OXYGEN CONCENTRATION WITHIN THE NITROGEN-FIXING ROOT NODULES OF MYRICA GALE L.¹

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ABSTRACT

Microelectrodes were used to study the oxygen concentration within Myrica gale L. nodules. Low oxygen concentrations were found only in the region of the mature, nitrogen-fixing endophyte, and appeared to correspond to clusters of infected host cells. The oxygen concentration in the remainder of the nodule was much higher. Interconnected intercellular air spaces were demonstrated by infiltration with India ink. Infiltration of the spaces with water greatly reduced oxygen concentration throughout the nodule, indicating that they function in supplying oxygen to the infected cells and remainder of the nodule. These results differ from those found previously for soybean nodules and provide evidence that legume and actinorhizal nodules have different mechanisms for protecting nitrogenase from oxygen.

PURIFIED NITROGENASE is rapidly denatured when exposed to oxygen, and thus requires protection from oxygen when present in organisms living in an aerobic environment. In the Rhizobium-legume symbiosis, there is evidence that the inner layer of the nodule cortex lacks intercellular air spaces and thus restricts diffusion of oxygen to the bacteroid-containing tissue in the center of the nodule (Tjepkema and Yocum, 1974). Measurements with an oxygen microelectrode show a sharp decrease in oxygen concentration at the nodule cortex and a low value in the nodule center. Thus, the nodule structure helps to provide the low oxygen concentration required for nitrogen fixation by the rhizobia.

Little is known about oxygen relations in actinorhizal (actinomycete-induced) root nodules. In Alnus nodules, lenticels allow oxygen to enter the nodules freely, and well-developed intercellular air spaces connect the lenticels directly to the cells containing the actinomycetal endophyte (Wheeler, Gordon and Ching, 1979; Tjepkema, 1979). Thus, there is no cortical barrier to oxygen as in legumes and the Po₂ in the air spaces adjacent to the infected cells is probably close to atmospheric Po₂. However, oxygen concentration within the nodule has not been measured.

In the present study, another actinorhizal plant, Myrica gale, was examined. Myrica gale fixes nitrogen actively (Schwintzer, 1979) and the literature concerning its root nodules has been recently summarized (Schwintzer, Berry and Disney, 1982). The internal oxygen concentration was measured with oxygen electrodes, and the distribution of intercellular air spaces was traced with India ink and examined in thin, resin-embedded sections. Both high and low oxygen concentrations were found in the region of endophyte-containing cells. The low oxygen concentrations apparently correspond to clusters of infected cells. As in Alnus nodules, there was no cortical barrier to oxygen diffusion.

MATERIALS AND METHODS—Seeds of Myrica gale L. were germinated in sand after cold treatment. The seedlings were transferred to aeroponics tanks (Zobel, Del Tredici and Torrey, 1976) containing nitrogen-free nutrient solution and then inoculated with suspensions of ground nodules. The studies were made 3 months after nodule initiation.

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.) were removed by centrifugation at approximately 12,000 g for 30 min.

Thin sections were obtained from nodule lobes fixed in 3% glutaraldehyde and embedded in Spurr’s resin as described by Schwintzer, Berry and Disney (1982). Sections (1 to 2 μm) were stained in 0.9% aqueous KMnO₄.

The acetylene reduction assay was used to measure nitrogenase activity in nodule seg-

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ments (Burris, 1974). Nodules were divided into segments with a razor blade and eight half lobes or four entire lobes were placed in each of the 5-ml plastic syringes used for assay containers. The assay volume was 2.5 ml, the gas mixture was 10% acetylene and 90% air, and the temperature was 25°C. Ethylene accumulation was measured after 2 hr by gas chromatography (Tjepkema, 1978).

