Chapter 7

Measurement of Nitrogenase Activity in N_2-Fixing Nodules of Soybean

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Introduction

Although N_2 fixation is a fundamental process in the Nitrogen Cycle, and essential for the maintenance of global soil fertility, it is rarely studied in undergraduate laboratory courses. The reason for this is that the acetylene reduction assay, the standard method of measuring nitrogenase activity *in vivo* , has several problems that make it unsuitable for use in undergraduate teaching laboratories:

(a) It requires the use of 10% acetylene, which is explosive.
(b) A gas chromatograph is required to measure the ethylene formed by acetylene reduction, and these are expensive and require careful calibration.
(c) The method is laborious, and does not allow students to measure nitrogenase activity in real time.
(d) Exposure to acetylene inhibits nitrogenase activity in many legume symbioses so the acetylene reduction assay underestimates true activity.

A much easier, safer and more accurate method of assaying nitrogenase activity is by measurement of H_2 evolution from nodulated roots of legumes (Hunt and Layzell, 1993). Reduction of protons to H_2 is an obligate part of the nitrogenase reaction, and the rate of H_2 release into the soil is directly related to nitrogenase activity. By attaching a plant to a gas exchange system incorporating a H_2 sensor, nitrogenase activity *in vivo* can be measured non-invasively, and variations in activity can be observed in real time. Recently, a simple H_2 sensor designed for undergraduate teaching has been developed at Queen's University, and students in introductory level, and advanced level, courses in plant science are using this with great success.

The purpose of the following experiment is to show students how nitrogenase activity may be assayed by measurement of H_2 evolution from nodulated legume roots and, in doing so, introduce them to the mechanism of the nitrogenase reaction. The H_2 assay provides only a measurement of apparent nitrogenase activity (ANA) when the root system of a legume is flushed with normal air, since most of the electron flow through nitrogenase is used for reduction of N_2 rather than for reduction of protons to H_2. However, if the air flowing past the root system is replaced by Ar:O_2, all the electron flow though nitrogenase that formerly went to N_2 reduction is diverted to proton reduction, and H_2 evolution rate increases. The maximum rate of H_2 evolution attained under these conditions is a measure of total nitrogenase activity (TNA). By comparing rates of H_2 production in air and in Ar:O_2, students can measure ANA, TNA, rate of N_2 fixation, and the relative flux of electrons through nitrogenase to N_2 and protons under a given set of conditions. In conducting this experiment, students also learn how gas exchange techniques may be used to measure physiological and biochemical processes without detriment to the organism studied.

In the second phase of the experiment nitrogenase activity is measured after detachment of nodules from the root. This treatment causes a rapid decline in activity which, one might guess, occurs because the nodules are separated from their supply of phloem sap. Since the nitrogenase reaction is very energy-demanding, reduction of carbohydrate supply might be expected to limit

enzyme activity. However, on supplying the nodules with an increased partial pressure of O_2 (pO_2), nitrogenase activity increases, showing that O_2, rather than carbohydrate is the major factor limiting activity. This exercise teaches students to look beyond the obvious answers to physiological problems, and also introduces them to concepts of metabolic regulation.

This laboratory has been conducted in the Introductory Biology course (Bio 101) at Queen's University, and students usually have little difficulty in completing the experiment in a 3 hour period. Set-up time for the laboratory is minimal since the gas exchange apparatus may be constructed in a matter of minutes. The instructor may decide to do this, or allow the students to piece together the equipment themselves . Data manipulation within the DataLogger program is simple, and data may also be down-loaded to any spreadsheet program for students to analyze using their own computers. Usually, the students complete the experiment in the lab, analyze their data at home, and then present their data with an explanatory seminar the following week.

Students at introductory level are given a step-by-step guide to complete the experiment, as shown in the "Student Outline" below. At more advanced levels, the students in week 1 of a 2 week laboratory course in N_2 fixation, may be given detailed instructions in use of the gas exchange system and data acquisition software, and directed to perform the experiment described in the Student Outline. In the second week they are challenged to design their own experiments and use the system to investigate other environmental effects on nitrogenase activity and N_2 fixation rate. Treatments which result in predictable effects on nitrogenase activity include application of 10 mM nitrate to nodulated roots, exposure of roots to changes in pO_2 or to an atmosphere of $Ar:O_2$ for extended periods, stem-girdling shoots, and disturbing the root systems (Hunt and Layzell, 1993). Students use H_2 analysis to measure nitrogenase activity during these treatments, and are required to formulate hypotheses concerning the basis for the changes in activity they observe. They then conduct further experiments to test their hypotheses.

Required Materials

Students work in groups of two to four, each group requiring:

(a) Two legume plants, preferably soybean (*Glycine max* L. Merr), inoculated with a H_2-evolving strain of *Rhizobium* bacteria (e.g. *Bradyrhizobium japonicum* USDA 16). The plants should be grown in silica sand (grade 16) in a plastic pot that can be sealed for gas exchange measurements using a silicon rubber sealant (Qubitac). Details about plant growth are provided in "Notes to Instructors".
(b) Two nylon-polyethylene gas bags.
(c) An air pump.
(d) Two in-line flow restrictors: a high range restrictor delivering 500 $ml.min^{-1}$, and a low range restrictor delivering 200 $ml.min^{-1}$.
(e) A plastic column containing magnesium perchlorate as a drying agent.
(f) A hydrogen sensor.
(g) An oxygen sensor.
(h) A syringe barrel cuvette for housing detached nodules.
(i) A bubble, or variable area, flow meter. If a bubble flow meter is used, a stop watch will also be required.
(j) 1/8 inch i.d. Tygon tubing with luer-lok connectors to join together the components of the gas exchange system.
(k) A means to monitor the voltage output of the H_2 and O_2 sensors. Ideally this is done using an A/D converter (serial box interface) connected to a Macintosh or PC computer, although chart recorders may also be used. Voltage outputs from the sensors can be read to the screen in real time, and saved for later data manipulation, using data acquisition software (e.g. DataLogger).
(l) A supply of $Ar:O_2$ (80:20).
(m) A supply of $Ar:O_2$ (70:30).

Although some of the items listed above may be available in undergraduate labs, all components of the laboratory (including seeds and bacterial inoculum) are available as a package from an educational supply company (Qubit Systems Inc. See "Notes to Instructors" for details). The procedures for the laboratory exercise detailed below assume that the Qubit Systems' N_2 Fixation Package is being used, and that data is being collected by a Macintosh computer using the Datalogger software included in the package. Very slight alterations in data handling procedures are necessary if an IBM PC computer is used. Identical experimental procedures, except for data handling, may be used if instructors prefer to construct their own gas exchange apparatus.

