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GAS EXCHANGE OF LEGUME NODULES AND THE REGULATION OF NITROGENASE ACTIVITY

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INTRODUCTION

Measuring the rates at which legume nodules exchange various gases with their environment allows a great deal of information to be obtained concerning the factors that regulate and limit N_2 fixation. However, studies over the past 10 years have shown that many of the gas exchange techniques used to measure activity of nitrogenase (the N_2-fixing enzyme) produce artifacts that

make the results difficult to interpret (99, 100). More recently, new gas-exchange methods have been developed for the measurement of nitrogenase activity and N_2 fixation rate in intact, undisturbed nodules under steady- and nonsteady-state conditions (68, 69, 92, 100). These methods have led to a better understanding of how nodules adapt to their environment (88, 89, 158) and have shown that actual and potential rates of N_2 fixation in legume nodules are much greater than was previously supposed (35, 69, 91, 99, 100). Also, empirical measurements of O_2 and H_2 concentrations within the infected zone of nodules (30, 34, 81, 90, 137, 157, 159), and estimation of these concentrations using mathematical models of nodule structure and gas diffusion (67, 101, 124, 131), have indicated that N_2 fixation rate may be inhibited by accumulation of H_2 within the nodule, and that nodule metabolism may be limited by sub-optimal O_2 concentrations in the infected cells. An appreciation of the factors that affect these nodule gas concentrations is therefore essential to the short-term goal of maximizing N_2 fixation rate in crop legumes, and the longer-term goal of introducing effective N_2-fixing systems into nonleguminous crops.

Our aim in this review is to assess critically interpretations of nodule gas-exchange measurements that have led to recent advances in our understanding of the physiology of N_2 fixation. We pay particular attention to studies that identify environmental conditions that inhibit N_2 fixation, and the physiological and biochemical processes that may be responsible for this inhibition. Since in vivo rates of N_2 fixation, and estimates of nitrogenase activity, may be affected by the assays used to measure these processes (35, 100), we begin with an evaluation of the advantages and disadvantages of different gas-exchange techniques.

METHODS FOR MEASURING NITROGENASE ACTIVITY IN LEGUME NODULES

Several techniques have been developed to provide integrated rates of N_2 fixation over periods of hours or days. These include measurements of whole-plant N increment (58), isotope techniques involving ^{15}N (8, 46, 85), and measurements of ureide accumulation in xylem sap or plant tissues (65, 141). Although these methods are valuable for seasonal measurements and field studies, they are not appropriate for physiological studies that require dynamic measurements of nitrogenase activity. Only two methods, the acetylene reduction assay and measurement of nodule H_2 evolution, allow short-term measurement of nitrogenase activity in undisturbed plant tissue.

The Acetylene Reduction Assay (ARA)

Nitrogenase is a promiscuous enzyme capable of catalyzing the reduction of a wide variety of substrates (13, 14), including the reduction of acetylene to ethylene (36). In the presence of 10% acetylene virtually all electron flux through nitrogenase is diverted to acetylene reduction, and measurement of ethylene production rate therefore provides an assay for total electron flow through nitrogenase, commonly referred to as total nitrogenase activity (TNA). A method frequently used for the ARA is the closed-system assay originally devised by Hardy and coworkers in 1968 (59), in which excised nodulated roots or detached nodules are sealed in an assay vessel with 5–20% acetylene and the amount of ethylene in the vessel is measured after a known incubation period. The assay is swift, simple, and sensitive, but it does have serious problems:

1. In several symbioses TNA is inhibited within 8 min of exposure to acetylene and reaches less than 50% of its initial activity within 30 min (100). Most closed-system ARA methods reported in the literature use incubation periods of 30 min or longer, resulting in TNA values that are grossly underestimated due to an acetylene-induced decline in nitrogenase activity.
2. The high solubility of acetylene promotes its retention in nodule tissues so that inhibition of TNA may remain, to some extent, long after the assay is complete. Repeated assays on the same plant material may therefore result in progressively lower estimates of TNA (116).
3. The closed-system ARA provides only a time-integrated measurement of TNA and does not allow rapid changes in activity to be observed.
4. Nodule detachment, or excision of nodulated roots, has been shown to cause rapid inhibition of TNA (99, 111, 135). Moreover, it has been demonstrated that nodule disturbance during removal of plants from their growth medium causes severe nitrogenase inhibition (99).
5. Gas mixtures containing more than 4% acetylene in air are explosive and must be used with extreme caution.

Several approaches have been used to overcome these problems. The development of an open-system gas-exchange apparatus for the ARA (100) allowed time-course measurements of TNA to be made on individual plants. This led to the discovery of the acetylene-induced decline in nitrogenase activity and the realization that most previous measurements of TNA were underestimated. However, the open-system ARA does not allow continuous, real-time measurement of TNA because discrete samples must be taken from the gas stream for analysis of ethylene by gas chromatography. Also, stable nitrogenase activity is maintained for a very short period before the onset of the acetylene-in-

duced decline; thus factors affecting TNA cannot be studied using non-inhibited nodules.

One approach that has been used to avoid errors resulting from the acetylene-induced decline has been to study symbiotic associations that are not inhibited by acetylene (41, 95, 100). However, in identifying these symbioses, the conditions under which the plants were cultured or assayed were likely to cause nodule disturbance that would inhibit nitrogenase activity and mask the acetylene-induced decline (99). While legume symbioses may exist that are not affected by long-term exposure to acetylene, it is essential to ensure that the apparent lack of an acetylene-induced decline is real and not due to inhibition of nitrogenase by other factors.

An alternative approach to circumventing complications with the ARA is to use low acetylene concentrations that do not inhibit nitrogenase (33). This produces measured nitrogenase activities that are subsaturated with respect to acetylene, but saturated activities can be estimated from an empirically determined relationship between acetylene concentration and TNA (31). However, this relationship is sensitive to changes in the nodules' permeability to gas diffusion which, in turn, is sensitive to changes in environmental conditions (70, 88, 158). The method is therefore of little use in studies requiring measurements of nitrogenase activity before and after environmental treatments.

Despite the problems associated with the ARA, the method has contributed greatly to our understanding of the physiology of N_2 fixation. Although the measurement of nodule H_2 evolution has many advantages as a method for determining nitrogenase activity (see below), the ARA can still provide useful physiological data if it is used with caution. For quantitative measurements of TNA an open-flow gas-analysis system should be used with intact, undisturbed (not uprooted) plants, and with a saturating (e.g. 10%) acetylene concentration in the gas stream. Ideally the pO_2 should be atmospheric and not reduced to approximately 18.8% by simple addition of 10% acetylene to air, since decreases in pO_2 inhibit nitrogenase activity and nodule metabolism (49, 68). The gas flow rate (ml/min) in the system should be at least twice the gas volume (ml) of the root chamber to ensure a low system time constant. This allows accurate measurement of the peak acetylene reduction rate that normally occurs within 2–10 min after exposure of the nodulated root to acetylene. Repeated assays on the same plant should either be avoided or separated by a time interval known to allow recovery of nitrogenase from the acetylene-induced decline.

Qualitative, or semi-quantitative, measurements of TNA can sometimes be obtained by use of the closed-system ARA, by continuous measurements of acetylene reduction in open-flow systems, or by using disturbed plants or detached plant parts. Although the results obtained using these methods may be difficult to interpret, in certain situations techniques that provide accurate

measurements of uninhibited TNA may not be available. In these cases, it must be recognized that the degree of inhibition or stimulation of nitrogenase activity in control and treated plants may be affected by the assay conditions. In general, valid comparisons may only be made if the assay conditions inhibit the control and treated plants to the same extent and if this inhibition is significantly less than that caused by the experimental treatments. It has been argued that the ARA can provide accurate measurements of TNA in the crop environment because nodules grown in the field are subjected to environmental conditions that inhibit nitrogenase activity to a greater degree than exposure to acetylene (129). As a consequence, these nodules would not show an acetylene-induced decline. While this may be the case during adverse conditions, it must be recognized that the soil environment varies on a daily and seasonal basis. Therefore, the investigator must demonstrate convincingly the absence of an acetylene-induced decline each time the ARA is used, and the results from individual assays should not be extrapolated to estimate N_2 fixation rate over an extended growth period.

Measurement of Nodule H_2 Evolution

H_2 production is an obligate part of the N_2 fixation reaction (13, 115, 130, 136), and several studies have examined the relationship among rates of N_2 fixation, H_2 evolution, and acetylene reduction (56, 100, 119, 144). H_2 evolution rate in air provides only a measurement of apparent nitrogenase activity (ANA) because a proportion of the total electron flux through nitrogenase is used for N_2 reduction. However, total nitrogenase activity (TNA) may be measured as H_2 evolution in an atmosphere lacking N_2 (e.g. Ar:O_2) (68, 69), and the rate of N_2 fixation may be calculated as (TNA – ANA)/e, where e = 3 and represents the number of electron pairs required to reduce N_2 to NH_3. The relative allocation of electrons by nitrogenase to H^+ and N_2 reduction can be calculated as 1 – (ANA/TNA), a value termed the electron allocation coefficient of nitrogenase (EAC) (44, 68).

Measurement of nitrogenase activity by H_2 analysis is best employed using an open-circuit gas-exchange system (68, 69) in conjunction with a flow-through H_2 analyzer (92). H_2 evolution rate from nodules (either attached or detached from roots) can then be measured during steady-state conditions or during environmental perturbations. ANA may be monitored continuously in N_2:O_2, and spot measurements of TNA can be obtained from the peak rate of H_2 evolution attained when N_2 is replaced by Ar in the flowing gas stream (68, 69). It is important to switch back to a N_2:O_2 atmosphere after peak TNA is obtained, since long-term exposure to Ar:O_2 causes nitrogenase inhibition (68, 82, 100). This inhibition has been termed the Ar-induced decline and seems to be similar to the acetylene-induced decline.

Monitoring H_2 evolution from nodules has several distinct advantages over the ARA and other methods of measuring nitrogenase activity:

1. The flow-through H_2 analyzer (92) is extremely sensitive, and is the only instrument that allows continuous, real-time measurements of nitrogenase activity (ANA). Also, with appropriate control of the gas composition surrounding the nodule, the H_2 analyzer provides measurements of ANA, TNA, EAC, and N_2 fixation rate. By comparison, the open system ARA provides only measurements of TNA.

2. Measurements of ANA, and short-term measurements of TNA, do not inhibit nitrogenase activity, so that measurements can be performed on the same plant material, either continuously or intermittently, over virtually any experimental period.

3. The method is less labor-intensive than the ARA and, unlike the ARA, does not pose any hazards to the experimenter.

4. The H_2 analyzer is inexpensive compared to the gas chromatograph required for the ARA.

There are also disadvantages associated with the H_2 evolution assay as a measure of nitrogenase activity, and with the use of the flow-through H_2 gas analyzer. These include:

1. Certain legume symbioses possess an uptake hydrogenase enzyme (HUP) that recycles some or all of the H_2 produced by nitrogenase (3, 47, 51). H_2 analysis cannot be used to estimate nitrogenase activity in these HUP^+ symbioses. However, many agronomically important legumes, including virtually all clover, alfalfa, and trefoil symbioses, as well as most soybean and pea symbioses, are HUP^-.