Oxygen microelectrode measurements were made as described previously for studies of soybean nodules (Tjepkema and Yocum, 1974). Model 721 microelectrodes from the Transidyne General Corporation (Ann Arbor, Michigan) were used which had a tip diameter of 2 μm and a 10-μm length of exposed platinum. Nodules were held against a calomel reference electrode in a small chamber, and electrode current was measured with a Keithley model 610C electrometer. The platinum microelectrodes were maintained at −0.50 V with respect to the reference electrode, and the apparatus was enclosed in an aluminum box. All measurements were made at room temperature, approximately 24°C. The electrodes were advanced into the nodule along a radial path, directed towards the vascular cylinder. The point of entry was in the basal portion of the youngest nodule lobe, or just below this. After measurements, the point of electrode entry was located and free-hand sections were cut along the electrode path. Measurements were then made of the location of endophyte-containing cells and the vascular cylinder. But the electrode path was visible only close to the point of entry, so there was uncertainty as to the exact path of the electrode.

Before and after each use the electrodes were calibrated in an air-equilibrated agar gel, containing 0.1 M KCl. The relationship between the electrode current in tissue and the current in 0.1 M KCl is only approximate, due to dis-

Table 1. The location of nitrogenase activity in root nodules of Myrica gale

<table>
<thead>
<tr>
<th>Location</th>
<th>Mu mol C2H4 h⁻¹ g⁻¹ fresh wt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Youngest lobe—apical half</td>
<td>0.29 ± 0.14</td>
</tr>
<tr>
<td>Youngest lobe—basal half</td>
<td>3.88 ± 0.46</td>
</tr>
<tr>
<td>Second youngest lobe—entire</td>
<td>2.50 ± 1.04</td>
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* Mean ± SE; N = 3.
solved substances and the intercellular air spaces in the nodule. These are unevenly distributed and their effect on the oxygen electrode current cannot be accurately calculated. Thus the electrode current was only qualitatively related to oxygen concentration in the nodule.

Results—After vacuum infiltration, India ink was found in all tissues of the nodule, except within the vascular cylinder and the periderm. The ink appeared to enter the nodule via the cut surface of the nodule root. The ink-filled spaces were largest just under the periderm and just exterior to the vascular bundle (Fig. 1, 2). The ink-filled spaces were smaller and less extensive in the infected region of the nodule. The spaces were larger at the base of the nodule (Fig. 2) than in the middle of a lobe (Fig. 1).

The pattern of intercellular spaces shown by India ink was confirmed in observations of thin sections of plastic-embedded nodules (Fig. 3). There were large intercellular air spaces under the periderm and just exterior to the vascular cylinder, and small intercellular spaces elsewhere. Air spaces were not observed between adjacent infected cells.

As in other actinorhizal nodules (Becking, 1977), the apical tissue of the nodule had little nitrogenase activity (Table 1), and thus oxygen electrode measurements were made in the older, more active tissue.

Low values of oxygen electrode current were found only in the region of the infected cells (Fig. 5–8). The pattern of electrode current was irregular; in 1 of 12 measurements there were no areas of low oxygen concentration (Fig. 4), while in the remainder there were from 1 to 4 regions of low oxygen concentration on the electrode path from the periderm to the vascular bundle. In the apical halves of the youngest nodule lobes there were no mature infected cells and also no regions of low oxygen concentration (Fig. 9). Thus areas of low oxygen concentration were almost always found in the region of mature infected cells, but not elsewhere in Myrica gale nodules.

Part of the oxygen electrode current was due
to reduction of substances other than oxygen. When measurements were made with argon flowing through the electrode chamber, the electrode current was about 100 times lower and was probably due to reduction of substances other than oxygen (Fig. 8). The higher electrode current near the nodule exterior during the measurements in argon was probably due to failure to completely exclude oxygen from the electrode chamber.

Since oxygen diffuses about 300,000 times faster through air than water, it mostly diffuses to the nodule interior via the intercellular air spaces. When these spaces were infiltrated with water, the oxygen concentration in the nodule interior was much lower (Fig. 10).

**Discussion**—The results show that low oxygen concentrations occur only in the region of the mature endophyte in root nodules of *Myrica gale*. Within this region, areas of both high and low oxygen concentrations were found (Fig. 4–8). The regions of low oxygen concentration varied from about 40 μm to 200 μm in width. They probably corresponded to the locations of individual infected cells or to several adjacent infected cells, since the oxygen electrode has a spatial resolution of approximately 30 μm (Tjepkema and Yocum, 1974), and the infected cells varied in diameter from about 30 to 40 μm. Uninfected cells were probably not responsible for the areas of low oxygen concentration since such cells were highly vacuolate (Fig. 3) and thus not capable of high rates of oxygen uptake. Furthermore, low electrode currents were not observed in the apical halves of nodule lobes, where there were only uninfected cells or immature infected cells (Fig. 9).