Student Outline

Introduction

Biological N_2 fixation is of fundamental importance to life on earth. Annually, 200 million tons of nitrogen are added to the soil by nitrogen fixing organisms - four times the amount provided by inorganic fertilizers. Most of the nitrogen entering the environment by fixation is derived from bacteria that form symbiotic associations with plants, and it has been known for centuries that these plants (especially legumes such as clover and alfalfa) improve the yield of crops when they are grown with them in rotation. For this reason legumes are an essential component of sustainable agricultural systems. Conversely, use of inorganic fertilizers has deleterious effects on the environment. A significant proportion of the fertilizer nitrogen that is applied to fields is leached into waterways where it contributes to the formation of algal blooms and the eutrophication of rivers and lakes. Also, the large energy expenditure required for the industrial manufacture of inorganic fertilizers (from ammonia in the Haber-Bosch process) requires the burning of fossil fuels, which results in atmospheric pollution. Clearly then, there are great environmental and economic benefits to the cultivation of leguminous crops (including soybeans, peas, lentils and peanuts), and it would benefit world agriculture if the ability to fix N_2 could be introduced into non-legume species such as rice and wheat. However, before this is possible, researchers must clearly understand the factors that control N_2 fixation in legumes.

Biological N_2 fixation in leguminous plants requires the development of a symbiotic relationship between soil bacteria and the plant root. The most common endosymbionts found in the roots of crop legumes are bacteria of the genera *Rhizobium* and *Bradyrhizobium.* An extremely complex recognition process has evolved between legumes and bacteria, so that the interactions between them are species specific. In the following laboratory, N_2 fixation and nitrogenase activity in the Soybean ∞ *Bradyrhizobium japonicum* symbiosis will be studied.

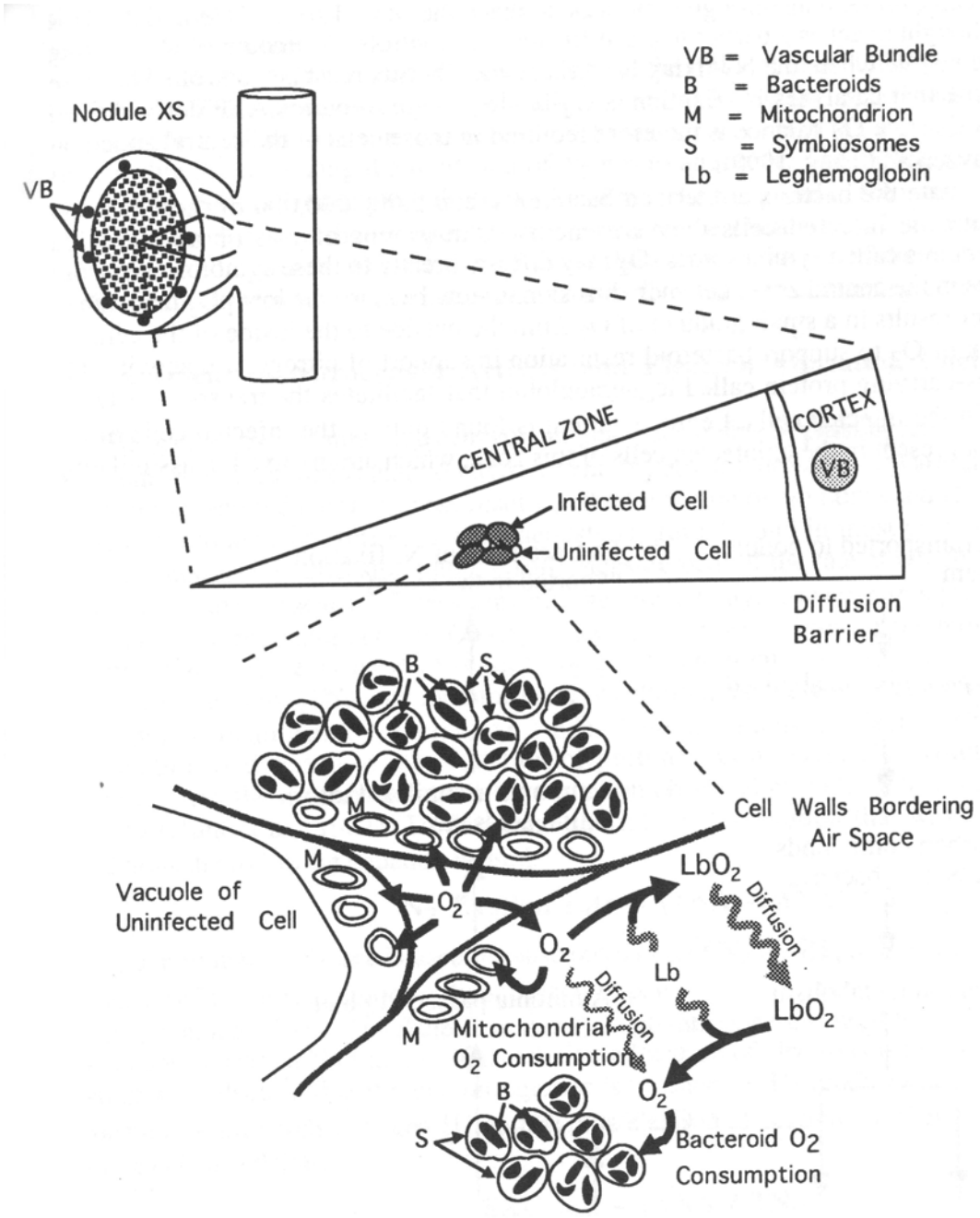

Figure 7.1. Structure of a soybean nodule.

As in all legume symbioses, *B. japonicum* inhabits swollen outgrowths of the soybean root called nodules. The root nodule can be subdivided into specific zones as shown in Figure 1. The outer periderm consists of loosely packed cells and lenticels through which gases diffuse into the outer cortical layer. These gases include the N_2 required for N_2 fixation, as well as O_2 required for plant and bacterial respiration. The cells of the outer cortex are also loosely packed, and contain large air spaces which offer little resistance to the diffusion of gases. However, in the inner cortex, the cells are smaller and much closer together, open intercellular spaces are infrequent, and the gases may have to diffuse through these cells, rather than through air spaces, to reach the central

zone of the nodule. This part of the nodule is thought to act as a barrier to gas diffusion that controls the amount of O_2 entering the central zone of the nodule where the N_2-fixing bacteria reside. O_2 flux must be controlled because the nitrogenase enzyme that catalyzes N_2 fixation is O_2-labile, and yet requires ATP derived from aerobic respiration. A delicate O_2 balance is therefore required in those cells of the central zone that house the bacteria (Layzell and Hunt, 1990).