2. The H_2 analyzer is very sensitive to water vapor, and its output for a given pH_2 changes with pO_2, with the nature of the balance gas (N_2 or Ar), and, to a certain extent, with time. It is therefore difficult and time-consuming to calibrate.

3. Extended exposure to an $Ar:O_2$ atmosphere causes a decline in TNA. However, brief exposure of nodules to $Ar:O_2$ is not inhibitory, and repeated assays of TNA can be made on the same plant material. This is a significant advantage over the ARA.

4. To date, a H_2 analyzer has not been developed for field studies. A potential problem with field use of the analyzer is the effect of H_2 oxidation by bacteria and abiotic factors that may be present in the soil (121). Studies are required to determine the extent to which this oxidation may cause underestimation of H_2 evolution rate from the nodules.

Despite these drawbacks, the advantages of the H_2 evolution assay in providing a broad range of physiological measurements make it the most

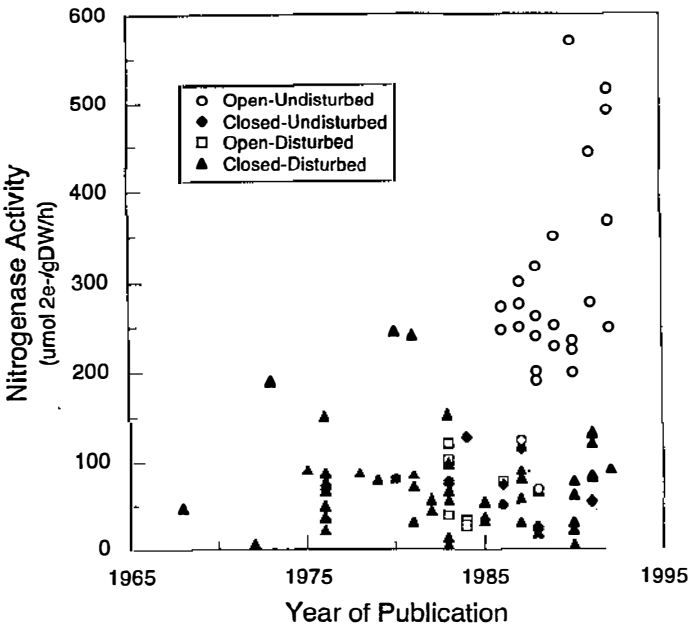

Figure 1 Values of nitrogenase activity measured in different legume symbioses between 1968 and 1992. The data were obtained from 83 published reports and have been normalized to units of μmol of electron pairs per g dry weight of nodules per hour. A fresh weight to dry weight ratio of 5:1 was used to convert values expressed originally on a fresh weight basis. The assay methods for nitrogenase activity were as follows: *unfilled circles*: open system ARA or H_2 evolution assay using undisturbed, intact, attached nodules; *filled diamonds*: closed system ARA using physically undisturbed, intact and attached nodules; *unfilled squares*: open system ARA or H_2 evolution assay using disturbed or detached nodules; *filled triangles*: closed system ARA using disturbed or detached nodules.

useful and adaptable method for instantaneous or continuous measurements of nitrogenase activity.

Measured Values of Nitrogenase Activity: Past and Present

At this point it is instructive to illustrate the progress that has been made in techniques for measuring nitrogenase activity by comparing the absolute rates reported for various legume symbioses since Hardy's original study in 1968 (59). The data presented in Figure 1 summarize published TNA values from various legume symbioses reported in 82 papers published since 1968. All TNA values were measured at pO_2 between 18 and 21% in control plants and have been normalized to units of total electron flow through the enzyme (μmol of electron pairs per g dry weight of nodules per hour).

Figure 1 shows that measured values of TNA have increased significantly as less invasive assay techniques have been developed and used. It also shows that the highest values are obtained using intact, undisturbed plants in an open gas-exchange system in which TNA is measured as peak acetylene reduction rate or peak rate of H_2 evolution in $Ar:O_2$. Although interpretation of the data may be complicated by different symbioses having different nodule-specific nitrogenase activities, it is interesting that the four highest activities presented are derived from experiments with four different legumes: clover (52), soybean (23), alfalfa (29), and pea (35).

The magnitude of nitrogenase inhibition apparent in the values obtained using disturbed plant material and/or closed system assays seriously call into question the validity of the studies from which these values were obtained. It may be argued that the methods are valid in providing relative, or comparative, measurements of nitrogenase activities between different experimental treatments or different symbioses. However, this would require that the assay methods inhibit a similar proportion of nitrogenase activity in control and treated plants, or in each symbiosis studied. It has been shown that this assumption is not valid (99, 100). Unfortunately, inhibitory techniques are still used to evaluate symbiotic associations or the effects of environmental or physiological treatments on nitrogenase activity.

IN VIVO NITROGENASE ACTIVITY: POTENTIAL AND LIMITATIONS

The data presented in Figure 1 raise an intriguing question: Do the highest measured values of nitrogenase activity represent the maximum rates attainable in legume nodules, or does potential exist for much greater activity? This question has important agronomic implications, since realization of full potential activity could circumvent the need to increase legume yield by the addition of fertilizer nitrogen. To determine the approaches by which nodules may be manipulated to increase N_2 fixation rate, we must first consider the factors that limit nodule activity under normal conditions and during environmental stresses.

H_2 Inhibition of N_2 Fixation

At least one H_2 is produced for every N_2 fixed by nitrogenase (130) so that at least 25% of the electron flux through the enzyme is diverted to proton reduction rather than N_2 fixation. The maximum EAC of nitrogenase is therefore 0.75, although EAC values between 0.40 and 0.70 are commonly reported (37, 44, 93). This suggests that H_2 production often accounts for a greater propor-

tion of TNA than is optimal for N_2 fixation. Higher EAC values have been reported in nodules exposed to reductions in phloem sap supply (37), temperature (141), and light intensity (43), and in nodules inhibited by nitrate fertilization (43). However, the low EAC values in control plants and the apparent increases in EAC following treatments that inhibit nitrogenase activity may be artifactual because in most studies the TNA values used in the calculation of EAC (i.e. EAC = 1 – (ANA/TNA)) were obtained using disturbed tissues or an inhibitory ARA. Inhibition of TNA by the ARA would produce underestimation of EAC if the corresponding measurements of H_2 evolution were relatively non-inhibitory.

H_2 evolution in air and in Ar:O_2, respectively (4, 15, 29, 68, 82), have resulted in EAC values between 0.59 and 0.70. These are less than the theoretical maximum EAC of 0.75 but are generally higher than those estimated using the ARA.

Two major factors may be responsible for sub-optimal values of EAC. First, $K_m(N_2)$ values for purified nitrogenase (112) indicate that atmospheric levels of N_2 gas within the nodule central zone would be insufficient to saturate the N_2-binding sites of nitrogenase, resulting in a maximum EAC value of 0.714 (101). Since mathematical models of gas diffusion into nodules (67, 124) predict that the pN_2 in the central zone is likely to be lower than atmospheric (71.4 kPa rather than 79 kPa), this maximum EAC value may be further reduced. Second, H_2 acts as an autocatalytic competitive inhibitor of the N_2 fixation reaction (37, 55, 91), and the Ki(H_2) of H_2 inhibition (~3 kPa) (112) is close to the pH_2 value that has been measured by microelectrodes in the central zone of HUP^- soybean nodules (157). A mathematical model of nodule gas diffusion and enzyme kinetics incorporating these data (91, 101) predicted that the maximum EAC in soybean nodules should be between 0.60 and 0.70, and empirical measurements of EAC and TNA at different external pN_2, pH_2, and pO_2 supported this prediction. Together, these data indicate that H_2 inhibition of N_2 fixation is a major factor regulating EAC and may therefore have a significant effect on legume N_2 fixation rate and yield.

Many legume symbioses avoid accumulation of nodule H_2 by oxidizing the gas via HUP (3, 47). This enzyme reduces the pH_2 within the central zone of these symbioses to negligible levels (157) and may therefore reduce autocatalytic H_2 inhibition of N_2 fixation. However, there is no clear evidence that HUP^+ symbioses allocate electrons more efficiently to the reduction of N_2 rather than protons, since there are no reports in the literature of EAC measurements in HUP^+ nodules. A potential advantage of HUP may be in the provision of reductant as a substrate for nitrogenase or for ATP production (45, 104). Also, the consumption of O_2 during H_2 oxidation could play a role in protecting nitrogenase from O_2 inhibition (45, 51). However, in vivo studies with HUP^+ and HUP^- symbioses have failed to show that expression of HUP

activity confers a clear advantage on a N_2-fixing symbiosis. This may reflect a balancing disadvantage of HUP in competing with bacteroid respiration for a limited pool of O_2 in the infected cells (91).

O_2 Limitation of Nitrogenase Activity

It is well known that nitrogenase is O_2-labile (115) yet requires large amounts of ATP synthesized by aerobic respiration. The mechanisms by which legume nodules protect the enzyme from O_2-inactivation, yet meet their ATP requirements, have been the subject of much recent research. As is discussed below, this research has shown that infected cell O_2 concentration (O_i) is regulated such that it limits nodule carbon metabolism and N_2 fixation. In the following sections we summarize our current understanding of O_2 regulation in legume nodules and identify areas for future research.

THE NODULE DIFFUSION BARRIER Most investigators agree that the nodule inner cortex, which surrounds the central zone of infected cells, contains a barrier to gas diffusion that limits the flux of O_2 to the bacteroids. Evidence for this was obtained by microelectrode studies which showed that pO_2 is much lower (137, 159) and pH_2 is much higher (157) in the central zone than in the cortical layers. Therefore, in addition to protecting nitrogenase from O_2 inactivation, the nodule diffusion barrier may also affect nitrogenase function by restricting H_2 efflux from the central zone and exacerbating H_2 inhibition of N_2 fixation (91, 157).

Pankhurst & Sprent (105) and later Ralston & Imsande (111) found that drought stress and nodule excision, respectively, caused an inhibition of nitrogenase activity that could be at least partially recovered by increasing external pO_2 (O_e). They suggested that these treatments may have caused a decrease in the nodule's permeability to O_2 diffusion. However, Minchin, Witty, and Sheehy (98, 100, 126) were the first to propose that legume nodules exercise physiological control over their permeability to O_2 diffusion and thereby regulate O_i and protect nitrogenase from O_2 inactivation. Many studies over the past 10 years have confirmed their original hypothesis, and several reviews have been written detailing the evidence for, and implications of, a variable diffusion barrier in legume nodules (70, 88, 158). Some of this evidence is summarized below:

1. Legume nodules can adapt to step increases in external pO_2 (O_e) and maintain high nitrogenase activity without significant increases in respiratory CO_2 evolution or O_2 consumption (68, 126, 154). Since O_i must remain low at elevated O_e to maintain active nitrogenase, and assuming that CO_2 evolution

reflects O_2 uptake (F), Fick's first law of diffusion ($P = F/(O_e - O_i)$) predicts that nodule permeability to O_2 diffusion (P) must decrease at the higher O_e.