This pattern of oxygen concentration is consistent with the distribution of intercellular air spaces in *Myrica gale* nodules (Fig. 1–3). Interconnected, ink-filled spaces were observed in all regions of the nodule except within the vascular cylinder. Air spaces provide a low resistance to oxygen diffusion, and one would thus expect cells connected by them to be at similar oxygen concentrations, as was observed. Intercellular spaces were lacking be-

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Fig. 8. Oxygen electrode current within a nodule in atmospheres of air and then argon. INF, zone of infected cells; V, vascular bundle. Electrode calibration (0.1 M KCl): 46 pA before measurements, 31 pA afterwards.

Fig. 9. Oxygen electrode current as the electrode is advanced into the apical half of a nodule lobe. V, vascular bundle. Electrode calibration (0.1 M KCl): 36 pA before measurements, 46 pA afterwards.

Fig. 10. Oxygen electrode current within a nodule after infiltration of the intercellular air spaces with water. INF, zone of infected cells, V, vascular bundle. Electrode calibration: 23 pA before measurements, 34 pA afterwards.
between adjacent infected cells (Fig. 3), thus increasing the resistance to oxygen diffusion into these cells. Combined with a high rate of respiration by the infected cells, this would explain the observed regions of low oxygen concentration.

The intercellular air spaces of the nodule are connected to those of the nodule root, which provides a large surface area for the absorption of oxygen (Tjepkema, 1978; Torrey and Callaham, 1978). The resistance to oxygen diffusion through the nodule root epidermis and nodule periderm appears to be relatively small (Tjepkema, 1978), so that the \( P_o \), in the intercellular air spaces is probably not substantially below that at the nodule exterior. This conclusion is supported by the observation of oxygen electrode currents in the nodule center that are almost as large as those observed at the nodule surface or in air-equilibrated agar.

These results are very different from those observed previously for the root nodules of soybeans, a legume. Using the same type of electrode, very low oxygen concentrations were observed throughout the endophyte-containing region of soybean nodules, even though uninfected cells were present (Tjepkema and Yocum, 1974). It was concluded that there is a cell layer in the inner nodule cortex of soybean nodules that restricts oxygen diffusion into the region of the endophyte. No such layer is present in the nodules of Myrica gale, and thus high oxygen concentrations as well as low oxygen concentrations occur in the infected region. This suggests that protection of nitrogenase occurs within the infected cells of Myrica gale, rather than relying on a diffusion barrier that entirely surrounds the infected cells, as is the case in soybean nodules.

There are two ways in which nitrogenase in Myrica gale nodules may be protected from oxygen. Figures 5–8 demonstrate that regions of low oxygen concentration exist in nodules and one possibility is that nitrogenase activity is localized in these regions. The second possibility is that the vesicles of the endophyte are analogous to the heterocysts of blue-green algae in providing protection of nitrogenase against oxygen inactivation. The vesicles are believed to be the site of nitrogen fixation and our results demonstrate that this is also true for cultures of the endophyte, Frankia (Tjepkema, Ormerod and Torrey, 1980, 1981). Nitrogen fixation by cultures occurs only when vesicles are present and maximum rates of nitrogen fixation occur at atmospheric \( P_o \) (20 kPa). Thus the endophyte itself is able to protect nitrogenase from oxygen and does not require a nodule structure that provides an environment of low oxygen concentration.

We conclude that the mechanisms for the protection of nitrogenase from oxygen are fundamentally different in legume and actinorhizal nodules. In legume nodules, nitrogen fixation by Rhizobium can take place only at low oxygen concentrations, and the nodule structure provides such a low oxygen environment. In actinorhizal nodules, nitrogen fixation by Frankia can occur at atmospheric \( P_o \), and no barrier to oxygen diffusion into the nodule is required.

**LITERATURE CITED**