In their symbiotic state the bacteria are termed bacteroids, and 2,000,000,000 of these infect a typical nodule. Within the infected cells they are enclosed, in groups of two or more, inside membrane-bound structures called symbiosomes. O_2 may diffuse directly to these symbiosomes from the intercellular spaces in the central zone, but such diffusion is slow because the low pO_2 maintained by the diffusion barrier results in a small gradient of O_2 from the outside to the inside of the cell. To provide sufficient flux of O_2 to support bacteroid respiration in support of nitrogenase activity, the nodule contains an O_2-carrying protein called leghemoglobin that facilitates the transport of O_2 to respiratory sites within the infected cells. Leghemoglobin is found only in the infected cells of the central zone, and is not present in the uninfected cells of this zone, which are involved in assimilating fixed nitrogen.

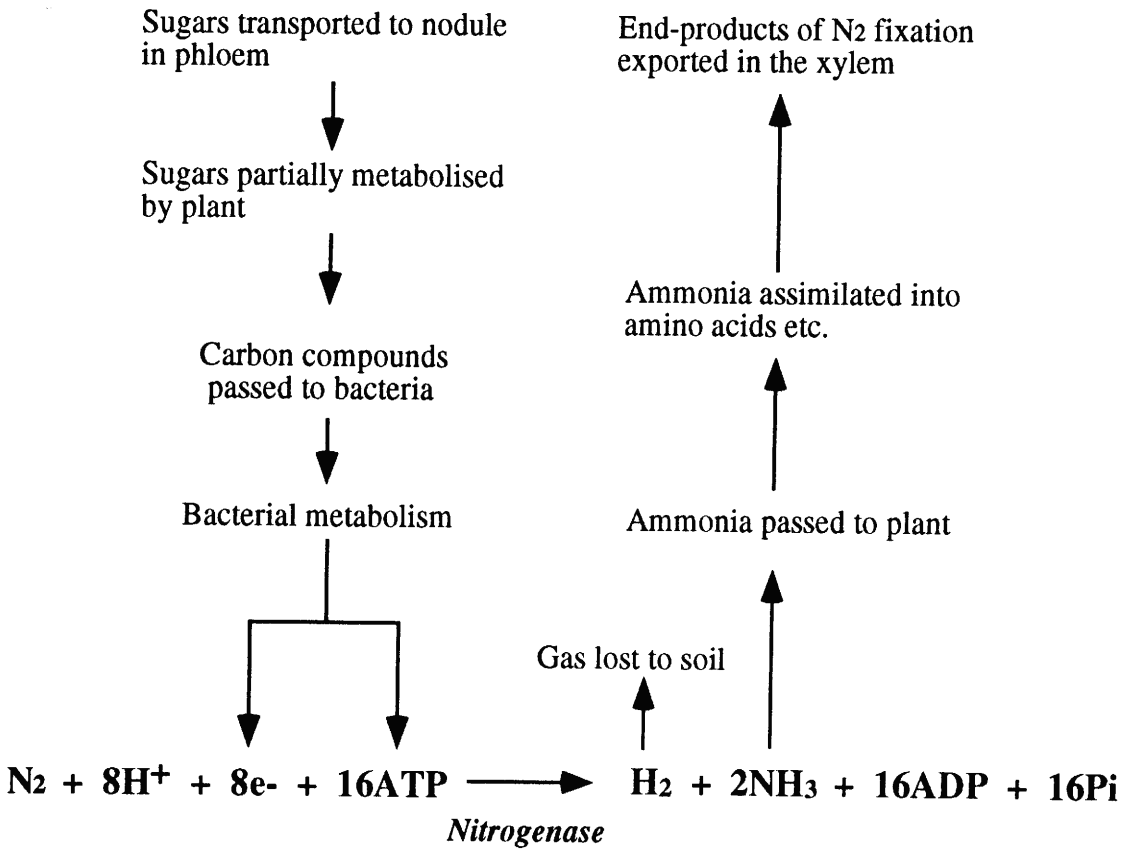

Figure 7.2. Exchange of materials between the plant and bacterial symbionts during N2 fixation.

The bacteroids are responsible for reducing the N_2 gas that diffuses into the central zone to ammonia, which can then be assimilated by the plant cells. Figure 2 shows the exchange of materials that must occur between the plant and the bacteroids to allow for the fixation of N_2 and the assimilation of ammonia.

A carbon source from the plant is supplied via the phloem to root nodules housing the N_2 fixing bacteroids. The carbon source is partially metabolized by the plant, and the resulting carbon

compounds are imported into the bacteria. The bacteria contain the enzyme nitrogenase which is responsible for catalyzing the reduction of N_2 gas to ammonia. The carbon compounds entering the bacteria are metabolized to produce the ATP and reductant required in the nitrogenase reaction, and the ammonia produced in the reaction is transported to the plant where it is assimilated into organic nitrogenous compounds. These are exported in the xylem to the rest of the plant, where they are used in the synthesis of amino acids, nucleic acids and other nitrogen-containing compounds.

Measurement of Nitrogenase Activity and Electron Allocation Coefficient

Nitrogenase is a promiscuous enzyme in that it is capable of passing electrons to (reducing) many different substrates. In the natural environment, N_2 gas and protons (H^+) are the major substrates for nitrogenase, and reduction of these results in the formation of ammonia and H_2, respectively. Both of these end-products are formed simultaneously (Figure 2), and in most crop legumes the H_2 is lost from the nodules by diffusion into the soil. Measurement of the rate at which H_2 is produced by the nodules provides a means for measuring nitrogenase activity. However, this provides only an estimate of apparent nitrogenase activity (ANA), since under most circumstances most of the electron flux through the enzyme is used to reduce N_2 gas to ammonia. To obtain a measurement of total nitrogenase activity (TNA) by monitoring H_2 evolution, it is necessary to replace the N_2 available to the nodule with an inert gas such as Ar. Under these conditions the electron flux previously used for N_2 fixation is diverted to the reduction of protons. As a result, H_2 evolution from the nodule increases, and the maximum rate of H_2 evolution observed provides a measure of total nitrogenase activity (Hunt et al., 1987). To determine the rate of N_2 fixation that was occurring under initial conditions the following equation is used:

$$\mathbf{N_2 \text{ fixation rate} = (TNA - ANA) / 3}$$

A denominator of three is used because reduction of N_2 to NH_3 requires 3 electron pairs, whereas reduction of H^+ to H_2 requires only one electron pair.