2. When O_i was monitored using a nodule oximeter, an increase in O_e from 20 to 25% O_2 resulted in a rapid rise in O_i, followed by a decline to its initial low level within 10 min (81). This study also confirmed the dependence of nitrogenase activity on a low O_i.

3. Changes in nodule permeability to O_2 diffusion have been measured indirectly using a nodule oximeter to monitor the time-course of the increase in O_i following a change in O_e from 0 to 100% O_2 (30). Permeability was calculated by fitting the empirical data to a mathematical model.

4. Increases in O_e (155) or inhibitory treatments such as drought stress (153) or extended exposure to $Ar:O_2$ (82) resulted in decreases in nodule permeability to acetylene and ethylene diffusion when measured by fitting mathematical models to either the relationship between acetylene concentration and nitrogenase activity (33) or the time-course of ethylene production following nodule exposure to acetylene (82, 155). These data suggest the presence of a physical barrier that can be altered in its permeability to all gases.

5. Measurements with H_2 microelectrodes have shown that the concentration of H_2 in the central zone of soybean nodules increases with increases in O_c (157), indicating decreased nodule permeability to gas diffusion after elevation of pO_2.

Gas exchange measurements from legume nodules have been incorporated into mathematical models of O_2 diffusion to make predictions of diffusion barrier structure (28, 67, 124, 131). These models are in general agreement that the permeability of nodules required to maintain O_i at a level suitable for nitrogenase activity may be controlled by an aqueous barrier of cells and intercellular spaces surrounding the nodule central zone, and that continuous air pathways between the rhizosphere and nodule central zone must be absent or limited to an extremely small number. Most studies of nodule structure indicate that intercellular spaces in the inner cortex are small and few in number (4, 10, 108, 152), but it is a matter of controversy whether or not these spaces are continuous with those in the outer cortex and central zone (10, 34, 38, 108, 152, 157). Water release into, and uptake from, the intercellular spaces would vary the path length of O_2 diffusion through the aqueous phase and thereby alter nodule permeability. Evidence in support of this hypothesis has been obtained by direct microscopic observation of a reduction in air spaces in cut nodules of pea and french bean during increases in external pO_2 (159). Movement of water to displace air from the intercellular spaces could occur by an osmotic mechanism, and various solutes, including inorganic ions (70, 126), nitrogenous metabolites (128), and sucrose (66) have been sug-

gested as regulatory osmoticants in diffusion barrier control. However, these suggestions have been merely speculative (70, 126, 128) or based on loose correlations among solute concentration, nodule osmolarity, and nodule permeability (66).

It should be noted that although the inner cortex may have structural modifications to provide a barrier to gas diffusion, it is only one of a series of resistances that O_2 encounters in its diffusion to the bacteroids. For example, facilitated diffusion of O_2 by leghemoglobin (2) may be another control point for the regulation of O_2 supply to the bacteroids in the infected cells.

THE O_2 OPTIMUM FOR IN VIVO NITROGENASE ACTIVITY Considerable attention has been devoted to determining the optimum O_e for in vivo nitrogenase activity, and values between 20 and 100% O_2 have been reported for nodules of soybean, sanfoin, lupin, and clover (17, 18, 24, 139). However, use of disturbed nodules and extended exposure to acetylene make the results of these studies difficult to interpret, particularly since it is known that the decline in nitrogenase activity due to these treatments is caused, at least in part, by a decrease in nodule permeability to O_2 (98, 99, 135). In addition, the discovery that nodules can adapt to step changes in O_e (68, 154) makes it difficult to determine whether the optimal O_e for nitrogenase activity reflects the O_2 requirements of the nodule or the speed with which the nodule can adapt to changing O_2 conditions.

To examine this question, Hunt and coworkers (69) developed a system for increasing O_e linearly with time around intact, undisturbed nodules and determined the rates of pO_2 increase that exceeded the rate at which nodules could increase their permeability to O_2 diffusion. Using this approach, studies with soybean (69), pea (35), and lupin (35) showed that high O_e stimulated nitrogenase activity by up to 25% of that measured at atmospheric pO_2. These results showed that diffusion barrier permeability is regulated in soybean, pea, and lupin nodules to maintain O_i at a value that is sub-optimal for nitrogenase activity. The peak TNA attained during the pO_2 increase has been termed the potential nitrogenase activity (PNA) and represents activity that can be attained under O_2-saturated conditions. The ratio of TNA at 20% O_2 to PNA has been termed the O_2-limitation coefficient of nitrogenase (OLC_N) and is a useful index of the degree of O_2 limitation of nitrogenase within legume nodules (35).

If the diffusion barrier maintains O_i at a value sub-optimal for nitrogenase activity, it may be possible to increase legume yield by genetic manipulation to produce a more permeable barrier. However, it has been proposed on a theoretical basis that nodules must be O_2 limited for them to exercise physiological control over O_i (88). If O_i did not limit infected cell respiration rate it would be virtually impossible for the variable diffusion barrier to control O_i with the precision required to prevent nitrogenase inactivation.

MEASUREMENT OF INFECTED CELL O_2 CONCENTRATION Although useful information can be obtained by measuring nitrogenase activity during increases in external pO_2, the pO_2 at which maximum activity is attained has little physiological relevance. More important is the relationship among nitrogenase activity, nitrogenase-linked respiration, and O_i. Although studies with microelectrodes (137, 159) have shown that O_i is maintained at a very low concentration, the electrodes are too insensitive to provide absolute measurements of O_i under normal atmospheric conditions. Therefore, indirect assays for O_i have been developed based on the spectroscopic properties of leghemoglobin (Lb). This O_2-binding heme-protein is found in the infected cells of all active legume nodules, where it facilitates the diffusion of O_2 to the sites of bacteroid respiration (2, 123). The spectral properties of Lb change depending on its oxygenation state (1, 7), allowing the fractional oxygenation of Lb (FOL) to be estimated spectrophotometrically. O_i may then be calculated from FOL if the rate constants are known for O_2 association and disassociation with Lb (11).

Absorbance spectroscopy of intact legume nodules is difficult because the cortical tissues surrounding the infected cells scatter most of the incident light away from the nodule central zone. To alleviate this problem, experiments have been performed on sliced nodules (1), flat, transluscent nodules (83), and nodules flattened artificially during growth (81). Reflectance spectroscopy has been used to measure the mean FOL in several detached nodules in air and aqueous media (103). Under atmospheric conditions, these methods have produced values of O_i between 3.2 and 21.5 nM.

More recently, a nodule oximeter has been developed (30, 90) that allows measurement of O_i in intact, attached spherical nodules. Unlike previous methods, the oximeter makes use of pulse-modulated spectroscopy so that all measurements can be made under ambient light conditions in the laboratory or the field. The oximeter also allows the estimation of several other physiological parameters related to the regulation of O_i. By monitoring the rate of Lb deoxygenation during exposure of a nodule to zero pO_2 (pure N_2 gas) it is possible to calculate the rate, apparent $K_m(O_i)$, and V_{max} of infected cell O_2 consumption within the nodule (30). The rate of increase of Lb oxygenation following a change in O_e from 0 to 100% O_2 allows estimation of the nodule's permeability to O_2 diffusion (30). Finally, if the optical path length through the nodule can be estimated, the extinction coefficient of Lb can be used in conjunction with oximetry measurements to calculate the Lb content of the nodule (29). The nodule oximeter is therefore an extremely powerful tool for studying factors that regulate in vivo nitrogenase activity, especially when used in conjunction with an open-circuit gas-exchange apparatus for concurrent measurements of nodule nitrogenase activity and respiration (86).

THE RELATIONSHIP AMONG RESPIRATION, NITROGENASE ACTIVITY, AND INFECTED CELL O_2 CONCENTRATION The observation that respiration rate and nitrogenase activity in legume nodules can be stimulated by increasing O_e has led to considerable interest in determining the relationships between O_i and these physiological parameters. Study of these relationships has involved measurement of O_i using the nodule oximeter, while a variety of approaches have been taken to monitor respiration (O_2 uptake or CO_2 evolution) and nitrogenase activity in the same nodule. For example:

1. In studies with birdsfoot trefoil, a plot of O_2 uptake (measured by nodule oximetry) against O_i showed Michaelis-Menten type kinetics for substrate limitation from 0 to 70 nM O_2 (30). Respiration was half-saturated at an O_i of ~15 nM O_2, and above 40 nM O_2 little stimulation was observed in the rate of O_2 consumption. The O_i measured in these nodules under ambient conditions was ~9 nM, which was significantly below that required to support the maximum rate of O_2 consumption (30). This observation is consistent with the O_2-limited nature of nodule metabolism. The saturation of O_2 uptake at O_i levels above 40 nM suggests that some factor(s), other than O_i, limit(s) respiration rate in the nodules.

2. Using soybean nodules, Denison and coworkers (34) coupled measurements of O_i with microelectrode measurements of central zone H_2 concentration (H_i). By making assumptions concerning nodule structure and H_2 diffusion characteristics, H_i values were converted to H_2 evolution rates as measurements of nitrogenase activity. The results showed that H_2 evolution rate remained relatively stable at O_i values between 15 and 150 nM, whereas respiration rate (O_2 consumption rate derived from oximetry measurements) increased with O_i up to 150 nM. This would suggest that infected cell respiration rate limits nitrogenase activity over a very small range of O_i values. This observation is difficult to reconcile with other studies (e.g. 69, 90) that show nitrogenase limitation by O_2 under ambient conditions when O_i is maintained between 19 and 35 nM (82, 90). This difficulty may indicate problems in the use of the H_2 electrode for estimating nitrogenase activity, because a more direct approach, as described below, has yielded significantly different results.

3. Recently a probe has been developed that measures O_i by oximetry in a single soybean nodule while either ANA, TNA, or PNA and CO_2 evolution are monitored in the same nodule by an open-flow gas-exchange system (86). A plot of TNA versus O_i showed O_2-limited kinetics at O_i levels between 0 and 57 nM O_2 with half-maximal TNA being attained at ~10 nM. This relationship was very similar to that obtained using intact nodulated roots (90), although TNA values in the nodulated roots were much greater than those in the single nodules. Therefore, although use of the probe may cause nitrogenase inhibition, it is still appropriate for investigating the relationship between TNA and

O_i. At O_i values between 68 and 280 nM, TNA in the single nodules declined. This decline was not due to the direct inhibition of the nitrogenase enzyme since declines in TNA at O_i values up to 250 nM are reversible (34, 81).

