \) in the intercellular air spaces required to saturate respiration of the infected cells is 0.23 kPa. This is small compared to the external \( P_o \) of 40 kPa required to saturate nodule respiration. Thus, the \( P_o \) gradients calculated for the lenticels, intercellular air spaces of the central tissue, and within the infected cells are all very small compared to the \( P_o \) gradient calculated for the inner cortex.

**DISCUSSION**

In the absence of evidence to the contrary, we assume that the rhizobia in *Parasponia* nodules require microaerophilic conditions for nitrogen fixation, as is the case for other rhizobia. Our calculations indicate that a large \( P_o \) gradient occurs across the inner cortex, but not the other components of the pathway of \( O_2 \) diffusion from the atmosphere to the rhizobia (see “Results” and Fig. 8).

This conclusion is supported by our measurements with \( O_2 \) microelectrodes. Electrode current decreased sharply as the electrode passed through the cortex, with only very low currents being observed throughout the bacterial zone. Thus, it is unlikely that there are regions of high \( P_o \) in the bacterial zone, which should be present if a significant part of the total \( P_o \) gradient occurs in this tissue.

If \( O_2 \) uptake by the bacterial zone is limited by \( O_2 \) diffusion through the inner cortex, one would expect \( O_2 \) uptake to follow equation 1 where \( D, A, \) and \( dx \) are properties of the inner cortex. This predicts that \( O_2 \) uptake would be directly proportional to external \( P_o \) as long as the \( P_o \) in the bacterial zone is small compared to external \( P_o \). Such a result is observed in Figure 7, with \( O_2 \) uptake being proportional to \( P_o \) between 0 and 40 kPa. Respiration becomes \( O_2 \) saturated at about 40 kPa.

Further evidence for diffusion limitation of bacterial zone respiration comes from the dependence of \( O_2 \) uptake and nitrogenase activity on temperature (Fig. 6). Between 18 and 28°C, there was little change in nitrogenase activity, while the \( Q_o \) of \( O_2 \) uptake was only 1.4. This is much closer to the \( Q_o \) of a diffusion process, which is 1.15 (18), than respiration of typical plant tissue which has a \( Q_o \) of 2 to 3. The fact that the \( Q_o \) was greater than 1.15 may have been due to \( O_2 \) uptake by cortical and vascular tissue exterior to the bacterial zone, where respiration is less diffusion limited. Nitrogenase activity is presumably more or less proportional to the rate of \( O_2 \) diffusion to the bacterial zone, and thus, the lack of temperature response between 18 and 28°C is consistent with diffusion limitation of \( O_2 \) uptake by the bacterial tissue. The decrease in nitrogenase activity below 18°C was most likely due to a marked increase in the \( P_o \) in the bacterial tissue as respiration became \( O_2 \) saturated.

All of the above observations are similar to previous results for soybean nodules (8, 9, 13, 14) and indicate that there are no fundamental differences in the means by which microaerophilic conditions are maintained in the bacterial zone. This suggests that the *Parasponia-Rhizobium* symbiosis has much more in common with the legume-*Rhizobium* symbiosis than with actinorhizal root nodules. In actinorhizal nodules, there are sometimes continuous intercellular air spaces from the atmosphere to the infected cells, nitrogenase activity is strongly temperature dependent, and microaerophilic conditions are not required for nitrogen fixation by the free-living endophyte (9, 11).

In view of the preceding, the absence of leghemoglobin from *Parasponia* nodules is puzzling (5). Leghemoglobin is apparently essential for \( O_2 \) transport in legume nodules and may serve to minimize the \( P_o \) within infected cells (4, 9, 13). A possible explanation for the absence of leghemoglobin in *Parasponia* nodules is that the average \( P_o \) in the infected cells may be lower than in legume nodules. Using equation 5, we estimate that, at an external \( P_o \) of 20 kPa, 75% of the rhizobia in *Parasponia* nodules are at \( P_o \) values below 0.018 kPa. Isolated soybean bacteroids show lactic inhibition due to excessive \( O_2 \) at this \( P_o \) value (4). Another possibility is that rhizobia from *Parasponia* are more tolerant of \( O_2 \) than are other rhizobia (1).

Because of the absence of leghemoglobin, one might hypothesize that there would be greater energy usage per unit of \( N_2 \) fixed in *Parasponia* nodules than in legumes, due to some form of respiratory protection. However, this was not observed. The ratio of \( CO_2 \) evolution to acetylene reduction can be taken as a measure of energy usage for nitrogen fixation. We observed a minimum ratio of 3.4 mol of \( CO_2 \) evolved per mol of acetylene reduced in *Parasponia* which compares to a minimum ratio of 2.5 in soybeans in the laboratory and ratios of 3.4 to 5.6 in field collections of other legumes (12). Although more measurements are needed, it seems that the energy efficiency of nitrogen fixation in *Parasponia* is similar to that in legumes.

In conclusion, the inner cortex in *Parasponia* nodules limits the rate of \( O_2 \) uptake and creates microaerophilic conditions in the bacterial zone. *Parasponia* infected legume nodules appear to be identical in this respect. But our results give no clear explanation as to why leghemoglobin is present in legume nodules and absent in *Parasponia* nodules. In spite of the absence of leghemoglobin, energy usage in *Parasponia* nodules is similar to that in legume nodules.

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