In air, at least 25% of total electron flux through nitrogenase is used for proton reduction while the remainder is used in N_2 fixation. This relative allocation of electrons is not constant, and often more than 25% of electron flux through nitrogenase is “wasted” in H_2 production. To determine the relative allocation of electrons between H^+ and N_2, the electron allocation coefficient of nitrogenase (EAC) may be calculated thus:

$$\mathbf{EAC = 1 - (ANA / TNA)}$$

EAC is an important parameter, because higher values of EAC mean that a greater proportion of nitrogenase activity is being used for the production of nitrogenous products for export to the plant. In the first part of this study, you will be measuring ANA and TNA and calculating EAC in soybean nodules.

Effects of Nodule Detachment on Nitrogenase Activity

In this part of the experiment you will be measuring nitrogenase activity after removing the nodules from the plant. In the past many researchers used detached nodules to investigate the effect of environmental perturbations on nitrogenase activity, since they are easier to manipulate than intact nodulated roots. Your experiment will test the validity of this approach, and will also involve an assessment of factors that may limit and regulate nitrogenase activity *in vivo*.

Procedures

Measurement of Nitrogenase Activity and Electron Allocation Coefficient

(1) Turn on the computer and click on the N_2Fix icon. This will activate the DataLogger software for data collection and analysis. A command box will appear asking "Do you want to load the calibration saved with this experiment? Click on the yes button.

(2)You will see 2 graphs. The upper graph shows the voltage output of the H_2 sensor and the lower graph shows the output from the O_2 sensor in $\%O_2$. Click on the maximum value displayed on the vertical (y) axis of the top graph and change this number to 3.0. Click on the maximum value displayed on the horizontal (x) axis on the graph and, if necessary, change the number to 100. You will then have 100 minutes to complete your experiment after it starts. The computer will still store data after the 100 minutes, but these data will not appear on the graph.

(3) Set up the components of the gas exchange system on the laboratory stand as shown in Figure 3, but do not attach the plant to the system at this time. Attach the high range flow restrictor to the outlet of the pump and then connect this to the inlet of the bubble flow meter. Turn on the pump and measure its output flow rate. To do this, squeeze the rubber bulb at the base of the flow meter and measuring the time it takes for a bubble to travel a set volume. Make several measurements over the same volume and take the mean of these. The flow rate should be approximately 500 $ml.min^{-1}$ (i.e. each bubble should take about 6 seconds to travel 50 ml). It is not important to obtain a flow rate of exactly 500 $ml.min^{-1}$, but it is important to measure the rate you obtain as accurately as possible.

(4) Detach the bubble flow meter from the pump and connect the flow restrictor directly to the magnesium perchlorate column. Attach the outlet of the magnesium perchlorate column to the O_2 sensor inlet, and the outlet of the O_2 sensor to the H_2 sensor inlet. Allow the outlet of the H_2 sensor to vent to atmosphere.

(5) Fill one gas bag with a mixture of $Ar:O_2$ (80:20), and the other with a mixture of $Ar:O_2$ (70:30) from the gas tanks in the lab. Attach the gas bag with the 80:20 mixture to the inlet of the pump and flush this gas through the system. Click on the START button on the computer screen to begin data collection. The START button will change to a STOP button. Do not click on the STOP button until you have completed your experiment.

(6) During data collection, the outputs of the H_2 and O_2 sensors are shown as traces on the displayed graphs, and also as numerical values on the bottom of the computer screen. At this point, the O_2 value should read 20%. If it does not, use a small screwdriver to adjust the gain control on the O_2 sensor amplifier box until a value of 20% O_2 is displayed. The O_2 sensor is now calibrated.