From these and related studies with various legume associations (29, 90), a number of patterns and generalizations have become apparent. First, respiration and nitrogenase activity are half-maximal when 20–45% of the Lb is oxygenated (FOL = 0.20 to 0.45, equivalent to an O_i of 12 to 40 nM O_2 in most associations). Also, in intact, undisturbed nodules in equilibrium with air, ~16–42% of the Lb is oxygenated (equivalent to an O_i of ~9–35 nM O_2). This observation is consistent with previous reports (69) and theoretical considerations (88) that O_2 is the primary factor limiting nitrogenase activity in legume nodules under normal conditions.

It is also interesting that the O_i levels supporting half-maximal activity are greater than the $K_m(O_2)$ for the terminal oxidase of soybean bacteroids [5–8 nM O_2, (12)], yet less than the $K_m(O_2)$ of the mitochondrial terminal oxidase in cowpea nodules [100 nM O_2, (114)]. If other symbioses have terminal oxidases with similar affinities for O_2, it seems reasonable that the apparent substrate kinetics observed in legume nodules are the product of both bacterial and plant respiration. These values also suggest that O_2 limitation in legume nodules may be determined by respiratory processes in the plant fraction rather than those in the bacterial fraction because the latter should be O_2 sufficient at ambient values of O_i.

Little is known about O_2-limited metabolism in legume nodules. However, it has been proposed that mitochondrial respiration in the plant fraction of the infected cells may be severely hypoxic and that the bacteroids may derive their carbohydrate from C_4 acids produced as the anaerobic end-products of glycolysis and "reverse" TCA cycle activity (70, 142, 158). At O_i levels less than ~40 nM, adenylate levels may decline in the plant fraction and limit the transport of these C_4 acids into the infected cells or across the symbiosome membrane separating the plant cytosol from the bacteroids. In support of this hypothesis it has been shown (27, 140, 160) that the plasma membrane of the infected cells and the symbiosome membrane contain both C_4 acid transporters and membrane ATPases. Also, adenylate levels in soybean nodules have been shown to increase rapidly with a step increase in O_e (49, 63) and decrease rapidly following a step decrease in O_e (49). The reversible inhibition of nitrogenase activity and nodule metabolism at O_i levels between 68 and 250 nM O_2 (86) may be due to a stimulation of mitochondrial respiration and a consequent reduction in the supply of carbon to the bacteroids. As yet there is no empirical evidence to support this hypothesis. Further research is required that combines biochemical studies with in vivo measurements of nodule gas

exchange, O_i, and metabolite levels in the plant and bacteroid fractions of the infected cells.

Although the biochemical basis for the narrow optimum O_i of nitrogenase activity (86) remains uncertain, it is clear that the mechanisms protecting nitrogenase from O_2-inactivation have a profound effect on nodule activity. Below we present evidence that O_i is not only regulated in nodules but is also a central regulator of nodule metabolism. We illustrate this assertion by referring to several environmental factors shown to inhibit nitrogenase activity.

ENVIRONMENTAL FACTORS AFFECTING NITROGENASE ACTIVITY

Restriction of Phloem Sap Supply

An extensive review concerning the effects of carbon supply on N_2 fixation has been published recently (142). We therefore present only the aspects of this area of research that relate to nodule gas exchange and O_2 limitation of nitrogenase activity.

The relationship between nitrogenase activity and photosynthate supply has been studied by growing plants at different light intensities and pCO_2 (40, 87, 125, 156), and by reducing phloem sap supply to nodules by stem-girdling (146, 151), defoliation (52, 60, 61, 117, 118), nodule excision (111, 135), and shoot removal (detopping) (29, 32, 35, 40, 99). Attempts to increase nodule-specific nitrogenase activity by maximizing photosynthetic rate have met with little or no success (40, 50, 87), but all treatments that reduce phloem sap supply to nodules cause rapid declines in nitrogenase activity and respiration rate. This inhibition is not associated with any apparent reduction in nodule carbohydrate reserves (61, 143, 151), indicating that nitrogenase activity is dependent on translocated photosynthate rather than on stored carbon. However, the primary limiting factor in photosynthate-deprived nodules is not carbohydrate but O_2, because elevation of external pO_2 brings about a significant (though not full) recovery of activity (29, 35, 40, 60, 99, 111, 135, 146). Also, it has been shown by oximetry that nodule excision or plant detopping causes a rapid decline in O_i associated with a reduction in nodule permeability (29, 32, 40, 90, 135).

From the studies cited above, the general response of nodules to phloem sap deprivation can be summarized as shown in Figure 2. Control plants have an O_i of ~25 nM, and this supports a level of O_2 uptake, or nitrogenase activity, as defined by response curve A (Figure 2). Following phloem sap deprivation, nodule permeability decreases, causing a decrease in O_i and a corresponding decrease in nodule respiration and nitrogenase activity (inhibited point, Figure 2). Elevation of O_e overcomes the decreased permeability, resulting in a stimu-

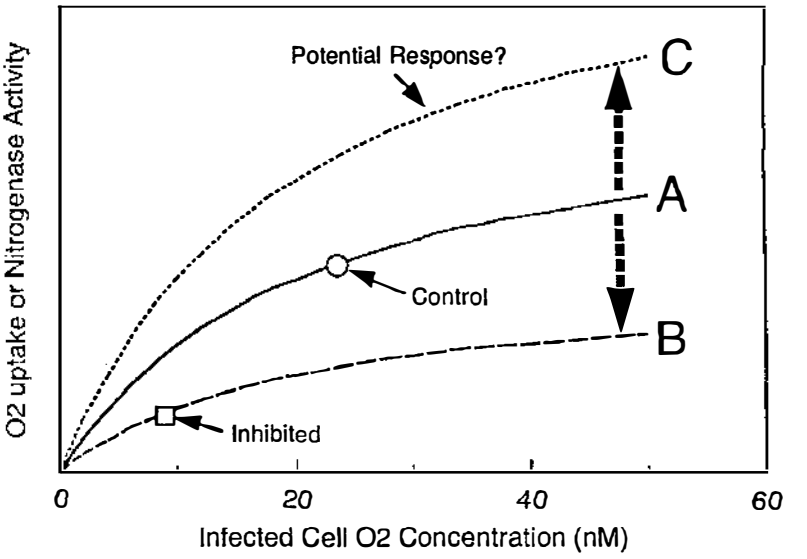

Figure 2 The form of the relationship that has been observed between infected cell O_2 concentration (O_i) and nitrogenase activity or O_2 uptake rate by the infected cells of a legume nodule (30, 82, 86, 90, 135). The solid line (A) shows the relationship under normal conditions in which O_i is maintained at a level (*unfilled circle*) that supports a sub-optimal metabolic rate in the nodule. The dashed line (B) shows the relationship typical of nodules inhibited by a variety of treatments (86, 90, 135). Note that O_i in the inhibited nodules is maintained at a level (*unfilled square*) that severely limits nodule metabolism. Also note that increases in O_i do not recover activity to the same level as that attained in the uninhibited nodule (i.e. the V_{max} is reduced). The dotted line (C) represents a hypothetical situation in which increased phloem sap to the uninhibited nodule may increase the V_{max} of the relationship so that infected cell activity at any given O_i is increased.

lation of O_2 uptake and nitrogenase activity along response curve B. Note that while the proportional increase in activity with O_e is greater in the inhibited plant than in the control plant (35), the maximum activity attained in the inhibited plant is less than that in the control. This decrease in the apparent V_{max} of the relationship between nodule activity and O_i is considered in more detail below.

The decrease in O_i following phloem sap deprivation may protect nitrogenase from O_2-inactivation during periods of reduced photosynthate transport because carbohydrate depletion by respiration would be reduced at low O_i and the period over which the nodule could maintain a level of O_2 consumption necessary to protect nitrogenase from O_2 inactivation would be increased (88). The mechanism by which reduced phloem sap supply decreases nodule permeability is not known, but because the phloem supplies water, sucrose, and other

carbohydrates, as well as growth regulators to the nodule, it is reasonable to propose that any one or a combination of these constituents may play a role in diffusion barrier regulation (113, 148, 149).

It is important to note that in addition to decreasing O_i and increasing the degree of O_2 limitation of nitrogenase activity, phloem sap deprivation causes a decline in the maximum nodule activity that can be attained by increasing O_e (response curve A to B, Figure 2). The magnitude of the decline in the V_{max} of this relationship varies with symbiotic association (29) and increases with time of phloem sap deprivation (29, 135). The specific factors that affect the V_{max} of the O_2 response curves are not known, but if phloem sap constituents are involved it is reasonable to propose that a greater V_{max} may be attainable at saturating O_i if phloem sap supply to the nodule is maximized (response curve C, Figure 2). To test this hypothesis, it would be necessary to determine the relationship between O_i and V_{max} in single nodules with known levels of available carbohydrate. To date such a study has not been attempted.

Nitrate Fertilization

Despite the large body of data accumulated on nitrate inhibition of nitrogenase activity, the precise mechanisms underlying this inhibition remain obscure (147). Bacteroids isolated from nitrate-inhibited nodules maintained nitrogenase activities similar to those isolated from control plants, indicating that inhibition was a function of the bacteroid environment (94) rather than a direct effect of nitrate. This interpretation was confirmed in several later studies (16, 63, 96, 97, 146) which showed that inhibition in nodules could be partially reversed by elevating rhizosphere pO_2. Also, oximetry measurements have shown that O_i in soybean nodules declines from its usual value of 20–30 nM (82, 90) to only 7 nM within 48 hr of applying 10 mM nitrate to attached nodulated roots (90). It is apparent, therefore, that nitrate fertilization has effects similar to those of phloem sap deprivation on O_i and the relationship between O_i and the V_{max} of nitrogenase activity (response curve A to curve B, Figure 2). Nitrate may cause a diversion of phloem sap supply from the nodule to the root tissue (133, 145, 146, 150). In support of this hypothesis it has been shown that sucrose concentration in white clover nodules declines after nitrate application (97) and that nodule starch pools decline in nitrate-treated nodules of pea (150) and soybean (145). Changes in nodule sucrose concentration may cause osmotic adjustment of the diffusion barrier resulting in decreased nodule permeability, and theoretical estimates (96, 97, 146) of permeability have indicated that such a decrease occurs during nitrate fertilization. However, direct measurements of permeability by the acetylene lag-phase method, or by nodule oximetry, have not been made on nitrate-treated nodules.

Several alternative hypotheses have been advanced to account for the nitrate-mediated O_2 limitation of nitrogenase activity. These include:

1. Nitrate reductase (NR) activity in the nodule may lead to the production of nitrite, which is a strong inhibitor of nitrogenase (138). Accumulation of nitrite requires a low nitrite reductase (NiR) activity in the nodule; and because NiR activity is dependent on O_2 concentration, a low value of O_i would contribute to nitrogenase inhibition whereas elevation of external pO_2 would relieve this inhibition (62). However, some doubts have been raised concerning the role of nitrite as a direct inhibitor of nitrogenase in vivo because nodules that have NR-deficient bacteroids still show inhibition by nitrate even though nitrite is present at non-inhibitory levels (134).