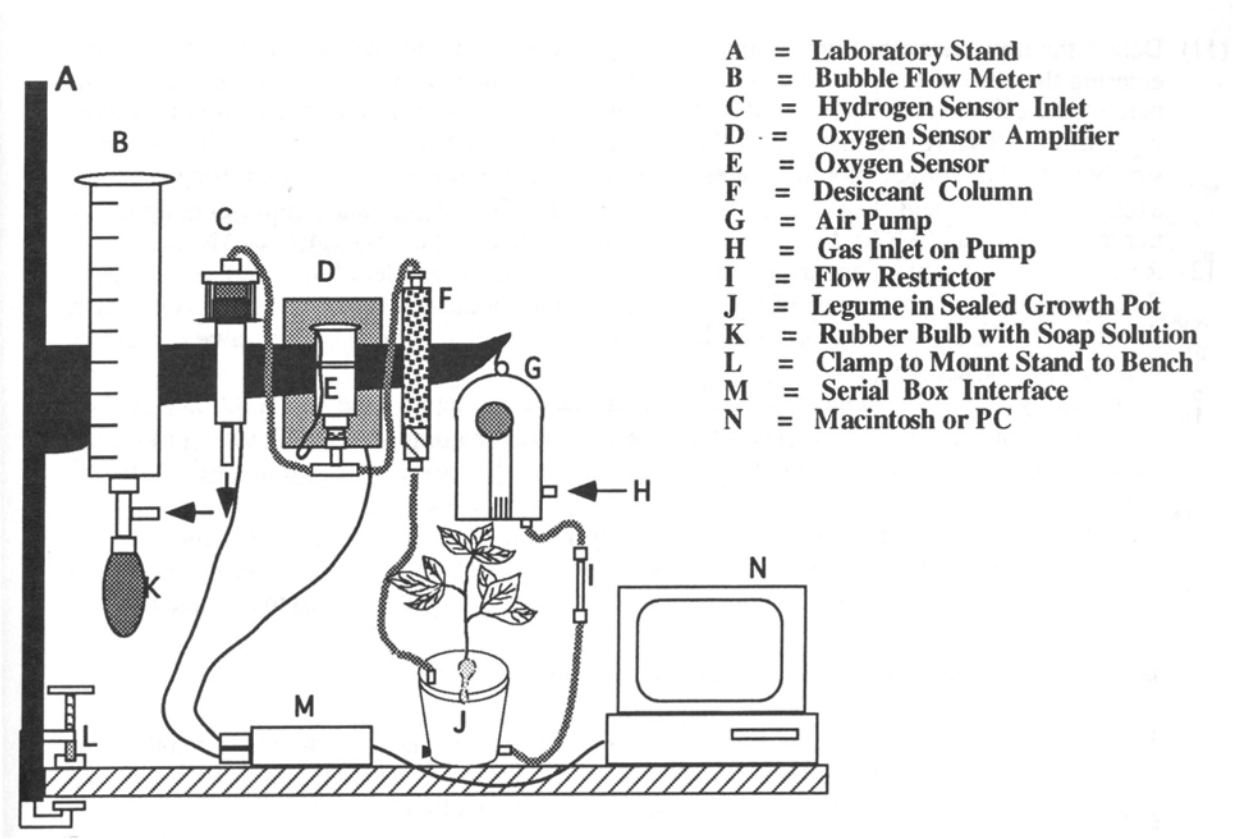

Figure 7.3. An Open-Flow Gas Exchange System for Measuring Nitrogenase Activity**.**

(7) The output from the H_2 sensor at this point represents its ZERO value in an $Ar:O_2$ (80:20) atmosphere. Note that this value is not 0.0, but a positive value that must be subtracted from your measured data. Allow the zero reading to proceed until the trace is stable (less than 5 minutes) and then make a note of the stable reading. While waiting for the reading to stabilize proceed with step #10 below.

(8) Having obtained a zero reading in $Ar:O_2$ (80:20), reseal the gas bag, and then attach the bag containing $Ar:O_2$ (70:30) to the system. The zero reading should change slightly. Make a note of this reading.

(9) Detach and reseal the gas bag, then pass room air through the system and allow the H_2 trace to reach a new zero value. Make a note of this third zero reading.

(10) Seal one of the soybean plants inside its growth pot using the lid provided. Be sure to treat the plant gently, since disturbance has been shown to cause inhibition of nitrogenase activity. The lid has a slit for accommodating the stem of the plant, and its rim clips down around the perimeter of the pot making a seal. The stem and slit are sealed by rolling a bead of flexible sealant (Qubitac) to a length greater than twice the length of the slit in the lid, and then wrapping this bead around the stem and molding it over the slit. Ensure that the sealant is wound tightly about the stem, so that air cannot leak from the pot, either through the slit, or through air spaces around the stem. Place a rubber bung in one of the holes at the base of the pot, and attach a gas line fitting to the other hole. Attach another gas line fitting to the hole in the lid.

(11) Detach the flow restrictor from the magnesium perchlorate column, and attach it to the gas line entering the base of the pot. Attach the gas line leaving the pot to the inlet of the

magnesium perchlorate column (see Figure 3). Room air is now being flushed through the pot to the O_2 and H_2 sensors. Observe the increase in the output of the H_2 sensor and wait for this to reach a steady state. The output should peak as H_2 accumulated in the pot is flushed through the gas exchange system, and then decline to a steady state. This steady state represents apparent nitrogenase activity (ANA). Make a note of the voltage output of the H_2 sensor at ANA.

(12) Refill the gas bag containing Ar:O_2 (80:20), and attach this to the inlet of the pump. As Ar:O_2 is flushed through the pot the rate of H_2 evolution from the nodulated root system will rise. When the output of the H_2 sensor reaches a plateau, record the value obtained. This value represents total nitrogenase activity (TNA).

(13) Immediately after measurement of TNA, turn off the pump, remove the Ar:O_2 (80:20) gas bag from the pump, seal it, and quickly attach the gas bag containing Ar:O_2 (70:30). Turn on the pump, and observe the changes in H_2 evolution from the nodulated root over the next 10 minutes

(14) Remove the gas bag from the inlet of the pump and seal it. Detach the plant from the gas exchange system. Click on the STOP button at the lower left of the screen. Select “File” and “Save data as” from the menu. Name your file “EAC Msmt”. It will be saved to the hard drive.

Effects of Nodule Detachment on Nitrogenase Activity

(1) Seal the other soybean plant in its pot and attach it to the gas exchange system between the flow restrictor and the magnesium perchlorate column. Click on the START button to begin data collection, and measure ANA and TNA as described in steps 11 and 12 above.

(2) Immediately after measuring TNA, detach the gas bag from the pump and seal it. Detach the pot from the gas exchange system. Replace the high range flow restrictor at the outlet of the pump with the low range flow restrictor. Attach the flow restrictor to the bubble flow meter and measure the flow rate of gas from the pump. This should have declined to approximately 200 $ml.min^{-1}$. Again, the absolute flow rate you obtain is not important as long as the rate is measured accurately.

(3) Detach the flow meter from the restrictor, and attach the restrictor to the inlet of the magnesium perchlorate column to pump room air through the O_2 and H_2 sensors.

(4) Remove the plant from its pot and shake the silica sand away from the root system. Swiftly detach **ALL** the nodules from the root and place them on moist filter paper in the nodule cuvette.

(5) Attach the nodule cuvette to the gas exchange system between the flow restrictor and the inlet of the magnesium perchlorate column. Observe the response of the H_2 sensor, and wait until a steady voltage output is reached. Make a note of this voltage. This represents the ANA value of the detached nodules.

(6) Attach the gas bag containing Ar:O_2 (80:20) to the pump, and observe the increase in H_2 sensor output. Record the value when this reaches a maximum, stable level. This represents the TNA value of the detached nodules.

(7) Attach the gas bag containing Ar:O_2 (70:30) to the pump and observe the change in H_2 sensor output. Record the value when this reaches stability.

(8) Click on the STOP button at the lower left of the screen. Select “File” and “Save data as” from the menu. Name your file “Detached Nodules”. It will be saved to the hard drive.

Data Analysis

Measurement of Nitrogenase Activity and EAC.

(1) Select "File" and "Open" from the menu. Open "EAC Msmt". A command box will appear on the screen asking you if you wish to load the calibration stored with your data file. Answer "yes". Your data will appear on the screen exactly as it appeared when you saved it at the end of the experiment.

(2) Select "Analyze" from the menu at the top of the screen by clicking and holding with the mouse. Select "Analyze Data A" and then release the mouse button. A vertical line will appear on the displayed graphs which can be moved along the data points by moving the mouse. Note that as you move the vertical line, the numerical display on the bottom of the screen will change to show you the exact voltage from the H_2 sensor at a particular pO_2, and the time value at the points on the H_2 and O_2 graphs where the line is situated.

(3) Using the mouse, select a series of H_2 measurements from each part of your experiment. Take a number of measurements in Air, Ar:O_2 (80:20) and Ar:O_2 (70:30). These measurements are shown in volts and should be converted to rates of H_2 production (equivalent to nitrogenase activity) using the following equation:

Nitrogenase activity (μmol $H_2.h^{-1}$) = Voltage × K × Flow Rate

K is a factor for converting voltage readings to measurements of H_2 concentration in units of μmol.L^{-1}. Flow rate has units of **liters per hour**. Since the H_2 sensor has a different response in N_2 and Ar, the value of K differs in each gas.

K = 0.75 μmol.L^{-1} per volt in air.

K = 1.0 μmol.L^{-1} per volt in Ar:O_2 (80:20).

K = 1.25 μmol.L^{-1} per volt in Ar:O_2 (70:30).

You must use the correct value of K in your calculations of nitrogenase activity in air and in the different Ar:O_2 mixtures. Also, remember that you must subtract either the AIR ZERO reading, or the appropriate Ar:O_2 ZERO reading from your H_2 voltage measurements before using the equation above.

(4) Calculate values of ANA and TNA from your experiment, and use these to calculate the EAC of your sample. What happened to the TNA measurement when the plant was exposed to 30% O_2 in Ar?

(5) Given that 2 electrons are required to reduce one proton to H_2, and 6 electrons are required to reduce one N_2 molecule to NH_3, use your data to calculate the rate of N_2 fixation in your sample in terms of μmol N_2 fixed.h^{-1}.

(6) How would changes in EAC affect the amount of N_2 fixed by the plant? What would be the likely effect on plant yield if EAC declined to a very low value?

Effects of Nodule Detachment on Nitrogenase Activity

(1) Select "File" and "Open" from the menu. Open "Detached Nodules". A command box will appear asking you if you wish to load the calibration stored with your data file. Answer "Yes". Your data will appear on the screen exactly as it appeared when you saved it at the end of the experiment.

(2) Select "Analyze" from the menu at the top of the screen by clicking and holding with the mouse. Select "Analyze Data A" and then release the mouse button. A vertical line will appear on your graphs which can be moved along the data points by moving the mouse. Note that as you move the vertical line, the digital display on the bottom of the screen will change to show you the exact voltage from the H_2 sensor at a particular O_2 concentration, and the time value at the points on the H_2 and O_2 graphs where the line is situated.

(3) Using the mouse, select and record a series of H_2 sensor voltages representing ANA in air, and TNA in Ar:O_2 (80:20) for the intact plant, then record a series of H_2 sensor voltages representing ANA in air, TNA in Ar:O_2 (80:20) and TNA in Ar:O2 (70:30) for the detached nodules. Calculate ANA and TNA for both the intact nodulated root and the detached nodules. Calculate TNA at 30% O_2 for the detached nodules, and EAC in both the intact root and the detached nodules.

Remember that you must subtract the zero reading from your H_2 voltage measurements before calculating nitrogenase activity.

(4) Compare the value of TNA measured in the intact nodulated root and the detached nodules. If you removed all the nodules from the root, should the TNA values be the same? Look at the scheme of metabolite transfer between plant and bacteria shown in Figure 2 and speculate on the factors that might change nitrogenase activity when the nodule is detached from the rest of the plant.

(5) In the detached nodules was it possible to calculate EAC in both air and 30% O_2? Did EAC change after nodule detachment?

(6) What happened when the pO_2 around the detached nodules was increased? Can you explain this response?

Notes for the Instructor

Measurement of ANA, TNA and EAC in Intact Nodulated Roots

The data collected by the students should be similar to that shown in Figure 4. After analysis of the data nitrogenase activities will be expressed on a "per plant" basis, and the values obtained will depend, to a great extent, on the number of nodules on the roots. As a result, variations in activity are likely to occur between the plants used by different groups of students. Instructors may wish to extend the experiment by asking the students to weigh the nodules after the experiment. Nitrogenase activities can then be expressed on a nodule weight specific basis (i.e. in units of μmol H_2. g^{-1} nodule fresh weight. h^{-1}). This should reduce the variations in activity seen within a plant population, and the extra procedure provides a useful exercise in data interpretation. For example, a larger plant may have a higher nitrogenase activity when this is expressed on a "per plant basis" because its root system may contain more nodules. However, if the larger plant has a significant number of senescent, or immature nodules, it may have a lower nitrogenase activity than others in the population when this activity is expressed per unit nodule fresh weight. Instructors may wish to discuss the most appropriate basis on which to express nitrogenase activity in different situations. An agronomist who wishes to know the effect of N_2 fixation on plant yield would be interested in measuring nitrogenase activity per plant, whereas a physiologist studying the regulation of N_2 fixation would need to express nitrogenase activity on a nodule-specific basis.

Students are asked to comment on the significance of the EAC values they measure. The theoretical maximum electron allocation coefficient (EAC) of nitrogenase is 0.75 because the nitrogenase mechanism requires that at least one H_2 molecule is produced for every N_2 molecule

fixed. Since 3 electron pairs are required for the reduction of one N_2 molecule, and one electron pair is required for the reduction of two protons, a minimum of 25% of the electron flux through nitrogenase must be used for proton reduction. The EAC of soybean nodules is usually between 0.6 and 0.7, which means that more than 25% of the electron flux through nitrogenase is used for proton reduction. This represents a "waste" of energy that could be used for N_2 fixation. The lower the EAC value the greater this waste, and plants with very low EAC values may produce less yield because of reduced nitrogen status.

Students are also asked to observe the effect on nitrogenase activity of increasing pO_2 from 20 to 30%. In the intact plant, this increase should cause a rapid inhibition of nitrogenase activity to almost zero. The detailed causes of this inhibition are complex (Hunt and Layzell, 1993) but for the purposes of this laboratory the effect may be used to illustrate the O_2-lability of the nitrogenase enzyme.