2. In symbioses that possess significant activities of NR, it has been proposed that cytosolic nitrite in the infected cells may combine with ferrous Lb to form nitrosyl-leghemoglobin (LbNO) and thereby reduce the amount of functional Lb available to facilitate O_2 diffusion to the bacteroids (75–79). In soybean nodules, LbNO formation was associated with a decline in ATP concentration and ATP/ADP ratio, consistent with increased O_2 limitation of nodule metabolism (76). Presumably, elevation of external pO_2 would increase the O_2 diffusion gradient within the infected cells of nitrate-treated nodules and ameliorate the effects of the reduced concentration of functional Lb. This hypothesis is not supported by mathematical models of nodule function, which suggest that nodules contain a large excess of Lb and that the site of O_i regulation is in the nodule cortex rather than in the central zone (28, 67, 124). However, many assumptions inherent in these models have not been tested empirically, and the LbNO hypothesis requires a re-evaluation of Lb as a potential factor in the regulation of O_i. The nodule oximeter (30), which is capable of measuring O_i, changes in nodule permeability to O_2 diffusion, and Lb concentration, would be an extremely useful tool in future studies.

3. NR activity has been proposed to inhibit nitrogenase activity by competing for reductant supply to bacteroids (63). So far only circumstantial evidence supports this hypothesis, because direct quantification has not been made of the relative reductant requirements of NR and the reactions supporting nitrogenase activity.

The three hypotheses outlined above all require the presence of nitrate or nitrite in the central zone of the nodule. However, within 3 days of applying 10 mM nitrate to nodulated roots of soybean, cowpea, and fava bean, Sprent and coworkers (132) failed to find appreciable concentrations of either nitrate or nitrite in the central zone of the nodules even though nitrogenase inhibition was observed throughout this period. Although LbNO (which could have sequestered available nitrite) was not assayed in these experiments, it was concluded that nitrate did not penetrate the central zone (132), and it was suggested that direct nitrite inhibition of nitrogenase activity and/or Lb function would therefore be unlikely. Instead, a two-stage inhibition process by

nitrate was proposed (96). In the first stage, nitrate is restricted to the nodule cortex, where it causes an increase in diffusion barrier resistance—perhaps by acting as an osmotically active ion (132), or by causing diversion of phloem sap away from the nodule (133, 145). In the second stage, reduced input of carbohydrate to the nodule may cause deterioration of the nodule cortex, allowing nitrate to penetrate to the infected zone. This would lead to nitrite-induced inhibition of nitrogenase, as well as nitrite effects on Lb (6, 75, 78) and degradative effects on other cytosolic proteins (5). This latter stage of nitrate inhibition would result in nodule senescence and irreversible inhibition of nitrogenase activity.

Given the disparities in the hypotheses described above, further studies are needed to determine the relative importance of NR, nitrite accumulation, functional Lb concentration, carbohydrate supply, and osmoticant levels on the inhibition of nitrogenase by nitrate. In addition, identification and characterization of legume symbioses that are not inhibited by nitrate provide an interesting approach to this problem, and some progress has been made in this direction using supernodulating mutants of soybean (26, 57, 122).

Drought Stress

An early study by Pankhurst & Sprent (105) reported that inhibition of nitrogenase activity in detached drought-stressed soybean nodules could be recovered by elevating pO_2. It was proposed that the primary effect of drought was to limit O_2 flux to the nodule central zone, perhaps by collapse of lenticels on the nodule surface (106). In intact field-grown soybeans, drought stress caused a decline in acetylene reduction activity that was linearly correlated with a decline in nodule conductance (153). It was also shown that elevated pO_2 increased acetylene reduction activity in the stressed plants to a level similar to that in control plants at 20% O_2. However, the control plants showed a much greater stimulation of activity with pO_2 than did the stressed plants, indicating that drought may have reduced the maximum nitrogenase activity (V_{max}) attainable at saturating O_i. This suggestion was supported by later studies, which showed that elevated pO_2 could only partially recover nitrogenase activity in drought-stressed nodules (42, 54).

Although O_2 limitation of nitrogenase activity and an apparent decline in V_{max} occur during both drought and phloem sap deprivation, it is unlikely that nitrogenase inhibition during drought results from reduction in photosynthate supply to the nodules. Nitrogenase activity declines rapidly with the onset of drought to an extent that cannot be accounted for by the relatively slow and small concurrent decline in photosynthetic rate (39, 42). Also, the concentration of soluble sugars in water-stressed nodules is similar to or greater than that in control nodules (25, 48, 71, 107). This finding indicates that carbohydrate is not limited in drought-stressed nodules; it also suggests that sucrose

does not act as a regulatory osmoticant in diffusion barrier control under drought conditions. Rather, sucrose accumulation, and that of proline, which has also been shown to accumulate in water-stressed nodules (71, 84), would tend to draw water from the intercellular spaces and thereby increase nodule permeability.

An alternative hypothesis for the mechanism of O_2 limitation of nitrogenase during drought is that Lb concentration, which declines in water-stressed nodules (53, 54, 80), restricts facilitated diffusion of O_2 to the bacteroids. The decline in Lb content may be due to an increase in protease activity associated with a more general disruption of cytosolic proteins (53, 54). This hypothesis is similar to that proposed by Kanayama and coworkers (75, 77–79) in their attempt to account for the mechanism of nitrate inhibition of nodule activity. It has also been suggested that the bacteroids themselves may lose respiratory capacity during drought stress (54).

More work is required to elucidate the mechanisms for short-term and long-term inhibition of nitrogenase activity during drought. The apparent decrease in nodule permeability requires further investigation, preferably by use of oximetry, which would provide associated measurements of O_i and Lb status. Among approaches that may be useful in the future is a study of the physiological basis for variations in sensitivity to drought that have recently been reported in different soybean cultivars (120).

DEVELOPMENTAL AND LONG-TERM ADAPTATION TO RHIZOSPHERE pO_2

Most of the research concerned with elucidating mechanisms of O_2 regulation in legume nodules has been done using mature nodules. However, it is important to note that the gas diffusion characteristics of the nodule may change during development. As spherical nodules expand, surface area to volume ratio decreases significantly. Since diffusion rate of O_2 into the nodule is dependent on nodule surface area, a smaller nodule requires a much greater resistance than a larger nodule to maintain the same O_i (67). This requirement is extremely important because expression of *nif* and *fix* genes, which code for the proteins required for N_2 fixation, are regulated by the *nif*A gene, which is only expressed in a low-O_2 environment (64). How then does the developing nodule, which has no nitrogenase-linked O_2 consumption, maintain O_i at a level low enough for *nif*A expression? A mathematical model (127) predicted that a developing nodule without intercellular spaces, and with a central zone respiration rate equivalent to that of a mature nodule, would require a minimum radius of 0.55 mm to provide the O_i necessary for *nif*A expression. Because soybean nodules of only 0.4 mm radius possess active nitrogenase (9), it has been proposed that developing nodules may reduce O_i by consump-

tion of O_2 in alternative chain respiration (127). However, there is little direct evidence for this hypothesis.

Morphological development of nodules is greatly influenced by the gradient of O_2 between the rhizosphere and the central zone. Several structural studies have indicated that nodules of soybean and cowpea grown at low pO_2 have cortical intercellular spaces that are more frequent and larger in cross-sectional area than those of air-grown nodules (4, 20, 22, 108). The reverse is true of nodules grown under supra-ambient pO_2, and the intercellular spaces of these nodules are frequently occluded with a glycoprotein that may further reduce ventilation (73). In addition to structural adaptations, Lb concentration may vary under different pO_2 regimes (19, 108), providing another potential mechanism for O_2 regulation. Although nodules grown under sub- and supra-ambient pO_2 can vary their permeabilities, the range of external pO_2 to which they can adapt is more limited than that in air-grown nodules (4, 21). This provides evidence that the mechanisms for long-term changes in nodule resistance are different from those controlling resistance of the variable diffusion barrier (4). Although long-term adaptation to supra-ambient pO_2 may have little relevance in the natural environment, low pO_2 values may be maintained for long periods in waterlogged or otherwise poorly aerated soils. Nodules growing in these environments, such as those of the aquatic legume *Neptunia,* possess structural modifications and physiological responses similar to those of nodules grown under sub-ambient pO_2 in the laboratory (72, 74).

CONCLUDING REMARKS

Despite the significant advances made in our understanding of the factors that regulate nitrogenase activity in legume nodules, several areas of doubt and controversy require careful research in the future:

1. The concept of a regulated barrier to gas diffusion in the nodule cortex is becoming dogma without adequate justification. Structural studies, which involve minimal tissue disturbance, are required to define clearly the geometry of gas diffusion pathways throughout the nodule. Data from these may then be incorporated into mathematical models of gas diffusion that can predict the relative effects on O_i of physical resistances in the cortex and central zone. In addition, the hypothetical osmotic mechanism for diffusion barrier regulation requires rigorous testing. Are changes in nodule permeability inversely correlated with apoplastic water content and nodule density? Can excised nodules, or cells isolated from the nodule cortex, be induced to modify their water relations in response to variations in physiologically relevant O_2 concentrations? Are changes in nodule permeability to O_2 correlated with changes in the turgidity of cells in the nodule cortex?

2. A reassessment is needed of the role of Lb as a potential regulator of O_i and O_2 flux throughout the infected cell. Can changes in the affinity of Lb for O_2, or in the concentration of functional Lb, account for the changes in nodule permeability or the degree of O_2 limitation of nodule activity observed during certain inhibitory treatments?

3. Further study is needed to characterize the relationships between O_i and nodule respiration or nitrogenase activity in control and inhibited nodules. What determines V_{max} at saturating O_i, and why should a lower V_{max} be attained at saturating O_i in nodules inhibited by adverse environmental conditions? Why are nitrogenase activity and nodule respiration inhibited by O_i levels only marginally higher than those that limit nodule metabolism?

4. The biochemical basis of O_2 limitation of nodule metabolism is poorly understood. Detailed analyses of respiratory metabolites in the bacteroid and plant fractions of infected cells during O_2 sufficiency and O_2 limitation are required to identify the pathways that support nitrogenase activity under normal and stress conditions.

A wide variety of new techniques are available to aid future research in these areas. Non-invasive measurements of ANA, TNA, PNA, O_i, nodule permeability, and the rate of nitrogenase-linked respiration can all be monitored continuously, or successively, in whole plants or in single attached nodules under a wide variety of experimental conditions. These techniques also allow nodules to be sampled for structural or metabolic analysis when their physiological state is well defined. The same techniques are adaptable for field studies and may be used to determine swiftly and accurately whether or not nodule metabolism in a selected plant is operating close to, or much below, its potential activity. Gas exchange measurements will therefore continue to provide essential information in future physiological and agronomic studies of N_2 fixation in legume nodules.

Literature Cited

1. Appleby, C. A. 1969. Properties of leghaemoglobin in vivo and its isolation as ferrous oxyleghaemoglobin. *Biochem. Biophys. Acta* 188:222–29
2. Appleby, C. A. 1984. Leghemoglobin and *Rhizobium* respiration. *Annu. Rev. Plant Physiol.* 35:443–78
3. Arp, D. J. 1990. H_2 cycling in N_2 fixation: past, present and future outlook. See Ref. 52a, pp. 67–76
4. Atkins, C. A., Hunt, S., Layzell, D. B. 1993. Gaseous diffussive properties of soybean nodules cultured with non-ambient pO_2. *Physiol. Plant.* In press
5. Becana, M., Minchin, F. R., Sprent, J. I. 1989. Short-term inhibition of legume N_2 fixation by nitrate. I. Nitrate effects on nitrate-reductase activities of bacteroids and nodule cytosol. *Planta* 180:40–45
6. Becana, M., Sprent, J. I. 1989. Effect of nitrate on components of nodule leghemoglobins. *J. Exp. Bot.* 40:725–31
7. Bergersen, F. J. 1962. Oxygenation of leghaemoglobin in soybean root-nodules in relation to the external oxygen tension. *Nature* 194:1059–61
8. Bergersen, F. J. 1980. Measurement of nitrogen fixation by direct means. In Methods for Evaluating Biological Nitrogen Fixation, ed. F. J. Bergersen, pp. 65–111. New York: Wiley Interscience
9. Bergersen, F. J. 1982. *Root Nodules of Le-*

gumes: Structure and Functions. New York: Research Studies Press
10. Bergersen, F. J., Goodchild, D. J. 1973. Aeration pathways in soybean root nodules. *Aust. J. Biol. Sci.* 26:729–40
11. Bergersen, F. J., Turner, G. L. 1979. Systems utilizing oxygenated leghemoglobin and myoglobin as sources of free dissolved O_2 at low concentrations for experiments with bacteria. *Anal. Biochem.* 96:165–74
12. Bergersen, F. J., Turner, G. L. 1980. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N_2-fixing bacteria grown in continuous culture. *J. Gen. Microbiol.* 118:235–52
13. Burgess, B. K. 1986. Substrate reactions of nitrogenase. In *Molybdenum Enzymes,* ed. T. G. Spiro, pp. 161–219. New York: Wiley & Sons
14. Burris, R. H. 1991. Nitrogenases. *J. Biol. Chem.* 266:9339–42
15. Caetano-Anollés, G., Gresshoff, P. M. 1991. Plant genetic control of nodulation. *Annu. Rev. Microbiol.* 45:345–82
16. Carroll, B. J., Hansen, A. P., McNeil, D. L., Gresshoff, P. M. 1987. Effect of oxygen supply on nitrogenase activity of nitrate and dark-stressed soybean (*Glycine max* (L.) Merr) plants. *Aust. J. Plant Physiol.* 14:679–87
17. Criswell, J. G., Havelka, U. D., Quebedeaux, B., Hardy, R. W. F. 1976. Adaptation of nitrogen fixation by intact soybean nodules to altered rhizosphere pO_2. *Plant Physiol.* 58:622–25
18. Criswell, J. G., Havelka, U. D., Quebedeaux, B., Hardy, R. W. F. 1977. Effect of rhizosphere pO_2 on nitrogen fixation by excised and intact nodulated soybean roots. *Crop Sci.* 17:39–44
19. Dakora, F. D., Appleby, C. A., Atkins, C. A. 1991. Effect of pO_2 on the formation and status of leghemoglobin in nodules of cowpea and soybean. *Plant Physiol.* 95:723–30
20. Dakora, F. D., Atkins, C. A. 1990. Morphological and structural adaptation of nodules of cowpea to functioning under sub- and supra-ambient oxygen pressure. *Planta* 182:572–82
21. Dakora, F. D., Atkins, C. A. 1990. Effect of pO_2 on growth and nodule functioning of symbiotic cowpea (*Vigna unguiculata* L. Walp). *Plant Physiol.* 93:948–55
22. Dakora, F. D., Atkins, C. A. 1991. Adaptation of nodulated soybean (*Glycine max* L. Merr) to growth in rhizospheres containing nonambient pO_2. *Plant Physiol.* 96: 728–36
23. Dalton, D. A., Post, C. J., Langeberg, L. 1991. Effects of ambient oxygen and of fixed nitrogen on concentrations of glutathione, ascorbate, and associated enzymes in soybean root nodules. *Plant Physiol.* 96:812–18
24. Davey, A. G., Simpson, R. J. 1989. Changes in nitrogenase activity and nodule diffusion resistance of subterranean clover in response to pO_2. *J. Exp. Bot.* 40:149–58
25. Davey, A. G., Simpson, R. J. 1990. Nitrogen fixation by subterranean clover at varying stages of nodule dehydration. I. Carbohydrate status and short-term recovery of nodulated root respiration. *J. Exp. Bot.* 41:1175–87
26. Day, D. A., Price, G. D., Schuller, K. A., Gresshoff, P. M. 1987. Nodule physiology of a supernodulating soybean (*Glycine max*) mutant. *Aust. J. Plant Physiol.* 14:527–38
27. Day, D. A., Yang, L. J. O., Udvardi, M. K. 1990. Nutrient exchange across the peribacteroid membrane of isolated symbiososmes. See Ref. 52a, pp. 219–26
28. Denison, R. F. 1992. Mathematical modelling of oxygen diffusion and respiration in legume root nodules. *Plant Physiol.* 98:901–7
29. Denison, R. F., Hunt, S., Layzell, D. B. 1992. Nitrogenase activity, nodule respiration and O_2 permeability following detopping of alfalfa and birdsfoot trefoil. *Plant Physiol.* 98:894–900
30. Denison, R. F., Layzell, D. B. 1991. Measurement of legume nodule respiration and O_2 permeability by noninvasive spectrophotometry of leghemoglobin. *Plant Physiol.* 96:137–43
31. Denison, R. F., Sinclair, T. R. 1985. Diurnal and seasonal variation in dinitrogen fixation (acetylene reduction) rates by field-grown soybeans. *Agron. J.* 77:679–84
32. Denison, R. F., Smith, D. L., Layzell, D. B. 1991. Noninvasive measurement of internal oxygen concentrations of field-grown soybean nodules. *Agron. J.* 83:166–69
33. Denison, R. F., Weisz, P. R., Sinclair, T. R. 1983. Analysis of acetylene reduction rates of soybean nodules at low acetylene concentrations. *Plant Physiol.* 73:648–51
34. Denison, R. F., Witty, J. F., Minchin, F. R. 1992. Reversible O_2 inhibition of nitrogenase activity in attached soybean nodules. *Plant Physiol.* 100:1863–68
35. Diaz del Castillo, L., Hunt, S., Layzell, D. B. 1992. O_2 regulation and O_2 limitation of nitrogenase activity in root nodules of pea and lupin. *Physiol. Plant.* 86:269–78
36. Dilworth, M. 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridum pasteurianum. Biochem. Biophys. Acta* 127:285–94
37. Dixon, R. O. D., Blunden, E. A. G. 1983. The relative efficiency of nitrogen fixation in pea root nodules. *Plant Soil* 75:131–38
38. Dixon, R. O. D., Blunden, E. A. G., Searl, J. W. 1981. Intercellular space and hydro-

gen diffusion in pea and lupin root nodules. *Plant Sci. Lett.* 23:109–16
39. Djekoun, A., Planchon, C. 1991. Water status effect on dinitrogen fixation and photosynthesis in soybean. *Agron. J.* 83:316–22
40. Drevon, J. J., Gaudillere, J. P., Bernoud, J. P., Jardinet, F., Evrard, M. 1991. Influence of photon flux density and fluctuation on the nitrogen fixing *Glycine max* (L. Merr)-*Bradyrhizobium japonicum* symbiosis in a controlled environment. *Agronomie* 11: 193–99
41. Drevon, J. J., Kalia, V. C., Heckmann, M. O., Pedelahore, P. 1988. In situ open-flow assay of acetylene reduction activity by soybean root nodules: influence of acetylene and oxygen. *Plant Physiol. Biochem.* 26:73–78
42. Durand, J. L., Sheehy, J. E., Minchin, F. R. 1987. Nitrogenase activity, photosynthesis and nodule water potential in soyabean plants experiencing water deprivation. *J. Exp. Bot.* 38:311–21
43. Edie, S. A. 1983. Acetylene reduction and hydrogen evolution by nitrogenase in a *Rhizobium*-legume symbiosis. *Can. J. Bot.* 61:780–85
44. Edie, S. A., Phillips, D. A. 1983. Effect of the host legume on acetylene reduction and hydrogen evolution by *Rhizobium* nitrogenase. *Plant Physiol.* 72:156–60
45. Emerich, D. W., Ruiz-Argueso, T., Ching, T. M., Evans, H. J. 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. *J. Bacteriol.* 137:153–60
46. Eskew, D. L., Gresshoff, P. M., Doty, M., Mora, C. 1992. Sealed tube combustion for natural ^{15}N abundance estimation of N_2 fixation and application to supernodulating soybean mutants. *Can. J. Microbiol.* 38:598–603
47. Evans, H. J., Harker, A. R., Papen, H., Russell, S. A., Hanus, F. J., Zuber, M. 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. *Annu. Rev. Microbiol.* 41:335–61
48. Fellows, R. J., Patterson, R. P., Raper, C. D., Harris, D. 1987. Nodule activity and allocation of photosynthate of soybean during recovery from water stress. *Plant Physiol.* 84:456–60
49. Fernando, S. M., Hunt, S., Smith, R., Turpin, D. H., Atkins, C. A., Layzell, D. B. 1990. O_2 induced changes in metabolite pool sizes in soybean nodules. See Ref. 52a, p. 347
50. Finn, G. A., Brun, W. A. 1982. Effect of atmospheric CO_2 enrichment on growth, nonstructural carbohydrate content, and root nodule activity in soybean. *Plant Physiol.* 69:327–31
51. Godfroy, A., Drevon, J. J. 1991. Hydrogen effect on nitrogenase (C_2H_2 reduction) response to oxygen in Bradyrhizobium japonicum Phaseoleae symbioses. *J. Exp. Bot.* 42:1379–85
52. Gordon, A. J., Kessler, W., Minchin, F. R. 1990. Defoliation-induced stress in nodules of white clover. I. Changes in physiological parameters and protein synthesis. *J. Exp. Bot.* 41:1245–53
52a. Gresshoff, P. M., Roth, L. E., Stacey, G., Newton, W. E., eds. 1990. *Nitrogen Fixation: Achievements and Objectives.* New York: Chapman & Hall
53. Guerin, V., Pladys, D., Trinchant, J. C., Rigaud, J. 1991. Proteolysis and nitrogen fixation in faba-bean (*Vicia faba*) nodules under water stress. *Physiol. Plant.* 82:1–7
54. Guerin, V., Trinchant, J. C., Rigaud, J. 1990. Nitrogen fixation (C_2H_2 reduction) by broad bean (*Vicia faba* L.) nodules and bacteroids under water-restricted conditions. *Plant Physiol.* 92:595–601
55. Guth, J. H., Burris, R. H. 1983. Comparative study of the active and inactive forms of dinitrogenase reductase from *Rhodospirillum rubrum. Biochem. Biophys. Acta* 749:91–100
56. Hansen, A. P., Pate, J. S., Atkins, C. A. 1987. Relationships between acetylene reduction activity, hydrogen evolution and nitrogen fixation in nodules of *Acacia* spp.: Experimental background to assaying fixation by acetylene reduction under field conditions. *J. Exp. Bot.* 38:1–12
57. Hansen, A. P., Peoples, M. B., Gresshoff, P. M., Atkins, C. A., Pate, J. S., et al. 1989. Symbiotic performance of supernodulating soybean (*Glycine max* (L.) Merrill) mutants during development on different nitrogen regimes. *J. Exp. Bot.* 40:715–24
58. Hardy, R. W. F., Holsten, R. D. 1977. Methods for measurement of dinitrogen fixation. In *Treatise in Dinitrogen Fixation,* ed. R. W. F. Hardy, A. H. Gibson, pp. 451–86. New York: Wiley & Sons
59. Hardy, R. W. F., Holsten, R. D., Jackson, E. K., Burns, R. C. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol.* 43:1185–207
60. Hartwig, U., Boller, B., Nosberger, J. 1987. Oxygen supply limits nitrogenase activity of clover nodules after defoliation. *Ann. Bot.* 59:285–91
61. Hartwig, U., Boller, B. C., Baur-Hoch, B., Nosberger, J. 1990. The influence of carbohydrate reserves on the response of nodulated white clover to defoliation. *Ann. Bot.* 65:97–105
62. Heckmann, M. O., Drevon, J. J. 1987. Nitrate metabolism in soybean root nodules. *Physiol. Plant.* 69:721–25
63. Heckmann, M. O., Drevon, J. J., Saglio, P., Salsac, L. 1989. Effect of oxygen and ma-