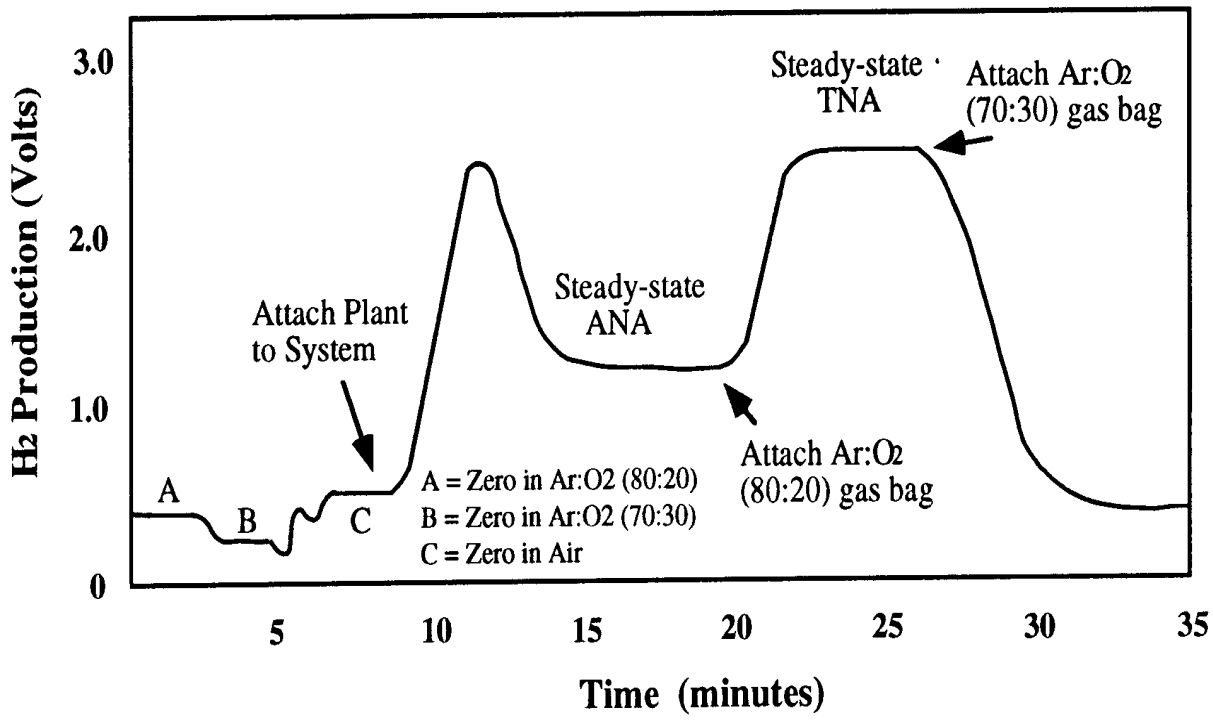

Figure 7.4. Typical Trace Obtained while Measuring ANA and TNA in Intact Nodulated Roots of Soybean.

Basis for the Calculation of Nitrogenase Activity

Instructors may wish to conduct a simple extra experiment to show how the calculation of nitrogenase activity by gas exchange analysis is dependent on the flow rate of gas through the cuvette. After H_2 evolution from a sample has reached a steady state at one flow rate, this is altered by changing the flow restrictor on the outlet of the pump. If flow increases, the H_2 content of the gas stream decreases, whereas decreased flow will cause the H_2 content to increase. The nitrogenase activity calculated at each flow rate will, of course, be identical. This approach shows the students the importance of measuring flow rate accurately, and will also demystify the equation for calculating nitrogenase activity.

Effects of Nodule Detachment on Nitrogenase Activity

The data collected by the students should be similar to that shown in Figure 5 below. When these data are used to calculate nitrogenase activities, it will be seen that nodule detachment causes a rapid inhibition of the enzyme. Typically, nitrogenase activity in detached nodules is less than 25% of the activity in the intact nodulated root of the same plant. To make this comparison it is not necessary to express activities on a nodule weight specific basis *but only if **all** the nodules are detached from the root and placed in the nodule cuvette*. Obviously, less activity will be measured in the cuvette if not all the nodules were harvested from the root. EAC measurements may be different in the detached nodules from those in the intact plant since this parameter is variable, and may alter with absolute nitrogenase activity. Note that EAC may **only** be measured at 20% O_2 in the detached nodules, since ANA was not measured at 30% O_2.

For many years it was assumed that the inhibition of nitrogenase activity after nodule detachment (or other treatments such as stem girdling, shoot excision or defoliation) was due to a reduction of carbohydrate supply from phloem sap (Hunt and Layzell, 1993). Nitrogenase has a high ATP demand, and phloem-derived carbohydrates are necessary to support bacterial oxidative phosphorylation to supply this demand. However, the fact that elevated pO_2 stimulates nitrogenase activity in detached nodules shows that carbohydrate cannot be the main limiting factor. In fact, when nodules are detached, they restrict the amount of O_2 diffusing to the bacteria so that bacterial respiration rate declines and less ATP becomes available for the nitrogenase reaction. The same response occurs when legumes are defoliated or detopped, and it may serve to slow down the rate of carbohydrate utilization and conserve resources until regrowth occurs.