late on NO_3^- inhibition of nitrogenase in soybean nodules. *Plant Physiol.* 90:224–29

64. Hennecke, H., Bott, M., Ramseier, T., Thony-Meyer, L., Fischer, H. M., et al. 1990. A genetic approach to analyze the critical role of oxygen in bacteroid metabolism. See Ref. 52a, pp. 293–99
65. Herridge, D. F., Bergersen, F. J., Peoples, M. B. 1990. Measurement of nitrogen fixation by soybean in the field using the ureide and natural ^{15}N abundance methods. *Plant Physiol.* 93:708–16
66. Hunt, S., Denison, R. F., King, B. J., Kouchi, H., Tajima, S., Layzell, D. B. 1990. An osmotic mechanism for diffusion barrier regulation in soybean nodules. See Ref. 52a, p. 352
67. Hunt, S., Gaito, S. T., Layzell, D. B. 1988. Model of gas exchange and diffusion in legume nodules. II. Characterization of the diffusion barrier and estimation of the concentrations of CO_2, H_2, and N_2 in the infected cells. *Planta* 173:128–41
68. Hunt, S., King, B. J., Canvin, D. T., Layzell, D. B. 1987. Steady and nonsteady state gas exchange characteristics of soybean nodules in relation to the oxygen diffusion barrier. *Plant Physiol.* 84:164–72
69. Hunt, S., King, B. J., Layzell, D. B. 1989. Effects of gradual increases in O_2 concentration on nodule activity in soybean. *Plant Physiol.* 91:315–21
70. Hunt, S., Layzell, D. B. 1991. The role of oxygen in nitrogen fixation and carbon metabolism in legume nodules. In *In Active Oxygen/Oxidative Stress and Plant Metabolism,* ed. E. J. Pell, K. L. Steffen, pp. 26–39. Rockville, MD: Am. Soc. Plant Physiol.
71. Irigoyen, J. J., Emerich, D. W., Sanchez-Diaz, M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiol. Plant* 84:55–60
72. James, E. K., Minchin, F. R., Sprent, J. I. 1992. The physiology and nitrogen-fixing capability of aquatically and terrestrially grown *Neptunia plena*—the importance of nodule oxygen supply. *Ann. Bot.* 69:181–87
73. James, E. K., Sprent, J. I., Minchin, F. R., Brewin, N. J. 1991. Intercellular location of glycoprotein in soybean nodules—effect of altered rhizosphere oxygen concentration. *Plant Cell Environ.* 14:467–76
74. James, E. K., Sprent, J. I., Sutherland, J. M., McInroy, S. G., Minchin, F. R. 1992. The structure of nitrogen fixing root nodules on the aquatic mimosoid legume *Neptunia plena. Ann. Bot.* 69:173–80
75. Kanayama, Y., Watanabe, I., Yamamoto, Y. 1990. Inhibition of nitrogen fixation in soybean plants supplied with nitrate. II. Nitrite accumulation and formation of nitrosylleghemoglobin in nodules. *Plant Cell Physiol.* 21:341–46
76. Kanayama, Y., Yamamoto, Y. 1990. Effects of nitrate on nucleotide levels in soybean nodules. *Plant Cell Physiol.* 31:893–95
77. Kanayama, Y., Yamamoto, Y. 1990. Inhibition of nitrogen fixation in soybean plants supplied with nitrate. II. Accumulation and properties of nitrosylleghemoglobin in nodules. *Plant Cell Physiol.* 31:207–14
78. Kanayama, Y., Yamamoto, Y. 1990. Inhibition of nitrogen fixation in soybean plants supplied with nitrate. III. Kinetics of the formation of nitrosylleghemoglobin and of the inhibition of oxyleghemoglobin. *Plant Cell Physiol.* 31:603–8
79. Kanayama, Y., Yamamoto, Y. 1991. Formation of nitrosylleghemoglobin in nodules of nitrate-treated cowpea and pea plants. *Plant Cell Physiol.* 32:19–24
80. Khanna-Chopra, R., Koundal, K. R., Sinha, S. K. 1984. A simple technique of studying water deficit effects on nitrogen fixation in nodules without influencing the whole plant. *Plant Physiol.* 76:254–56
81. King, B. J., Hunt, S., Weagle, G. E., Walsh, K. B., Pottier, R. H., et al. 1988. Regulation of O_2 concentration in soybean nodules observed by in situ spectroscopic measurement of leghemoglobin oxygenation. *Plant Physiol.* 87:296–99
82. King, B. J., Layzell, D. B. 1991. Effect of increases in O_2 concentration during the argon-induced decline in nitrogenase activity in root nodules of soybean. *Plant Physiol.* 96:376–81
83. Klucas, R. V., Lee, K. K., Saari, L. 1985. Factors affecting functional leghemoglobin in legume nodules. In *Nitrogen Fixation and CO_2* Metabolism, ed. P. W. Ludden, J. E. Burris, pp. 13–20. New York: Elsevier
84. Kohl, D. H., Kennelly, E. J., Zhu, Y. X., Schubert, K. R., Shearer, G. 1991. Proline accumulation, nitrogenase (C_2H_2 reducing) activity and activities of enzymes related to proline metabolism in drought-stressed soybean nodules. *J. Exp. Bot.* 42:831–37
85. Kohl, D. H., Shearer, G., Harper, J. E. 1980. Estimates of N_2 fixation based on differences in the natural abundance of ^{15}N in nodulating and nonnodulating isolines of soybeans. *Plant Physiol.* 66:61–65
86. Kuzma, M. M., Hunt, S., Layzell, D. B. 1993. The role of O_2 in the limitation and inhibition of nitrogenase activity and respiration rate in individual soybean nodules. *Plant Physiol.* 101:161–69
87. Lawn, R. J., Brun, W. A. 1974. Symbiotic nitrogen fixation in soybeans. I. Influence of photosynthetic source-sink manipulations. *Crop Sci.* 14:11–16

88. Layzell, D. B., Hunt, S. 1990. Oxygen and the regulation of nitrogen fixation in legume nodules. *Physiol. Plant.* 80:322–27
89. Layzell, D. B., Hunt, S., Moloney, A. H. M., Fernando, S. M., Diaz del Castillo, L. 1990. Physiological, metabolic and developmental implications of O_2 regulation in legume nodules. See Ref. 52a, pp. 21–32
90. Layzell, D. B., Hunt, S., Palmer, G. R. 1990. Mechanism of nitrogenase inhibition in soybean nodules. Pulse-modulated spectroscopy indicates that nitrogenase activity is limited by O_2. *Plant Physiol.* 92:1101–7
91. Layzell, D. B., Moloney, A. H. M. 1993. Dinitrogen fixation. In *Physiology and Determination of Crop Yield,* ed. K. J. Boote, T. Sinclair, pp. 1-40. Madison, WI: Am. Soc. Agronomy
92. Layzell, D. B., Weagle, G. E., Canvin, D. T. 1984. A highly sensitive flow through H_2 gas analyzer for use in nitrogen fixation studies. *Plant Physiol.* 75:582–85
93. Mahon, J. D., Nelson, L. M. 1986. The relationship between H_2 evolution and acetylene reduction in *Pisum sativum-Rhizobium leguminosarum* symbioses differing in uptake hydrogenase activity. *Plant Physiol.* 82:154–59
94. McNeil, D. L., Carroll, B. J., Gresshoff, P. M. 1984. The nitrogen fixation capacity of bacteroids extracted from soybean nodules inhibited by nitrate, ammonia, or dark treatments. *Symbiotic Nitrogen Fixat.* 1:79–88
95. McNeill, A. M., Sheehy, J. E., Drennan, D. S. H. 1989. The development and use of a flow-through apparatus for measuring nitrogenase activity and photosynthesis in field crops. *J. Exp. Bot.* 40:187–94
96. Minchin, F. R., Becana, M., Sprent, J. I. 1989. Short-term inhibition of legume N_2 fixation by nitrate. II. Nitrate effects on nodule oxygen diffusion. *Planta* 180:46–52
97. Minchin, F. R., Minguez, M. I., Sheehy, J. E., Witty, J. F., Skot, L. 1986. Relationships between nitrate and oxygen supply in symbiotic nitrogen fixation by white clover. *J. Exp. Bot.* 37:1103–13
98. Minchin, F. R., Sheehy, J. E., Minguez, M. I. 1984. Acetylene-induced changes in the oxygen diffusion resistance and nitrogenase activity of legume root nodules. *Ann. Bot.* 53:13–20
99. Minchin, F. R., Sheehy, J. E., Witty, J. F. 1986. Further errors in the acetylene reduction assay. Effects of plant disturbance. *J. Exp. Bot.* 37:1581–91
100. Minchin, F. R., Witty, J. F., Sheehy, J. E., Muller, M. 1983. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. *J. Exp. Bot.* 34:641–49
101. Moloney, A. H. M., Guy, R. D., Layzell, D. B. 1990. In vivo regulation of nitrogenase electron allocation. See Ref. 52a, p. 357
102. Deleted in proof
103. Monroe, J. D., Owens, T. G., LaRue, T. A. 1989. Measurement of the fractional oxygenation of leghemoglobin in intact detached pea nodules by reflectance spectroscopy. *Plant Physiol.* 91:598–602
104. Nelson, L. M., Salminen, S. O. 1982. Uptake hydrogenase activity and ATP formation in *Rhizobium leguminosarum* bacteroids. *J. Bacteriol.* 151:989–95
105. Pankhurst, C. E., Sprent, J. I. 1975. Effects of water stress on the respiratory and nitrogen-fixing activity of soybean root nodules. *J. Exp. Bot.* 26:287–304
106. Pankhurst, C. E., Sprent, J. I. 1975. Surface features of soybean root nodules. *Protoplasma* 85:85–98
107. Pararajasingham, S., Knievel, D. P. 1990. Nitrogenase activity, photosynthesis and total nonstructural carbohydrates in cowpea during and after drought stress. *Can. J. Plant Sci.* 70:1005–12
108. Parsons, R., Day, D. A. 1990. Mechanism of soybean nodule adaptation to different oxygen pressures. *Plant Cell Environ.* 13:501–12
109. Deleted in proof
110. Deleted in proof
111. Ralston, E. J., Imsande, J. 1982. Entry of oxygen and nitrogen into intact soybean nodules. *J. Exp. Bot.* 33:208–14
112. Rasche, M. E., Arp, D. J. 1989. Hydrogen inhibition of nitrogen reduction by nitrogenase in isolated soybean nodule bacteroids. *Plant Physiol.* 91:663–68
113. Raven, J. A., Sprent, J. I., McInroy, S. G., Hay, G. T. 1989. Water balance of N_2-fixing root nodules: Can phloem and xylem transport explain it? *Plant Cell Environ.* 12:683–88
114. Rawsthorne, S., LaRue, T. A. 1986. Preparation and properties of mitochondria from cowpea nodules. *Plant Physiol.* 81:1092–96
115. Robson, R. L., Postgate, J. R. 1980. Oxygen and hydrogen in biolgical nitrogen fixation. *Annu. Rev. Microbiol.* 34:183–207
116. Rosendahl, L., Jakobsen, I. 1988. Effects of age, supra-ambient oxygen and repeated assays on acetylene reduction and root respiration in pea. *Physiol. Plant.* 74:77–82
117. Ryle, G. J. A., Powell, C. E., Gordon, A. J. 1985. Short-term changes in CO_2 evolution associated with nitrogenase activity in white clover in response to defoliation and photosynthesis. *J. Exp. Bot.* 165:634–43
118. Ryle, G. J. A., Powell, C. E., Gordon, A. J. 1986. Defoliation in white clover: nodule metabolism, nodule growth and maintenance, and nitrogenase functioning during growth and regrowth. *Ann. Bot.* 57:263–71