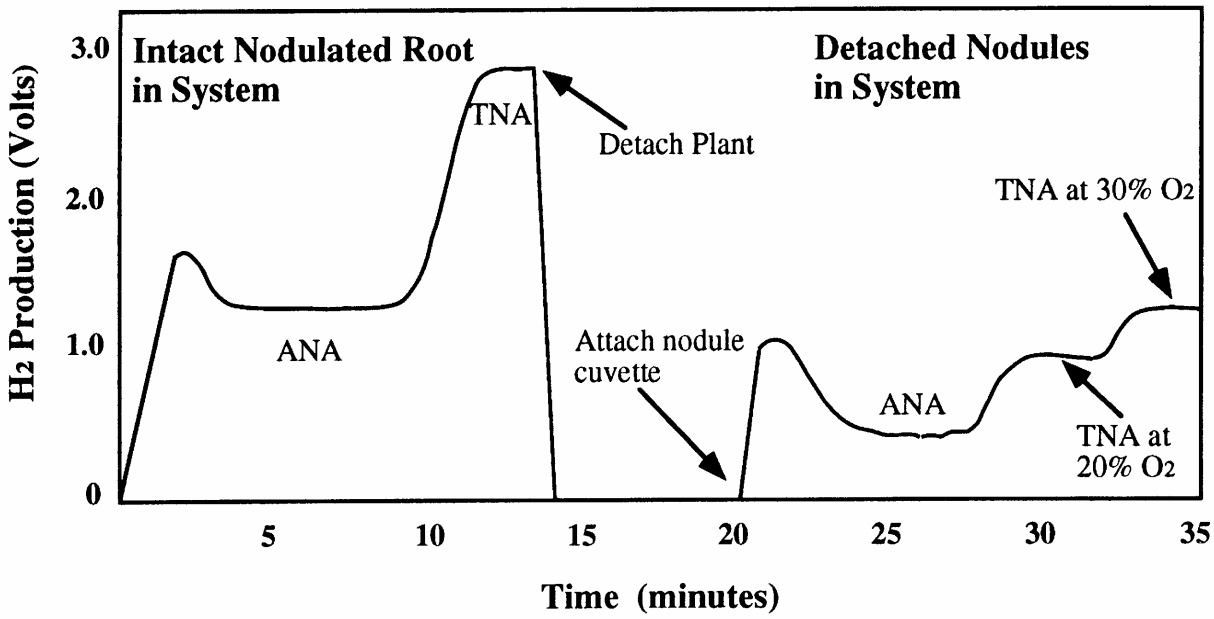

Figure 7.5. Typical Trace Obtained when Measuring ANA and TNA in Detached Nodules of Soybean.

The flux of O_2 into nodules is controlled by a barrier to gas diffusion thought to occur within the nodule cortex, and which may operate in a manner analogous to that of stomata in the leaf. Under normal conditions, in an intact nodulated root maintained at air levels of O_2, the diffusion barrier regulates the delicate balance of O_2 required for optimal respiration and optimal nitrogenase activity. A sudden increase in pO_2 (e.g. from 20 to 30%, as in this experiment) destroys this balance causing a sudden accumulation of O_2 in the nodule which inhibits nitrogenase. In contrast, when the nodule is placed under certain stress conditions, such as detachment from the root, the diffusion barrier closes. This restricts O_2 flux to the central zone, reducing respiration rate and limiting nitrogenase activity. By elevating external pO_2 from 20 to 30%, the greater O_2 diffusion gradient between the atmosphere inside and outside the nodule causes more O_2 to enter, stimulating respiration and nitrogenase activity. Therefore, increasing pO2 from 20 to 30% has opposite effects on attached and detached nodules. That the inhibition and stimulation of nitrogenase activity is related to respiratory activity can easily be demonstrated by attaching an infra-red gas analyzer to the gas exchange system. It will be seen that H_2 evolution from nitrogenase activity, and CO_2 evolution from respiration, will change in parallel during the experiment.

Instructors may wish to discuss the regulation of nitrogenase by O_2 and describe how this affects the design of experiments investigating aspects of nodule physiology. Although detached nodules may be easier to use in such experiments, the results obtained with these may be very different from those obtained with intact nodulated roots. This is because the detached nodules are O_2-limited and nitrogenase-inhibited, while the nodulated roots are usually O_2 sufficient and have higher nitrogenase activities. Researchers must therefore be very cautious in extrapolating physiological responses measured in disturbed tissues to those occurring in intact plants.

Literature Cited

Hunt, S., King, B.J., Canvin, D.T., and Layzell, D.B. 1987. Steady and nonsteady state gas exchange characteristics of soybean nodules in relation to the oxygen diffusion barrier. Plant Physiology, 84:164-172.

Hunt, S. and Layzell, D.B. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annual Review of Plant Physiology and Molecular Biology, 44:483-511.

Layzell, D.B., and Hunt, S. 1990. Oxygen and the regulation of nitrogen fixation in legume nodules. Physiologia Plantarum, 80:322-327.

APPENDIX
Supply, Preparation and Use of Materials

Equipment Supply

All the equipment necessary to conduct the experiment described above (as well as soybean seeds and bacterial inoculum) is available as a "N_2 Fixation Package" developed by Qubit Systems Inc. This package also contains manuals for students and instructors describing several other experiments that can be performed using H_2 analysis to measure nitrogenase activity. For further information about the Nitrogen Fixation Package, and other "Packages" designed for undergraduate biology laboratories in plant and animal biology contact:

Qubit Systems Inc.
134 Albert Street
Kingston, Ontario
Canada K7L 3V2
Phone: (613) 545-6961
Fax: (613) 545-6853
email: qubit@biology.queensu.ca
Website: www.queensu.ca/parteq/qubit.html

Cultivation of Plants

Nitrogenase activity can only be measured by H_2 evolution if the legume symbiosis lacks the enzyme uptake hydrogenase (HUP). It is essential, therefore, that the legume is inoculated with the correct strain of bacteria. Several strains of HUP^- bacteria are available as inoculants for soybean, and these are supplied in a peat-based medium for easy application to the soybean seeds. To cultivate H_2-evolving symbioses of soybean × *Bradyrhizobium japonicum*, the following procedure should be followed:

(a) Sterilize the plastic growth pots with a 5% bleach solution and then rinse.
(b) Fill pots to 3 cm with coarse gravel to cover the holes in the base of the pot. Fill the rest of the pot with coarse silica sand (grade 16).
(c) Surface sterilize soybean seeds with a 5% bleach solution for 2 min, and then rinse in water for 10 min.
(d) Water the silica sand and make 3 holes, each 3 cm deep in the sand. Place a seed in each hole and add a very small pinch of *Bradyrhizobium japonicum* peat inoculum. Cover the holes with the sand.
(e) For the first 10 days after planting, water the plants twice daily with a half strength Hoagland's solution containing 0.5 mM KNO_3 as the only nitrogen source. After 10 days, water with nitrogen-free half strength Hoagland's solution. Plants should be ready for experiments 28 days after sowing.

Since nitrogenase activity is affected by excessive disturbance of the root system, and by wetting the nodules, plants should not be watered within 3 hours of conducting an experiment. They should also be brought into the lab from the growth cabinet, or greenhouse, 2 hours before beginning experiments.

Use of the H_2 Sensor

The H_2 sensor should be warmed up for at least two hours before use, and it is best left plugged in if it is to be used in consecutive labs. To optimize response time, it should be exposed to a high H_2 concentration before use (1% H_2 is sufficient), although the sensor will work effectively without this conditioning. Conditioning may be done by filling a 5 ml syringe with 1% H_2 and flushing it through the inlet of the sensor. For teaching purposes it is not necessary to calibrate the sensor. If the sensor is required for research purposes in which absolute nitrogenase activities must be measured very accurately, it can be calibrated by passing gas mixtures through it containing known H_2 concentrations. All gases must be dried with magnesium perchlorate, and must contain the same O_2 concentration, since sensor output varies with the water and O_2 content of the gas.