119. Saito, S. M. T., Matsui, E., Salati, E. 1980. $^{15}N_2$ fixation, H_2 evolution and C_2H_2 reduction relationships in *Phaseolus vulgaris*. *Physiol. Plant.* 49:37–42
120. Sall, K., Sinclair, T. R. 1991. Soybean genotypic differences in sensitivity of symbiotic nitrogen fixation to soil dehydration. *Plant Soil* 133:31–37
121. Schuler, S., Conrad, R. 1991. Hydrogen oxidation in soil following rhizobial H_2 production due to N_2 fixation by a *Vicia faba-Rhizobium leguminosarum* symbiosis. *Biol Fertil. Soils* 11:190–95
122. Schuller, K. A., Minchin, F. R., Gresshoff, P. M. 1988. Nitrogenase activity and oxygen diffusion in nodules of soybean cv. Bragg and a supernodulating mutant: effects of nitrate. *J. Exp. Bot.* 39:865–77
123. Sheehy, J. E., Bergersen, F. J. 1986. A simulation study of the functional requirements and distribution of leghaemoglobin in relation to biological nitrogen fixation in legume root nodules. *Ann. Bot.* 58:121–36
124. Sheehy, J. E., Bergersen, F. J., Minchin, F. R., Witty, J. 1987. A simulation study of gaseous diffusion resistance, nodule pressure gradients and biological nitrogen fixation in soybean nodules. *Ann. Bot.* 60:345–51
125. Sheehy, J. E., Fishbeck, K. A., DeJong, T. M., Williams, L. E., Phillips, D. A. 1980. Carbon exchange rates of shoots required to utilize available acetylene reduction capacity in soybean and alfalfa root nodules. *Plant Physiol.* 66:101–4
126. Sheehy, J. E., Minchin, F. R., Witty, J. F. 1983. Biological control of the resistance to oxygen flux in nodules. *Ann. Bot.* 52:565–71
127. Sheehy, J. E., Thornley, J. H. M. 1988. Oxygen, the *nifA* gene, nodule structure and the initiation of nitrogen fixation. *Ann. Bot.* 61:605–9
128. Sheehy, J. E., Webb, J. 1991. Oxygen diffusion pathways and nitrogen fixation in legume root nodules. *Ann. Bot.* 67:85–92
129. Sheehy, J. E., Woodward, F. I., Gosse, G. 1991. Measurements of nitrogen fixation (C_2H_2), photosynthesis and respiration using an open system in the natural environment. *Ann. Bot.* 67:131–36
130. Simpson, F. B., Burris, R. H. 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. *Science* 224:1095–97
131. Sinclair, T. R., Goudriaan, J. 1981. Physical and morphological constraints on transport in nodules. *Plant Physiol.* 67:143–45
132. Sprent, J. I., Giannakis, C., Wallace, W. 1987. Transport of nitrate and calcium into legume root nodules. *J. Exp. Bot.* 38:1121–28
133. Streeter, J. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. *CRC Crit. Rev. Plant Sci.* 7:1–23
134. Streeter, J. G. 1985. Nitrate inhibition of legume nodule growth and activity. I. Long term studies with a continuous supply of nitrate. *Plant Physiol.* 77:321–24
135. Sung, L., Moloney, A. H. M., Hunt, S., Layzell, D. B. 1991. The effect of excision on O_2 diffusion and metabolism in soybean nodules. *Physiol. Plant.* 83:67–74
136. Thorneley, R. N. F., Lowe, D. J. 1985. Kinetics and mechanism of the nitrogenase enzyme system. In *Molybdenum Enzymes*, ed. T. G. Spiro, pp. 221–84. New York: Wiley & Sons
137. Tjepkema, J. D., Yocum, C. S. 1974. Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. *Planta* 119:351–60
138. Trinchant, J. C., Rigaud, J. 1982. Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids. *Appl. Environ. Microbiol.* 44:1385–88
139. Trinick, M. J., Dilworth, M. J., Grounds, M. 1976. Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. *New Phytol.* 77:359–70
140. Udvardi, M. K., Day, D. A. 1989. Electrogenic ATPase activity on the perbacteroid membrane of soybean (*Glycine max* L.) root nodules. I. *Plant Physiol.* 90:982–87
141. Van Berkum, P., Sloger, C., Weber, D. F., Cregan, P. B., Keyser, H. H. 1985. Relationship between ureide N and N_2 fixation, above ground N accumulation, acetylene reduction, and nodule mass in greenhouse and field studies with *Glycine max* L. (Merr). *Plant Physiol.* 77:53–58
142. Vance, C. P., Heichel, G. H. 1991. Carbon in N_2 fixation: limitation or exquisite adaptation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:373–92
143. Vance, C. P., Heichel, G. H., Barnes, D. K., Bryan, J. W., Johnson, L. E. 1979. Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. *Plant Physiol.* 64:1–8
144. Van Kessel, C., Burris, R. H. 1983. Effect of H_2 evolution on $^{15}N_2$ fixation, C_2H_2 reduction and relative efficiency of leguminous symbionts. *Physiol. Plant.* 59:329–34
145. Vessey, J. K., Walsh, K. B., Layzell, D. B. 1988. Can a limitation in phloem supply to nodules account for the inhibitory effect of nitrate on nitrogenase activity in soybean? *Physiol. Plant.* 74:137–46
146. Vessey, J. K., Walsh, K. B., Layzell, D. B. 1988. Oxygen limitation of N_2 fixation in stem-girdled and nitrate-treated soybean. *Physiol. Plant.* 73:113–21
147. Vessey, J. K., Waterer, J. 1992. In search of the mechanism of nitrate inhibition of nitrogenase activity in legume nodules: re-

cent developments. *Physiol. Plant* 84:171–76
148. Walsh, K. B. 1990. Vascular transport and soybean nodule function. III. Implications of a continual phloem supply of carbon and water. *Plant Cell Environ.* 13:893–901
149. Walsh, K. B., Canny, M. J., Layzell, D. B. 1989. Vascular transport and soybean nodule function. II. A role for phloem supply in product export. *Plant Cell Environ.* 12: 713–23
150. Walsh, K. B., Carroll, B. J. 1992. A reappraisal of nitrate inhibition of nitrogenase in A317, a nitrate reductase-deficient mutant of pea *(Pisum sativum). Physiol. Plant.* 84:283–91
151. Walsh, K. B., Vessey, J. K., Layzell, D. B. 1987. Carbohydrate supply and N_2 fixation in soybean. The effect of varied daylength and stem girdling. *Plant Physiol.* 85:137–44
152. Webb, J., Sheehy, J. E. 1991. Legume nodule morphology with regard to oxygen diffusion and nitrogen fixation. *Ann. Bot.* 67: 77–83
153. Weisz, P. R., Denison, R. F., Sinclair, T. R. 1985. Response to drought stress of nitrogen fixation (acetylene reduction) rates by field-grown soybeans. *Plant Physiol.* 78: 525–30
154. Weisz, P. R., Sinclair, T. R. 1987. Regulation of soybean nitrogen fixation in response to rhizosphere oxygen. I. Role of nodule respiration. *Plant Physiol.* 84:900–5
155. Weisz, P. R., Sinclair, T. R. 1988. A rapid nondestructive assay to quantify soybean nodule gas permeability. *Plant Soil* 105:69–78
156. Williams, L. E., DeJong, T. M., Phillips, D. A. 1982. Effect of changes in shoot carbon-exchange rate on soybean root nodule activity. *Plant Physiol.* 69:432–36
157. Witty, J. F. 1991. Microelectrode measurements of hydrogen concentrations and gradients in legume nodules. *J. Exp. Bot.* 42:765–71
158. Witty, J. F., Minchin, F. R., Skot, L., Sheehy, J. E. 1986. Nitrogen fixation and oxygen in legume root nodules. *Oxford Surv. Plant Mol. Cell Biol.* 3:275–314
159. Witty, J. F., Skot, L., Revsbech, N. P. 1987. Direct evidence for changes in the resistance of legume root nodules to O_2 diffusion. *J. Exp. Bot.* 38:1129-40
160. Yang, L. J. O., Udvardi, M. K., Day, D. A. 1990. Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. *Planta* 182:437–44